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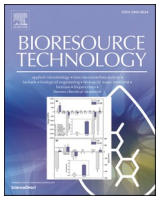

# Industrial biotechnology goes thermophilic: Thermoanaerobes as promising hosts in the circular carbon economy

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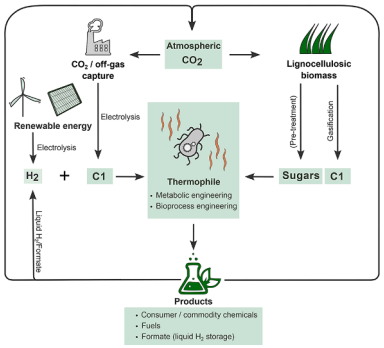
## HIGHLIGHTS

- "Second-" and "third-generation" (2G, 3G) feedstocks available today are addressed.
- Thermophiles converting renewable feedstocks for circular bioeconomy are reviewed.
- Current strategies for metabolic engineering of key thermoanaerobes are discussed.
- Bioprocess engineering considerations and fermentation parameters are highlighted.
- Several scenarios for C1 or LCB conversion to value-added products are showcased.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Transitioning away from fossil feedstocks is imperative to mitigate climate change, and necessitates the utilization of renewable, alternative carbon and energy sources to foster a circular carbon economy. In this context, lignocellulosic biomass and one-carbon compounds emerge as promising feedstocks that could be renewably upgraded by thermophilic anaerobes (thermoanaerobes) via gas fermentation or consolidated bioprocessing to value-added products. In this review, the potential of thermoanaerobes for cost-efficient, effective and sustainable bioproduction is discussed. Metabolic and bioprocess engineering approaches are reviewed to draw a comprehensive picture of current developments and future perspectives for the conversion of renewable feedstocks to chemicals and fuels of interest. Selected bioprocessing scenarios are outlined, offering practical insights into the applicability of thermoanaerobes at a large scale. Collectively, the potential advantages of thermoanaerobes regarding process economics could facilitate an easier transition towards sustainable bioprocesses with renewable feedstocks.

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## 1. Introduction

Global warming primarily results from the accumulation of greenhouse gas (GHG) – such as $CO_2$ – in the atmosphere and has been tightly linked with the linear, anthropogenic use of fossil resources (Emanuel, 2012). In this context, global carbon demand for the production of chemicals and other materials (~1,000 Mt carbon $a^{-1}$ by 2050, (Nova Institut, 2023)) as well as plastics (~1,200 Mt carbon $a^{-1}$ by 2050, (Nova Institut, 2020)) have been projected to increase significantly, where roughly 50 % of the required carbon would be derived from $CO_2$ or biomass, with the rest obtained through recycling technologies. To that end, transitioning away from fossil feedstocks for manufacturing carbon-containing products is required, which entails providing vast amounts of renewable, low-carbon footprint energy as well as sustainably sourced biomass to foster the implementation of a circular carbon economy.

Exploiting renewable feedstocks to produce fuels and commodity chemicals has been a key driver for the expansion of industrial biotechnology in the last few decades, starting with the transformation of agricultural resources – grown from light and $CO_2$ – into the "first-generation" fuel bioethanol (Liu et al., 2020b). Over time, concerns about land usage and the question of the food/feed vs. fuel competition gradually sparked the development of "second-generation" (2G) bioprocesses aiming at upgrading lignocellulosic biomass (LCB), a non-food plant component found in vast quantities in agriculture and forestry waste streams (Zuliani et al., 2021). Industrial bioproduction from LCB is currently dominated by ethanol and multiple commercial plants operating in the range of 30 to 90 ktons/a have been established (e.g., in the US or Brazil) (Lynd et al., 2017). More recently, focus has increasingly shifted towards "third-generation" (3G) fuels and chemicals, stemming directly from $CO_2$ and derived one-carbon (C1) compounds (i.e., methane, methanol, carbon monoxide, formate) (Liu et al., 2020b).

Both 2G and 3G feedstocks are not readily fermentable by typical mesophilic microbial cell factories (e.g., *Escherichia coli* or *Saccharomyces cerevisiae*), which therefore require extensive metabolic engineering to make the industrial process economically feasible (Francois et al., 2020; Jiang et al., 2021; Liu et al., 2020a). In this context, the use of alternative, non-model microbial workhorses, naturally suited for LCB or C1 fermentation, has attracted considerable attention (Hu et al., 2023; Olson et al., 2012; Orsi et al., 2023; Sun and Alper, 2020). The success story of *"Clostridium autoethanogenum"*, currently in use for $CO_2$ and CO upgrading into bioethanol at industrial scale (by Lanzatech, USA), is a remarkable example of this trend (Köpke and Simpson, 2020).

Anaerobic thermophiles – or thermoanaerobes – include many, largely under-used, facultatively or obligately anaerobic bacteria and archaea that grow optimally at high temperatures. Thermophiles are typically classified as moderate (optimal growth between 50 °C and 60 °C), extreme (60 °C to 80 °C) and hyperthermophiles (80 °C to 110 °C) (Zuliani et al., 2021). These relatively high temperatures – compared to bioprocesses operated at 30–37 °C – present multiple advantages, including reduced cooling costs (Abdel-Banat et al., 2010; Yang et al., 2008), lower contamination risks (Zeikus, 1979), higher catalytic turnover rates (Zeikus, 1979), facilitated recovery of volatile products (Vane, 2008), higher solubilization of LCB (Lynd et al., 2017), higher gas–liquid mass transfer rates for gas fermentation (Gorter de Vries et al., 2024; Jin et al., 2014).

