



Perspective

Synthetic Biology of Thermophiles: Taking Bioengineering to the Extremes?

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Abstract: Synthetic biology applications rely on a well-characterized set of microbial strains, with an established toolbox of molecular biology methods for their genetic manipulation. Since there are no thermophiles with such attributes, most biotechnology and synthetic biology studies use organisms that grow in the mesophilic temperature range. As a result, thermophiles, a heterogenous group of microbes that thrive at high (>50 °C) temperatures, are largely overlooked, with respect to their biotechnological potential, even though they share several favorable traits. Thermophilic bacteria tend to grow at higher rates compared to their mesophilic counterparts, while their growth has lower cooling requirements and is less prone to contamination. Over the last few years, there has been renewed interest in developing tools and methods for thermophile bioengineering. In this perspective, we explain why it is a good idea to invest time and effort into developing a thermophilic synthetic biology direction, which is the state of the art, and why we think that the implementation of a thermophilic synthetic biology platform—a thermochassis—will take synthetic biology to the extremes.

Keywords: synthetic biology; thermophiles; biotechnology; industrial microbiology; *Geobacillus* spp.



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1. Introduction—Life at the Extremes, Thermophilic Microorganisms

What is extreme? From an anthropocentric standpoint, the term “extreme” is defined as anything that is not inextricably linked to the “normal” and “typical” conditions under which our species thrives. For a long time, extremophiles were *terra incognita*, since the environments with extreme physico-chemical and climatic parameters were considered, *a priori*, as dead zones. As a result, extremophiles are considered a distinct microorganism class, only because they are found in regions where most organisms cannot typically live unassisted (Figure 1). These microbes have evolved a set of adaptations that make them “dominant” and not merely surviving as the “last organism standing” while waiting for more favorable conditions—this refers to extremophile as opposed to extremotolerant species. After all, these “extreme” environments are their preferred niche.

Thermophiles are a heterogenous group of heat-loving microbes, with optimum growth temperatures higher than 50 °C, which, during the recent decades, have attracted significant interest within the biotech community [1]. They have tremendous potential in microbial and enzyme technology applications, due to their unique metabolic characteristics, ability to catalyze specific reactions, and their potential industrial use under extreme thermal conditions [1,2]. Thermophilic bacteria, from various diverse genera, such as *Clostridium* spp., *Thermoanaerobacter* spp., *Caldicellulosiruptor* spp., *Thermococcus* spp., *Geobacillus* spp., and several others, have been evaluated and found to have a number of advantages over mesophilic bacteria, such as fast growth, low contamination risks, and the ability to produce robust, thermostable enzymes suitable for industrial processes [3–5]. Among the above genera, *Geobacilli* are among the most popular thermophiles used in biotechnology. The genus comprises a group of Gram-positive thermophilic bacteria,

including obligate aerobes, denitrifiers, and facultative anaerobes that can grow over a range of 45–75 °C [6]. Their catabolic versatility, particularly in the degradation of hemi-cellulose and starch, and their rapid growth rates, have raised their profile as organisms with potential to be used as second-generation (lignocellulosic) biorefineries for biofuel or chemical production [7,8]. The continued development of genetic tools to facilitate both fundamental research and metabolic engineering is now helping to realize this potential, for both metabolite production and optimized catabolism [6,9,10].



Figure 1. Common environmental niches that harbor thermophilic microbial consortia.

This perspective focuses on the potential advantages of thermophilic bacteria as biotechnology hosts, on the current synthetic biology and genetic engineering tools, and on why we think that the implementation of a thermophilic synthetic biology platform—a thermochassis [11–13]—will offer new options to biotechnologists and take synthetic biology to the extremes.

2. Thermophilic Biotechnology and Biocatalysis

2.1. Physiology and Metabolism

Microorganisms are significantly affected by the chemical and physical composition of their environment, with temperature being the most important environmental factor controlling their physiology. Not all temperatures are equally suitable for the growth of living organisms; thus, at very low or high temperatures, microorganisms are unable to grow and may even die. The minimum and maximum temperatures that favor growth vary widely between species, and usually reflect the range and the average value of the temperature at which they thrive.