This review focuses on the application of thermoanaerobes for conversion of second- (2G) and third-generation (3G) feedstocks for the production of economically relevant bioproducts. To that end, all relevant aspects including feedstocks, microbial catalysts, bioprocess, and metabolic engineering strategies are outlined and subsequently discussed in the context of selected bioproduction scenarios to showcase how thermoanaerobes could be used in practice in an industrial setting. Combined with a description of potential future research avenues and current knowledge gaps, this review aims to highlight the potential of thermoanaerobes for industrial biotechnology as a key transformative technology toward a circular bioeconomic system.

## 2. Feedstocks and bioprocess engineering considerations

### 2.1. General considerations for thermophilic bioprocessing

A major advantage of using extremophiles for bioprocessing is arguably the reduced risk of contamination, a major industrial hazard that systematically lowers yields and, in some cases can lead to plant shutdowns, resulting in drastic losses of productivity (Skinner and Leathers, 2004). Mesophilic bioprocesses are therefore typically equipped with sealing and sterilization procedures that can considerably reduce contamination risks (Chen and Jiang, 2018). Despite these precautions, contaminations are difficult to entirely prevent and are considered endemic in many cases (Skinner and Leathers, 2004). Harsh conditions, such as high temperatures, are expected to lower contamination rates, simply because the vast majority of microbes are not adapted to grow in such environments (Chen and Jiang, 2018; Zeldes et al., 2015). Viral contamination is still a considerable risk, that could however be mitigated by tuning the overly abundant CRISPR systems found in thermophiles (Elmore et al., 2013).

Since bioreactors can be easily insulated, the cost of heating these reactors is minimal and can be sustained by the metabolic heat produced by the microorganisms or by low-grade heat that can be acquired from the waste streams of many processing facilities (Keller et al., 2014; Liew et al., 2016). On the other hand, the cooling costs of mesophilic processes can be significant. At the industrial scale, heat produced by metabolically active cells is not efficiently dissipated into the environment, requiring intensive cooling (Yang et al., 2008; Zeldes et al., 2015). Significant cooling and heating costs can also derive from processes run in multiple steps, with different shifts in temperatures. For corn-based bioethanol production, starch liquefaction, mesophilic fermentation and ethanol distillation are all run at different temperature, with fermentation being by far the lowest point (Abdel-Banat et al., 2010). In such a scenario, running fermentation at higher temperatures would substantially reduce costs. Process modeling showed that heating of an industrial-scale reactor to 70 °C contributed to less than 2 % of the total process cost (Bing et al., 2022). Additionally, the temperature difference between the reactor and the ambient air is sufficient to keep the process at a constant temperature (Keller et al., 2014). Therefore, controlling temperatures in thermophilic processes could offer significant benefits, with active air circulation replacing expensive cooling methods used for mesophilic fermentations.

Higher temperatures can also be beneficial for downstream processing. Process costs could be drastically reduced for volatile products, such as alcohols and ketones by, e.g., *in situ* product recovery via gas stripping (i.e., ethanol, acetone) (Gorter de Vries et al., 2024; Kato et al., 2021).

Finally, bioprocessing of thermoanaerobes requires consideration of the ATP yield microbes can obtain from their respective target feedstocks. While cellulolytic strains achieve rather high ATP yields (e.g. 5 ATP for equimolar formation of ethanol/acetate by *Acetivibrio thermocellus,* formerly *Clostridium thermocellum*), acetogens gain little ATP from gaseous substrates (e.g. ~ 0.28 mol ATP per acetate for $H_2/CO_2$ conversion by *Thermoanaerobacter kivui*) (Basen and Müller, 2017). ATP yields can directly affect biomass formation, as described by the biomass yield ($Y_{X/S}$ in $g_{biomass}$ $mol^{-1}_{substrate}$). Multiplying $Y_{X/S}$ with the biomass-specific substrate uptake rate $q_s$ (mmol $g^{-1}h^{-1}$) gives the specific growth rate μ ($h^{-1}$) as the product. To achieve a target growth rate, therefore either qs or $Y_{X/S}$ need to be adjusted by the cell. In the case of cellulolytic thermoanaerobes with high ATP yields, it has been speculated that there might be a high selective pressure to maximize biomass yields rather than $q_s$ which is typically limited by $k_{cat}$ (Lynd et al., 2022). In contrast, for low ATP and therefore low biomass yields, as in thermophilic acetogens, the $q_s$ might be preferentially adjusted, which in turn shifts substrate utilization in favor of a higher product-to-biomass

ratio. This observation was suggested to be due to a significantly increased non-growth associated maintenance energy requirement at higher temperatures which is accounted for by the higher $q_s$ (Gorter de Vries et al., 2024). Overall, these factors need to be considered for bioprocess design to achieve high volumetric productivities by, e.g., using cell retention systems to increase the number of biocatalysts in the system.

### 2.2. Gaseous and liquid one carbon feedstocks, $H_2$ and gas fermentation

Table 1 summarizes the main 2G and 3G feedstocks available and their general characteristics.

#### 2.2.1. Sources of gaseous and liquid one-carbon feedstocks

$CO_2$ is a vastly abundant carbon source, with an estimated 3,000 gigatons available in the atmosphere (National Oceanic and Atmospheric Administration, 2023), increasing at a current rate of 37 billion tons per year (Friedlingstein et al., 2023). In addition, $CO_2$ is enriched in many waste streams e.g., in power plants or ethanol biorefineries (Köpke and Simpson, 2020). Whether $CO_2$ is captured from industrial off-gases or directly from the atmosphere, sustainable $CO_2$ storage or utilization requires renewable energy input (Takors et al., 2018).

Likewise, the utilization of $CO_2$ as a carbon source in bioprocesses requires an input of renewable energy. In case $CO_2$ is directly fed to a gas fermentation, a process that involves autotrophic microbes, $H_2$ is typically required as an electron source. $H_2$ can be generated renewably by electrolysis, whereas $CO_2$ electrolysis yields CO or syngas (a mixture of $CO/H_2/CO_2$) (Herranz et al., 2020), which may also serve as a feedstock for microbial gas fermentation. Syngas or CO are currently also available from steel mills and other industrial production plants or via biomass gasification (chapter 2.3.3).

For transport and storage, gaseous carbon and/or energy sources such as CO or $H_2$ are largely inconvenient. Moreover, their conversion via gas fermentation i) relies on a small set of microorganisms capable of using these gaseous C1 sources effectively and ii) complicates bioprocessing because of their low solubility in aqueous media, thus requiring reactors with high gas–liquid mass transfer rates (chapter 2.2.3). To circumvent these issues, the liquid C1 compounds formate and methanol may alternatively be used as feedstocks for microbial bioproduction. Formate can be produced by reduction of $CO_2$ either with light, electricity or $H_2$ as the energy source, with a current price of $ 200–500 ton$^{-1}$ (Pan et al., 2023; Zhang et al., 2022). Methanol ($E^{\circ\prime} \sim -420$ mV) can be produced from natural gas, syngas, or $H_2/CO_2$ via chemical catalysts or electrochemically, with a price lower than that for sugars ($ 150–300 ton$^{-1}$ compared to $ 300–400 ton$^{-1}$) (Jiang et al., 2021; Pacholik et al., 2021).

#### 2.2.2. $H_2$: Energy carrier

In this review, $H_2$ is described as a feedstock for microbial gas fermentation using thermophilic acetogens as well as a product of dark fermentation. Generally, $H_2$ is an attractive energy carrier with potential for renewable energy production and storage. The energy density per unit mass of $H_2$ exceeds that of petroleum by a factor of 3 with no direct carbon emissions when used for energy production (Rittmann et al., 2015). However, if $H_2$ has a high density by mass, it also has a low energy density by volume, which significantly hinders many of its potential applications. Currently, the global demand for $H_2$ is met mainly through fossil fuels (e.g., by steam reforming of methane (Braga et al., 2017; Yukesh Kannah et al., 2021)), which considerably limits its interest, as fossil-based production emits high amounts of GHG.

$H_2$ can alternatively be produced sustainably in various ways with low GHG emissions, e.g., through water electrolysis powered by renewable energy (Pan et al., 2023; Slobodkin et al., 2024). $H_2$ can also be produced biologically, either through "light" (biophotolysis, photosynthesis by algae and cyanobacteria) or "dark" fermentation with bacteria and archaea (Braga et al., 2017). Dark fermentation so far has proven more efficient than photosynthesis, as it can run without the need for light and hydrogenases are not inhibited by oxygen, since the process runs anaerobically (Cao et al., 2022). Furthermore, the $H_2$ yield for both routes is similar (up to 49 g kg$^{-1}$ feedstock) (Agyekum et al., 2022).

The Thauer limit of 4 mol $H_2$ mol$^{-1}$ glucose (equivalent to 45 g kg$^{-1}$ glucose) represents the maximum theoretical yield of $H_2$ by dark fermentation, which is constrained by the need for carbon and redox balancing within the cell (Thauer et al., 1977). It can be achieved only if a sugar is converted solely into acetate, $CO_2$ and $H_2$ but not into other common metabolic products of thermoanaerobes such as lactate and ethanol. Nevertheless, a recent study using artificial microbial consortia exceeded the Thauer limit by 40 % resulting in 5.6 mol $H_2$ per mol glucose (62 g $H_2$ kg$^{-1}$ glucose). This unexpected result was attributed to an unknown synergistic effect of the two strains responsible for $H_2$ production improvement (Ergal et al., 2020). The Thauer limit is at the lower end of the $H_2$ yield range typically observed for steam reforming (40–130 g kg$^{-1}$ feedstock) or biomass gasification (40–190 g kg$^{-1}$ feedstock) (Agyekum et al., 2022). Consequently, the cost of $H_2$ is similar between steam reforming ($ 2.27 kg$^{-1}$), biomass gasification ($ 1.77–2.05 kg$^{-1}$), and dark fermentation ($ 2.57 kg$^{-1}$), indicating that further advancements in dark fermentation could demonstrate industrial competitiveness (Kayfeci et al., 2019). Thermophilic $H_2$ production shows higher yields ($Y_{H2/substrate}$) compared to mesophilic, due to higher feedstock conversion efficiency, better feedstock solubilization at higher temperatures and decreased inhibition by $H_2$ partial pressure ($p_{H2}$) (O-Thong et al., 2019; Rittmann et al., 2015).