While most well-studied microorganisms are mesophiles, with optimum growth temperatures between 25 and 40 °C, microbial life also flourishes in environments with higher temperatures (Figure 1). These ecosystems are diverse, and range from soil and water tanks, heated by the sun, to boiling hot springs. Understanding the unique attributes of microbial adaptations to thermal environments and their evolutionary strategies that enable them to grow at high temperatures, provides some insight into the prediction and utilization of potential thermophilic microbial technologies.

2.1.1. Molecular Mechanisms

Thermophiles are constantly exposed to heat stress; thus, the answer to why thermophilic bacteria thrive at extreme temperatures that kill other life forms lies in the way that they have adapted all macromolecules (DNA, lipids, and proteins) and macromolecular complexes (cell surface, ribosomes, and so on) to remain functional [14]. Protein data are amongst the most studied aspects of adaptation for thermophiles. Thermophilic enzymes and other proteins are much more thermostable than those of mesophiles, and work optimally at high temperatures [15]. Surprisingly, however, studies of several thermostable enzymes have shown that they often differ slightly in their amino acid sequence from the corresponding heat-sensitive forms of the same mesophilic enzymes. Critical amino acid substitutions at only a few sites of the enzyme appear to allow it to fold in a unique thermostable manner [16]. The thermal stability of the proteins in thermophilic microorganisms is also enhanced by the additional networks of hydrogen bonds, decreased length of surface loops, increased number of ionic bonds between basic and acidic amino acids, and their often highly hydrophobic interior. This combination makes the protein more resistant to unfolding [17].

To enable life at high temperatures, thermophiles might have developed strategies to protect their nucleic acid molecules from denaturation [18]. Nucleotides are the basic building blocks of nucleic acid molecules, and they contain either a purine (adenine/guanine) or a pyrimidine base (cytosine/thymine). Since adenine and thymine connect through two hydrogen bonds, whereas cytosine and guanine are connected by three hydrogen bonds, it is assumed that a high genomic GC content in specific regions (stem-loops) confers greater stability, and, thus, this might be the main mechanism of protecting double-stranded DNA from denaturation in extremophiles [19]. However, many extremophiles do not have higher GC contents when compared with genomes from mesophiles, which draws us to the conclusion that nucleic acid stability at high temperatures is rather a matter of the chemical structure of the molecules that might be modified or of the specialized stabilizing structures that may be formed.

In addition to enzymes and other cellular macromolecules, the cytoplasmic membrane of thermophilic microorganisms must also be thermostable. Typically, heat denatures the lipid bilayer that makes up the cytoplasmic membrane. In thermophilic organisms, this is counteracted by the biosynthesis of membranes that contain more saturated and straight-chain fatty acids, and less unsaturated fatty acids. Saturated fatty acids form a stronger hydrophobic environment than unsaturated fats, and long-chain fatty acids have a higher melting point than shorter-chain fatty acids [20]. Overall, these two parameters increase the stability of the membrane.

2.1.2. Metabolism

Microbial metabolic activity can be significantly influenced by the physical and chemical composition of the environment, creating opportunities for metabolic engineering strategies that take advantage of these variations in metabolism. The plethora of information and data gathered from experimental and genomic studies leads to the conclusion that thermophilic bacteria have nearly all the essential metabolic networks found in mesophilic bacteria [21,22], conveyed, of course, by the necessary elevated temperature adaptations of the relevant macromolecules mentioned in the previous paragraph.