#### 2.2.3. Gas fermentation at high temperatures

The main limiting factor in gas fermentation processes is the gas–liquid mass transfer rate of the poorly soluble gaseous substrates such as $H_2$ and CO that can be achieved with a bioreactor system. The ability to transfer gases into the liquid phase directly affects the performance of the biocatalyst in terms of gas turnover rates as well as productivity and product titers. Generally, gas supply to the liquid can be increased by higher driving forces (higher partial of the gas or total pressure in the bioreactor) or by increasing the volumetric mass transfer coefficient $k_La$, which describes the efficiency with which a gas can be

**Table 1**
Renewable feedstocks for the circular bioeconomy derived from $CO_2$ and renewable energy.

| Feedstock | Characteristics | Source | Utilization |
|---|---|---|---|
| LCB/ Biomass | Recalcitrant, difficult to deconstruct | Crop residues, residual wood, municipal solid waste | Saccharification and fermentation, consolidated bioprocessing, biomass gasification and gas fermentation |
| $H_2$ | Gaseous, low solubility, explosive, high energy density | $H_2O$ electrolysis with renewable electricity, biomass gasification | Aerobic and anaerobic gas fermentation, chemical conversion, fuel cells |
| CO/syngas | Gaseous, low solubility, toxic | $CO_2$ electrolysis with renewable electricity, industrial waste gas, biomass gasification | Aerobic and anaerobic gas fermentation, chemical conversion (e.g. Fischer-Tropsch, synthetic natural gas) |
| Formate | High solubility, low energy density | $CO_2$ electrolysis with renewable electricity, chemical/ biological production from $H_2/CO_2$ | Aerobic and anaerobic fermentation, chemical synthesis |
| Methanol | High solubility, high energy density | $CO_2$ electrolysis with renewable electricity, chemical production from $H_2/CO_2$ | Aerobic and anaerobic fermentation, chemical synthesis |

delivered to a bioreactor. Bioreactor design must therefore aim at enhancing mass transfer rates by realizing high $k_La$ values. At the same time, low operational costs are needed for large-scale operation of gas fermentation which require a low power input per unit volume. Continuous stirred tank reactors (CSTR) used on a lab scale commonly achieve high mass transfer rates but are economically challenging to operate at a large scale due to a high volumetric power input (Takors et al., 2018). Consequently, the volumetric mass transfer coefficient per unit power input ($k_La/P_g$) has been used to model and compare the performance of bioreactor systems (Liew et al., 2016; Puiman et al., 2022). In addition to the $k_La$ value, higher driving forces by increased gas partial or total pressure can increase gas availability in a bioreactor. Commercial high-productivity syngas-to-ethanol fermentation has been established by LanzaTech employing an external loop gas-lift reactor (EL-GLR) (Puiman et al., 2022). Running gas fermentation processes at high temperatures additionally affects the gas–liquid mass transfer rate: gas solubility decreases at higher temperatures ($H_2$: −20 % and CO: −37 % at 60 compared to 30 °C), while diffusion rates increase ($H_2$: +61 %, CO: +134 % at 60 °C compared to 30 °C) (Gorter de Vries et al., 2024).

### 2.3. Lignocellulosic biomass, consolidated bioprocessing and biomass gasification

#### 2.3.1. Lignocellulosic biomass: Sources and availability

Biomass is an excellent renewable carbon and energy source with many benefits and applications in carbon sequestration and as a promising feedstock for the bioproduction of fuels and chemicals. Annual global production of lignocellulosic biomass (LCB) is estimated at 181.5 billion tons, making it the most abundant biomass on Earth (Ashokkumar et al., 2022). LCB includes herbaceous and woody plants, grasses, harvest residues from food crops (e.g., corn stover, sugar cane bagasse) and lignocellulosic crops not suitable for human consumption (Haberzettl et al., 2021; Rajesh Banu et al., 2021). Therefore, utilizing LCB as a feedstock for biotechnological production systems is considered highly economical and, if selected carefully, the use of LCB should not create competition with land use for food or feed production. Indeed, roughly 1 billion tons of LCB is projected to be sustainably available in the United States, European Union and China (Han et al., 2020; Lynd et al., 2022; Ma et al., 2020; Turhollow et al., 2014).

As LCB is characterized by a recalcitrant structure its valorization by microbial fermentation is impeded. Two main biotechnological approaches are pursued to circumvent this limitation:

1. Lignocellulose saccharification with mechanical and enzymatic treatment, coupled with sugar fermentation and
2. Biomass gasification with subsequent gas fermentation.

#### 2.3.2. Biomass saccharification

Two major LCB components, cellulose and hemicellulose, are polymers of various fermentable sugars. While enzymatic hydrolysis of cellulose yields glucose, the monomeric composition of hemicellulose varies depending on the feedstock (typically containing a mixture of pentoses and hexoses). Lignin provides structural rigidity to LCB and hinders deconstruction by cellulolytic enzymes by limiting their access to cellulose. Pretreatment of the biomass by physicochemical means is therefore usually coupled to the fermentation process, to increase the overall accessibility towards cellulases and overall increase saccharification yields (Ma et al., 2020).

Three main strategies currently couple feedstock pretreatment with fermentation: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF) and consolidated bioprocessing (CBP) with or without co-treatment (Lynd et al., 2022). SHF typically includes steam explosion to expose the molecular surface of LCB combined with enzymatic digestion, before fermentation of the solubilized sugars (Prasad et al., 2022). In SSF, the addition of catalytically-active carbohydrate-active enzymes (CAZymes, (Filiatrault-Chastel et al., 2021)) and/or co-treatment with steel ball milling of the biomass is coupled to fermentation (Gupta et al., 2009). Compared to pretreatment, mechanical co-treatment might be advantageous as steel ball milling can reach efficiencies in digesting LCB similar to those of termites (Bing et al., 2022).

In CBP, saccharification of the biomass and fermentation of the sugars is performed in a single step by a cellulolytic microbe or a microbial community, potentially resulting in substantial cost reduction (Liu et al., 2020a; Lynd et al., 2017). For LCB saccharification in general and CBP in particular, fermentation carried out at higher temperatures can considerably ease LCB solubilization (Singh et al., 2018), and (hemi-)cellulolytic thermophiles such as *Acetivibrio thermocellus* (formerly *Clostridium thermocellum*) are therefore ideal CBP hosts. Potential cellulolytic workhorses however generally need to be genetically modified to tailor their metabolism for high titer–rate–yield (T-R-Y) synthesis of a specific product (Herring et al., 2016).

#### 2.3.3. Biomass gasification

To overcome the hurdles rooted in the recalcitrant structure of LCB, gasification can alternatively be used to produce syngas as a uniform feedstock for subsequent bioprocessing from biomass. Syngas from biomass gasification is typically composed of CO, $CO_2$, $H_2$ and $CH_4$, with proportions typically dependent on the gasifier and operation conditions used (Benedikt et al., 2018, 2017; Munasinghe and Khanal, 2010; Schmid et al., 2021). Biomass gasification, as a thermochemical process for LCB deconstruction, is advantageous, as it is highly flexible and can accommodate many feedstock types, with high energy conversion efficiency and low costs (Periyasamy et al., 2023). Biomass gasification, utilizing temperatures of 800–1000 °C and pressures of 1–30 bar, employs various technologies and configurations for large-scale syngas production (Mauerhofer et al., 2021). Fluidized bed gasifiers, notably dual fluidized bed (DFB) technology, are preferred commercially due to their ease of up-scaling, isothermal operation, and high feedstock conversion efficiencies, offering means to convert different LCB into nitrogen-free syngas while controlling $H_2$:CO ratios (Mauerhofer et al., 2019). In addition to LCB (whose characteristics vary considerably depending on the source) other renewable feedstocks of interest, suitable for gasification, comprise municipal solid waste (2.1 billion tons per year) and sewage sludge (40–50 million metric tons per year) (Kumar et al., 2023).

Traditionally, syngas as a feedstock is further upgraded through inorganic chemical catalysis such as Fischer-Tropsch synthesis. In contrast, biotechnological syngas conversion via gas fermentation might be advantageous as microorganisms are more resilient to gas impurities and generate fewer byproducts, potentially saving costs on gas clean-up and product recovery. Major impurities from industrial off-gases include tars, nitric oxide, ammonia and $H_2S$ and compared to typical chemical catalysts, the resistance of biocatalysts tends to be much higher. $H_2S$, for instance, severely inhibits Fischer-Tropsch synthesis at concentrations above 0.1 ppm, whereas anaerobic acetogens can tolerate up to 12,000 ppm of $H_2S$ (Daniell et al., 2012). Key impurities that need to be removed from syngas are cyanide and $O_2$. Cyanide can inhibit crucial metallo-enzymes and will accumulate in the reaction medium. $O_2$ is highly problematic for anaerobes and needs to be eliminated with copper or palladium catalysts (Liew et al., 2016).

Gas solubility and gas–liquid mass transfer are important factors for maximizing productivity and titers in gas fermentation (Vees et al., 2020). At industrial scale, bubble columns are the preferred vessel for their cost-efficiency (low volumetric power input) and ease of operation (Munasinghe and Khanal, 2010). These gas fermenters are ideally operated over long periods in a continuous process mode with or without measures for process intensification (e.g., cell retention systems).

#### 2.3.4. Gasification or saccharification?

Choosing between saccharification or gasification of LCB depends on various factors, such as location, desired product and feedstock.

Comparing feedstock and product lower heating values (LHV, a variable that quantifies the combustion energy of a given substance), can approximate the overall energy efficiency of process chains using either LCB saccharification or biomass gasification coupled to a bioproduction system. Biomass gasification has an energy efficiency of ~64 %, based on the LHV of the feedstock compared to the product syngas (Schmid et al., 2012). Combined with an energy efficiency of 80 % in gas fermentation (syngas to liquid fermentation product) (Köpke and Simpson, 2020), ~51 % of the LHV from the biomass feedstock can be preserved in the final product. Additionally, biomass gasification has the benefit that it can include substrates rich in lignin. In CBP, while the lignin fraction is typically inaccessible by the microbe, the microbial removal of cellulose and hemicellulose can enhance the value of the remaining lignin fraction, which can be utilized as fuel or for generating high-value products such as vanillin (Lynd et al., 2022).

For LCB saccharification in consolidated bioprocessing, energy efficiencies of 77 % and 24 % for soy hulls and poplar wood, respectively, can be calculated, based on the mass fraction of solubilization of the feedstock (Bing et al., 2022) and the lower heating value of the solubilized sugars (Mourad and Walter, 2011). Combined with an energy efficiency of 82 % (soyhull) and 55 % (poplar) in the fermentation (Bing et al., 2022), soy hull and poplar wood bioconversion results in 63 % and 19 % energy efficiency overall. This comparison shows that both process chains can be comparable in energy conversion efficiency if sufficient feedstock solubilization can be achieved.

## 3. Strains, metabolic engineering, ALE and genetic tools

Thermophilic microorganisms form a heterogenous family spanning multiple *phyla* in the bacterial and archaeal domains, with a broad range of physiological, genetic and metabolic traits. In this wealth of microbes, only a limited number of species have received considerable attention as microbial catalysts for efficient industrial bioproduction scenarios in the circular carbon economy (Table 2). These hand-picked microorganisms are mostly anaerobes and can be classified as saccharolytic, cellulolytic, hemicellulolytic, and/or autotrophic based on the feedstocks they use. In most cases, these thermophilic strains have sparked interest for their natural capacity to produce simple chemicals or fuels (e.g., $H_2$, ethanol, lactate, acetate, butyrate, 1,2-propanediol) (Jiang et al., 2021; Straub et al., 2018). Although relatively narrow, this product spectrum could be expanded through metabolic engineering, (eg., butanol, acetone) as efficient genetic tools become gradually available for these strains (Dai et al., 2022; Lanahan et al., 2022; Yang et al., 2023).