However, thermophiles express certain unique growth and metabolic properties, featuring, firstly, a multitude of electron donors and acceptors. Among the reported electron donors for thermophilic and hyperthermophilic bacteria and archaea are H_2 , Fe^{2+} , H_2S , S , $S_2O_3^{2-}$, sulfide minerals, CH_4 , various mono-, di- and hydroxy-carboxylic acids, alcohols, amino acids, and complex organic substrates, while the electron acceptors include O_2 , Fe^{3+} , CO_2 , CO , NO_3^- , NO_2^- , NO , N_2O , SO_4^{2-} , SO_3^{2-} , and S . Although this multitude mainly reflects the increased chemical diversity in thermophilic environments, it also denotes increased metabolic wealth for their microbial inhabitants, which may be of

significance for thermophilic industrial applications—both under aerobic and anaerobic conditions [23–25].

Even though the metabolic pathways for primary and secondary metabolite production in thermophiles are still largely unresolved, considerable progress has been made in identifying the variety of carbon and/or energy sources that thermophilic bacteria can utilize. These include the most common biopolymers (starches, cellulose, xylan, pectin, etc.), sugars (mono- and di-saccharides), poly- and oligopeptides, amino acids, aromatics, hydrocarbons, carbon monoxide, and inorganics (H_2 and S^0) [5,26]. Although significant gaps still exist in elucidating the kinetic and regulatory properties of thermophilic carbon metabolism in general, this wide array of growth substrates demonstrates the significant potential for using thermophiles in industrial biotechnology applications.

2.2. Thermophiles in Industry

Thermophiles have several advantages that make them beneficial industrial biotechnology biocatalysts (Table 1). Thermophilic microorganisms tend to be robust, fast-growing microorganisms that are resistant to stresses. Their enzymes are thermostable and, thus, suitable for industrial processes that take place at high temperatures, such as single-vessel heat pretreatment and enzyme processing steps. Elevated temperatures accelerate biochemical reaction rates, while simultaneously minimizing microbial contamination risks—a significant problem in many biotechnological applications. In addition, high temperatures contribute to the reduction in energy input, since they promote solubility and the efficient mixing of substrates. Finally, they facilitate downstream product recovery, since solvent/product recovery processes mainly include distillation (gas and steam stripping) and permeation membrane separation [4].

Table 1. Advantages and disadvantages of using thermophilic organisms in industrial processes.

Advantages	Disadvantages
Reduced need for cooling	Poorly understood genetics and metabolism
Fewer contaminations	Lack of bioengineering tools
Thermostable enzymes	Infrastructure and fermentation expertise built around mesophiles
High growth rates	Not suitable for all heterologous protein expression (denaturation risks)

However, thermophiles are not yet ready to replace mesophiles as generic biotech hosts. These bacteria are not as well studied as the model microbes used in biotech, and they lack tools for efficient bioengineering. The current fermentation infrastructure and expertise is built around mesophilic microorganisms, and the industry might be reluctant to adapt to higher fermentation temperatures, unless there is a compelling reason to do so. Moreover, thermophiles are unsuitable to express proteins normally found in mesophilic organisms, due to the risks of protein denaturation or poor enzymatic performance at the high temperatures of thermophilic growth. This makes them unable to become generic protein expression vessels or metabolic engineering hosts for several heterologous metabolic pathways. Such proteins would need to be previously engineered to increase their thermostability, without affecting their functional characteristics. This is possible; however, it is tedious and adds another level of complexity in such metabolic engineering endeavors.

One of the low-hanging fruits of thermophile biotechnology may be the decomposition of complex lignocellulosic biomass—a common byproduct of agriculture—which is an essential process in second-generation biorefineries. Thermophiles already possess the enzymes needed to extract carbohydrates from their environment. The process may be facilitated by high temperatures, which help loosen the complex biopolymer biomass matrix, thus assisting enzymatic attack [23,26,27]. A large number of studies have reported the isolation of cellulose- and hemicellulose-degrading bacteria from high-temperature environments,

such as compost systems, soils, wastewaters, springs, marine hydrothermal vents, and the deep biosphere of gold mines. These microorganisms belong to distinct thermophilic and hyperthermophilic microbial genera, such as *Geobacillus*, *Parageobacillus*, *Rhodothermus*, *Caldicellulosiruptor*, *Clostridium*, *Thermotoga*, and many others [1,3,7,8]. Several of these microorganisms are efficient producers of cellulases and hemicellulases that can withstand extreme conditions, such as high temperature and the presence of toxic inhibitors, while, in parallel, they hydrolyze the corresponding carbohydrate polymers at significantly high rates [28–30].