Shuttle vectors with thermophilic origins of replication and thermostable markers are readily available (Zeldes et al., 2015). A significant number of thermophiles are naturally competent (Shaw et al., 2010), which could be a feature originating from their "extreme" lifestyle, for which fast adaptation is needed and could potentially be mediated by exogenous DNA uptake (Zeldes et al., 2015). This ability makes transformation protocols faster and less laborious compared to other microbes, where conjugation or electroporation is needed (Table 3). The selection markers differ between bacteria and archaea. While for bacteria the use of thermostable variants of antibiotic resistance proteins is established, archaea are resistant to common bacterial antibiotics. Hence, the use of alternative drugs for selection has therefore been investigated in archaea (Crosby et al., 2019; Zeldes et al., 2015). The most prevalent, simvastatin, inhibits HMG-CoA reductase, which is responsible for archaeal membrane lipid generation (Matsumi et al., 2007; Waege et al., 2010). Overexpression of HMG-CoA reductase in the vector acts as a positive selection, similar to a bacterial antibiotic

**Table 2**
Physiology and metabolism of selected thermophilic bacteria (B) and archaea (A).

| Name (B/A) | $T_{range}$ ($T_{opt}$) [°C] | Lifestyle | Feedstocks | Natural products | Selected reference |
|---|---|---|---|---|---|
| *Acetivibrio thermocellus* (B) | 50–68 (60) | Cellulolytic, saccharolytic | LCB, various hexoses | Ethanol, lactate, formate, acetate, $CO_2$, $H_2$ and secreted amino acids | (Akinosho et al., 2014; Xiong et al., 2016) |
| *Aquifex aeolicus* (B) | 85 | Autotrophic | $CO_2$, $O_2$, $H_2$, $S_0$ | Acetate, $H_2O$, sulfuric acid | (Monsalve et al., 2015) |
| *Bacillus smithii* (B) | 37–63 (55) | Saccharolytic | Glucose, sucrose, xylose | Lactate, acetate, succinate, ethanol | (Mougiakos et al., 2017) |
| *Caldicellulosiruptor bescii* (B) | 42–90 (79) | Cellulolytic, hemicellulolytic, saccharolytic | LCB, various hexoses and pentoses | Acetate, lactate, $H_2$, $CO_2$, ethanol | (Bing et al., 2022) |
| *Caldicellulosiruptor saccharolyticus* (B) | 45–80 (70) | Cellulolytic, hemicellulolytic, saccharolytic | LCB, various hexoses and pentoses | Acetate, lactate, ethanol | (Talluri et al., 2013) |
| *Carboxydothermus hydrogenoformans* (B) | 40–78 (71) | Autotrophic, carboxydotrophic | CO, pyruvate, lactate, formate, glycerol | $H_2$, $CO_2$ | (Parshina et al., 2005) |
| *Moorella thermoacetica* (B) | 45–65 (58) | Autotrophic, saccharolytic | $H_2/CO_2$, xylose, fructose, glucose, glycolate, glycerol, glyoxylate, methanol | Acetate | (Kato et al., 2024) |
| *Parageobacillus thermoglucosidasius* (B) | 55–65 (62) | Saccharolytic | Sugars including cellobiose | Ethanol, isobutanol | (Cripps et al., 2009) |
| *Pyrococcus furiosus* (A) | 70–103 (100) | Amylolytic, saccharolytic | Sugars, starch, tryptone, peptides | Acetate, $CO_2$ | (Lipscomb et al., 2023) |
| *Thermoanaerobacter kivui* (B) | 50–72 (66) | Autotrophic, saccharolytic | $CO_2/H_2$, CO, formate, glucose, mannose, fructose, pyruvate | $H_2$, acetate, formate | (Regis et al., 2024) |
| *Thermoanaerobacter italicus* (B) | 45–80 (70) | Amylolytic, hemicellulolytic, saccharolytic | Various hexoses and pentoses, xylan, starch, glycogen, pectin, pectate | Ethanol, lactate, acetate, succinate | (Andersen et al., 2015) |
| *Thermoanaerobacterium aotearoense* SCUT27 (B) | 35–70 (55) | Hemicellulolytic, saccharolytic | Various hexoses and pentoses, cellobiose, xylan, dextran | $H_2$, lactate, acetate | (Yang et al., 2013) |
| *Thermoanaerobacterium saccharolyticum* (B) | 45–70 (55) | Hemicellulolytic, saccharolytic, | Various hexoses and pentoses, xylan, cellobiose | Ethanol, acetate, lactate, $CO_2$ and $H_2$ | (Herring et al., 2016) |
| *Thermococcus kodakarensis* KOD1 (A) | 60–100 (85) | Saccharolytic | α-, β- glucans, peptides, H2, pyruvate | Acetate, H2, alanine, mevalonate | (Scott et al., 2021) |
| *Thermococcus onnurineus* NA1 (A) | 63–90 (80) | Autotrophic, amylolytic | CO (e.g. steel mill off gas), starch, formate | H2 | (Lee et al., 2022) |
| *Thermotoga maritima*/RQ7/ *neapolitana* (B) | 55–90 (80) | Saccharolytic, amylolytic | Glucose, xylose, maltose, starch | Acetate, CO2, H2, lactate | (Nguyen et al., 2010) |

**Table 3**
Selected tools for metabolic engineering in thermophilic bacteria and archaea and application examples.

| Tool | Description | Application | References |
|---|---|---|---|
| **Selection marker** | | | |
| Kanamycin | Evolved thermostable (80 °C) variant of a kanamycin resistance marker (*knt*) originally found in *Staphylococcus aureus*, codon optimized for *C. bescii* (Cb*htk*) | Use of Cb*htk* to generate a Δ*pyrE* strain in *C. bescii* | (Lipscomb et al., 2016) |
| Erythromycin | Resistance to erythromycin by expressing the adenine methylase gene (*ermB*) of *Streptococcus faecalis* plasmid pAMβ1 | Knock-out of *ldh* in *T. saccharolyticum* | (Shaw et al., 2008) |
| Thiamphenicol | Resistance to thiamphenicol by expressing the *cat* gene in the vector | Deletion of *pta* in *A. thermocellus* by selection of *cat* integration in the genome | (Argyros et al., 2011) |
| Simvastatin/mevinolin | Overexpression of *hmgA* (3-hydroxy-3-methylglutaryl coenzyme A reductase) gene in the donor DNA element confers resistance to simvastatin | Simvastatin selection in *P. furiosus* for Δ*pyrE* mutant generation | (Lipscomb et al., 2011) |
| Origin of replication | | | |
| pMU131 | Gram-positive thermophilic replicon from the native plasmid of *T. saccharolyticum* B6A-RI (pMU131) | Overexpression of *ech2C* in *T. kivui* to study the *in vivo* assembled Ech2 complex | (Katsyv and Müller, 2022) |
| pJGW37 | Gram-positive thermophilic replicon from the native plasmid of *C. bescii* (pBAS2) | Construction of a shuttle vector for *A. thermocellus* that replicates at 60 °C in multiple copies | (Groom et al., 2016) |
| pMU102 | Gram-positive thermophilic replicon derived from pNW33N | Development of a heterologous 2-step CRISPR/Cas genome editing tool in *A. thermocellus* | (Walker et al., 2020) |
| **Transformation** | | | |
| Natural competence | Development of a transformation protocol for *Thermotoga* spp. | Transformation of *Thermotoga* sp. RQ7 with shuttle vector | (Han et al., 2014) |
| Electroporation | Transforming a plasmid that contains a resistance marker between the homology arms of the *pta-ack* genes to disrupt the acetate formation pathway | Production of L-lactic acid in *T. aotearoense* SCUT 27 from xylan or glucose | (Yang et al., 2013) |
| Overcoming the restriction modification barrier | Methylation of donor DNA in *E. coli* with an endogenous α-class N4-Cytosine methyltransferase (M. CbeI) is required for transformation of *C. bescii* | Development of a uracil auxotrophic strain by methylating the donor DNA with CbeI by deleting the *pyrECB* genes | (Chung et al., 2012) |
| Liposome-mediated transformation | Transformation of pRQ7 native plasmid in *T. neapolitana* and *T. maritima* by converting the cells into spheroplasts prior to transformation | | (Yu et al., 2001) |
| **Gene expression/Reporter system** | | | |
| Inducible promoter | Anhydrotetracycline-inducible promoter based on the TetR repressor. | Dose-dependant mRuby2 fluorescence output at 52 °C in *P. thermoglucosidasius* | (Jensen et al., 2023) |
| pFAST fluorescent reporter | Thermostable fluorescent anaerobic reporter system developed for *T. kivui* | Determining promoter strength based on fluorescence | (Hocq et al., 2023) |
| β-galactosidase reporter | β-galactosidase from *Geobacillus stearothermophilus* (BgaB) produces distinct black colonies when S-gal is added to the cells in aerobic and anaerobic conditions | Determining promoter strength based on colorimetric assay | (Jensen et al., 2017) |
| **Genome editing based on auxotrophy** | | | |
| *pyrE/F* / 5′-FOA | Negative selection: *pyrE/F* deficient strains cannot grow without uracil and are resistant to 5′-FOA. Reintroduction of *pyrE/F* restores FOA sensitivity | Strategy for 2-step markerless genome editing applied in *T. kivui* for several gene knock-outs. | (Basen et al., 2017) |
| *tdk* / FUDR | Negative selection: *tdk* (thymidine kinase) deficient strains are resistant to FUDR (5′-fluoro-2′-deoxyuridine). Reintegration of *tdk* in the donor DNA restores FUDR sensitivity | Identification of the genes responsible for ethanol production in *T. saccharolyticum* by multiple gene deletions with the tdk genome editing tool | (Shao et al., 2016) |
| *hpt* / AZH | Negative selection: *hpt* (hypoxanthine phosphoribosyltransferase) deficient strains are resistant to 8-aza-2,6-hypoxanthine (AZH). Reintroduction of *hpt* restores AZH sensitivity | Deletion of *ldh* and *pta* genes in *A. thermocellus hpt*-deficient strain for ethanol production and reduction of by-product formation. | (Argyros et al., 2011) |
| *trpE* / 6-MP | Negative selection: *trpE* deficient strains cannot grow without tryptophan and are resistant to 6′-MP (6′-methylpurine). Reintroduction of *trpE* restores 6′-MP sensitivity | Deletion of cytosolic hydrogenase (TK2069-72) in *T. kodakarensis* prevents $H_2$ consumption and uncouples $H_2$ production from growth | (Santangelo et al., 2011) |
| **CRISPR-based genome editing – Large-scale genome editing** | | | |
| CRISPR/Cas9 | Overexpression of SpCas9 under an inducible promoter in *B. smithii*. Activation of SpCas9 takes place at 37 °C, while homologous recombination occurs at 45–55 °C | Deletion of *pyrF,* integration of *ldh* in *B. smithii* using SpCas9 | (Mougiakos et al., 2017) |
| CRISPR-IB | Plasmid-borne expression of guide RNA compatible with endogenous Type I-B system. Use of *tdk* as a negative selection | Deletion of *ldh* and *argR* increases ethanol production in *T. aotearoense* SCUT27 | (Dai et al., 2022) |
| CRISPR-IB | A combination of a target mutation with silent mutations on the spacer allows for single base pair mutagenesis. Homology arms with target mutation are introduced first, followed by sgRNA | A single mutation at DNA polymerase III of *A. thermocellus* created a hypermutator phenotype, which can reduce the time needed for ALE | (Lanahan et al., 2022) |
| CRISPR/Cas9 | Heterologous expression of GeoCas9 with guide RNA and exo/Beta recombineering machinery from *Acidithiobacillus caldus* | Introducing a nonsense mutation to *pyrF* by CRISPR Type-II in *A. thermocellus* with an editing efficiency of 94 % | (Walker et al., 2020) |
| BAC | Cloning of 16.9 kb into a bacterial artificial chromosome | The 18-gene cluster of formate hydrogen lyase from *T. onnurineus* was integrated in *P. furiosus* for formate utilization at 95 °C. | (Lipscomb et al., 2014) |