In conjunction with their biorefinery applications, thermophiles have also been evaluated as biofuel producers, where they could be used to produce easy-to-separate, volatile bioalcohols, such as bioethanol. Due to the high fermentation temperatures, the use of thermophilic microorganisms could greatly facilitate product recovery and reduce downstream costs. However, the efforts to produce bioethanol have been hampered by the limited biochemical knowledge and genetic tools, poorly understood host transformation systems, and scarcity of sequencing data for these microorganisms [9,10,31].

3. Synthetic Biology Chassis and the Need for a “Thermochassis”

The term “chassis” is used within the synthetic biology (SB) field to denote a microbial species that can be used as a versatile and efficient carrier and processor of any exogenous genetic information that codes for the production of specific metabolites (Figure 2), but what makes an organism suitable to become a biotechnological chassis?

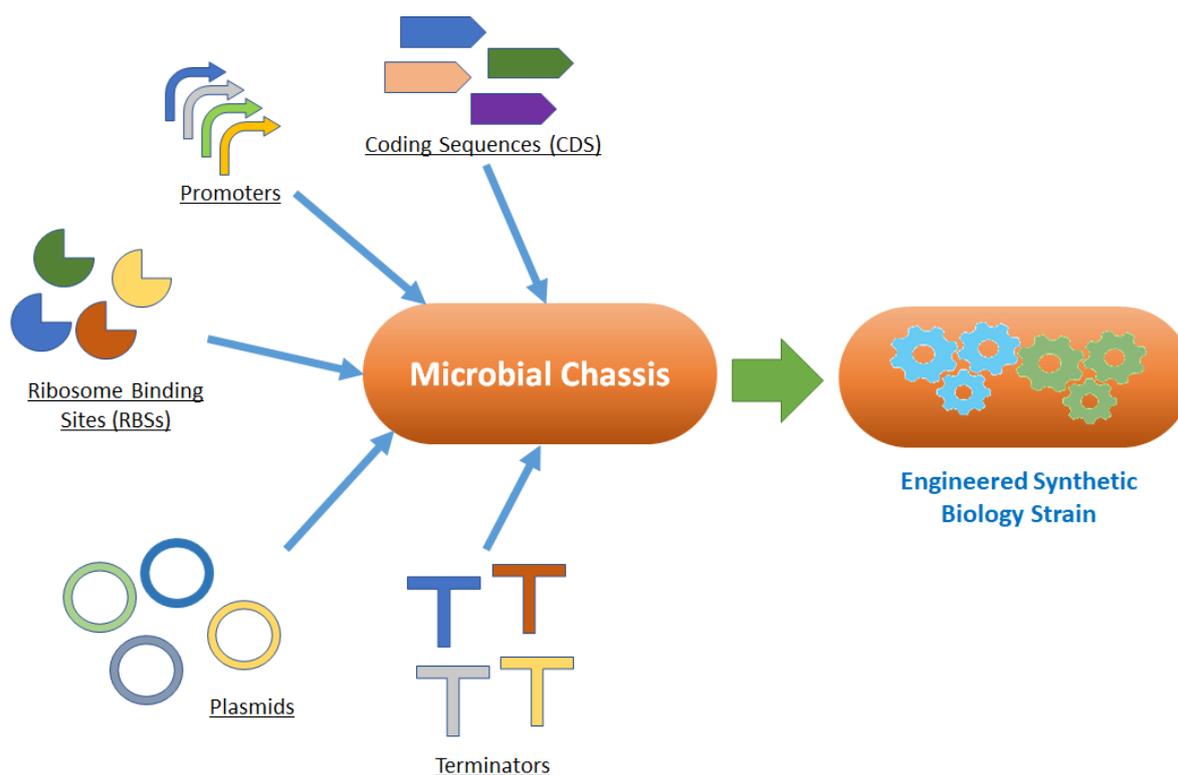


Figure 2. Schematic representation of the characteristics of a synthetic biology chassis.

SB applications rely heavily on design–build–test–learn circles. A strain to be used for industrial production needs to go through iterative processes of improvement. The bioengineering design needs to be tested and improved repeatedly to reach the desired result. Consequently, a chassis organism needs to have the following characteristics:

- Ease of genetic manipulation;
- Sequenced genome;
- Availability of standardized genetic parts, with predictable and reported characteristics.

These three features generate a frame of known factors, within which the bioengineering efforts can take place with a fair amount of predictability. The term evokes the basic frame of a car, to which a number of components can be added in response to specifications and/or the customer's desires [13]. The word started being used in 2000, by the incipient synthetic biology community of the time, as a humorous and engineering-sounding description of the biological host used as the recipient of recombinant DNA, which, by that time, nearly exclusively meant *Escherichia coli* [32]. Since then, only a few microorganisms have been proposed as SB chassis, with only *E. coli*, *Pseudomonas putida* (bacteria), and *Synechococcus/Synechocystis* spp. (cyanobacteria) being able to comply with most requirements of the term [33–35]. The above strains, as well as an additional few that are currently being investigated as potential SB chassis, can grow in the mesophilic temperature area, with an optimum growth range between 25 and 40 °C [12]. This fact represents a significant limitation for SB applications at the industrial level, where higher operating temperatures are a plus [11]. Based on the favorable traits of thermophilic microorganisms described in the previous paragraphs, the development of a thermophilic SB chassis that is able to function at higher temperatures (>55 °C) is missing from the SB microorganism palette, and is highly needed.

4. Future Perspectives in the Synthetic Biology of Thermophiles

The majority of metabolic engineering endeavors take place in well-known, standard laboratory organisms. *E. coli* and *Saccharomyces cerevisiae* are the main synthetic biology workhorses. However, as more applications develop and SB finds its way into the industry, it becomes apparent that model organisms may not be suitable for every kind of industrial approach [11]. As a result, we observe studies using a more varied selection of microbial hosts. In the last few years, increased use of organisms such as *Pseudomonas putida* [33], *Yarrowia lipolytica* [36], the extremely fast-growing bacterium *Vibrio natriegens* [37], and the photosynthetic microbes *Synechococcus*, *Synechocystis*, and *Chlamydomonas* [38,39] has been observed. This list becomes more comprehensive, but there is something consistently missing. There are neither thermophiles nor extreme thermophiles as standardized synthetic biology vessels. Therefore, SB cannot be used when applications require heat-demanding conditions.

The main reason for the above paucity lies in the fact that thermophilic bacteria are considered to be more recalcitrant to genetic manipulations. A small number of thermophilic genera, though, can be extensively engineered with heterologous sequences harbored in plasmids or incorporated into their genomic DNA [40]. The scientific literature only contains a few relevant studies that involve members of the *Geobacillus*, *Clostridium*, *Thermus*, and *Sulfolobus* genera (Table 2).

Geobacillus spp., having the additional advantage of utilizing a wide range of carbohydrates as feedstock, at significantly higher rates than other thermophiles, represents a promising bioproduction candidate for biorefinery approaches. Genetic manipulation of *Geobacillus* via the conjugative transfer of self-replicating vectors is possible and remains the most popular method used in the literature [41]. However, this method has the following two serious drawbacks: it has reduced transformation efficiency and does not allow for the targeted incorporation of heterologous DNA into the genome. Thus, it is very difficult to genetically study *Geobacillus* strains (e.g., by performing gene knockouts). One initial attempt to create a genetic engineering toolbox took place in 2016, where Reeve et al. characterized a set of bacterial promoters and ribosome binding sites for *G. thermoglucosidasius* [42]. Reeve et al. used an electroporation method to transform *G. thermoglucosidasius*, achieving higher efficiency with several self-replicating vectors. The reported transformation efficiency was in the range of 10^3 – 10^4 cfu/ μ g DNA, compared to 10^6 reported for the *E. coli* control.

Table 2. Synthetic biology works involving thermophiles.

Description	Organism	References
Plasmid vectors for transformation; reporter genes; origins of replication	<i>G. thermoglucosidasius</i>	[42]
Transformation vectors, knock-in/knockout system	<i>G. thermoglucosidasius</i>	[31]
Library of semi-synthetic constitutive promoters	<i>G. thermoglucosidasius</i>	[43]
Riboswitches that work at high temperatures	<i>G. thermoglucosidasius</i> , <i>Clostridium thermocellum</i>	[44]
Heterologous expression of glycoside hydrolases to degrade lignocellulosic biomass	<i>G. thermoglucosidasius</i>	[45]
Counterselection system for introduction of genomic point mutations or deletions	<i>T. thermophilus</i>	[46]
Modular vector toolkit for gene expression	<i>T. thermophilus</i>	[47]
ThermoCas9-based genome-editing tool	<i>T. thermophilus</i>	[48]
Thermostable Cas9	<i>T. thermophilus</i>	[49]
CRISPR-mediated genetic transformation	<i>Sulfolobus islandicus</i>	[50]

Since then, little progress has been achieved. The biotechnological arsenal of thermophiles was enriched by a set of expression vectors [31] and riboswitches that allowed for inducible transcription [44]. This lack of tools and standard laboratory practices is holding back the research community from using thermophiles to their full potential. One of the few works where genetic engineering was used to express a heterologous pathway was published in 2019, where the researchers expressed glycoside hydrolases to degrade a wheat straw-derived lignocellulosic substrate [45]. Cellulosic biomass degradation is an obvious application area in which thermophiles have an advantage, as high temperatures increase the solubility of the complex carbohydrates, allowing single-pot processes to occur, where heat pre-treatment and microbial degradation take place simultaneously.

The thermophile *Thermus thermophilus* is another microbe that has attracted the interest of metabolic engineers. *T. thermophilus* is an excellent source of thermophilic enzymes (including thermostable polymerases, which are widely used in PCR applications), and has found applications in the thermic degradation of biomass. The synthetic biology toolset of *T. thermophilus* is also limited; however, research groups around the world have enriched the toolset with elements for insertions/deletions [46], a modular vector toolkit for gene expression—which includes promoters, thermosensors and origins of replication [47]—and thermostable CRISPR Cas9 systems that work at 65 °C [48,49].

Besides the *Geobacillus* and *Thermus* genus, thermophilic archaea have attracted interest as synthetic biology platforms [51]. *Sulfolobus* species are both thermo- and acidophiles, making them suitable for industrial processes that take place in otherwise hostile conditions for bioengineering applications. There are established genetic manipulation techniques for *S. acidocaldarius* [52], as well as a more recent study on the CRISPR-mediated transformation of *S. islandicus* [50].

One recent idea finding its way into microbial biotechnology applications is the use of microbial consortia in challenging bioprocesses. The same can be applied in thermophiles, where different strains, with different genetic manipulations, can undertake different components of complex bioprocesses, as in the degradation of lignocellulosic biomass [53].

5. Conclusions

Thermophiles have a niche place in the industry; they have a wealth of biodiversity and biomolecules that can potentially be exploited, with significant advantages as biotechnology hosts. The lack of tools and bioengineering techniques is limiting the potential of thermophiles, whether we refer to single-organism or microbial consortia applications. We believe that the development of a synthetic biology thermochassis can rejuvenate the scientific community's interest in the field, and allow us to take synthetic biology to the extremes. Although still limited in volume, the research efforts described in this perspective pave the way for an efficient “synthetic biology future” for thermophiles.

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