resistance gene (Waege et al., 2010).

Current genome editing tools are for the most part based on selective/counter-selective nutritional markers (Basen et al., 2017; Straub et al., 2018; Zeldes et al., 2015) (Table 3). The most frequently used genome editing technique is based on pyrimidine metabolic marker genes (e.g., *pyrE*, *pyrF*), and homologous recombination, similar to what was first described in the fungus *Histoplasma capsulatum* (Krooth et al., 1979; Worsham and Goldman, 1988). Deletion of such genes results in a dual phenotype, i.e., auxotrophy for uracil and resistance to the toxic analog 5′-fluoroorotic acid (5-FOA). Further genome editing typically involves a two-step process during which a single crossover is selected for by prototrophy for uracil, and a double crossover via 5-FOA resistance (Krooth et al., 1979). With this method, markerless mutant strains have been created in thermophilic bacteria and archaea (Straub et al.,

2018; Zeldes et al., 2015). In hosts naturally auxotrophic for uracil (i.e., *Thermoanaerobacter ethanolicus*), the use of other auxotrophic markers has been explored, such as thymidine kinase (Shao et al., 2016).

Thermophilic CRISPR/Cas systems have more recently been developed for genome editing, which can be based either on a native or on a heterologous CRISPR machinery (Le and Sun, 2022). In the latter case, a heterologous thermostable Cas nuclease needs to be expressed in a replicative vector along with a targeting guide RNA and a homologous recombination template. GeoCas9 or CaldoCas9 mediated genome editing was successful in *A. thermocellus*, *Bacillus smithii*, *T. ethanolicus, Parageobacillus thermoglucosidasius* and *Thermus thermophilus,* with efficiencies reaching almost 100 % (Le and Sun, 2022). Endogenous CRISPR type I-B systems have also been successfully implemented in *A. thermocellus, Thermoanaerobacterium aotearoense* SCUT27 and *P. thermoglucosidasius* (Dai et al., 2022; Walker et al., 2020; Yang et al., 2023). Endogenous systems are particularly interesting, as they take advantage of the CRISPR machinery of the host, alleviating the thermostability and toxicity challenges typically faced with heterologous expression systems, while simultaneously increasing the cargo capacity of the editing vector.

Compared to auxotrophy-based genome editing, CRISPR-based tools are more straightforward and less laborious, as double crossover recombination events are immediately selected. On the other hand, the prevalence of off-targets as well as the thermostability of Cas proteins can all limit the applicability of CRISPR systems in thermophilic hosts (Le and Sun, 2022).

Rational genome editing is often coupled with random approaches such as adaptive laboratory evolution (ALE, (Dragosits and Mattanovich, 2013) to improve the overall phenotype or physiology of a strain. As in most cases ALE takes a long time, having a hypermutator phenotype, such as the one created in *A. thermocellus* can speed up the process (Lanahan et al., 2022). For most products of interest, anaerobic production is growth-coupled, so that higher performances typically result from selecting strains with higher growth rates (Herring et al., 2016; Holwerda et al., 2020; Svetlitchnyi et al., 2022). Similarly, tolerance for high product and/or feedstock concentrations can be obtained using long-term cultivation strategies (Herring et al., 2016; Holwerda et al., 2020; Svetlitchnyi et al., 2022). In turn, genome sequencing can reveal insights into the mutations generating the desired phenotype, pinpointing the underlying mechanisms and enabling their transfer to other strains (Tian et al., 2019).

## 4. (Hyper)thermophilic bioprocessing scenarios

To achieve a circular carbon bioeconomy, the ability to efficiently utilize diverse waste compounds/gases, occurring from industry, forestry and agriculture and convert them to value-added chemicals or gases will be pivotal. Hereafter, selected scenarios where a biocatalyst is converting carbon compounds to products of interest are described (Fig. 1). A few "model" thermoanaerobes chosen from Table 2 are discussed to illustrate these bioprocessing scenarios. Table 4 recapitulates the advances made for those selected microbes. Successful industrialization of a thermophilic conversion unit mainly depends on a few critical parameters. In particular, sufficient process metrics (T-R-Y, titer: ≥50 g/L, rate: ≥3 g/Lh$^{-1}$ and yield: ≥80 %) are required to achieve economic feasibility, ideally in a continuous bioproduction system (Van Dien, 2013; Vees et al., 2020).

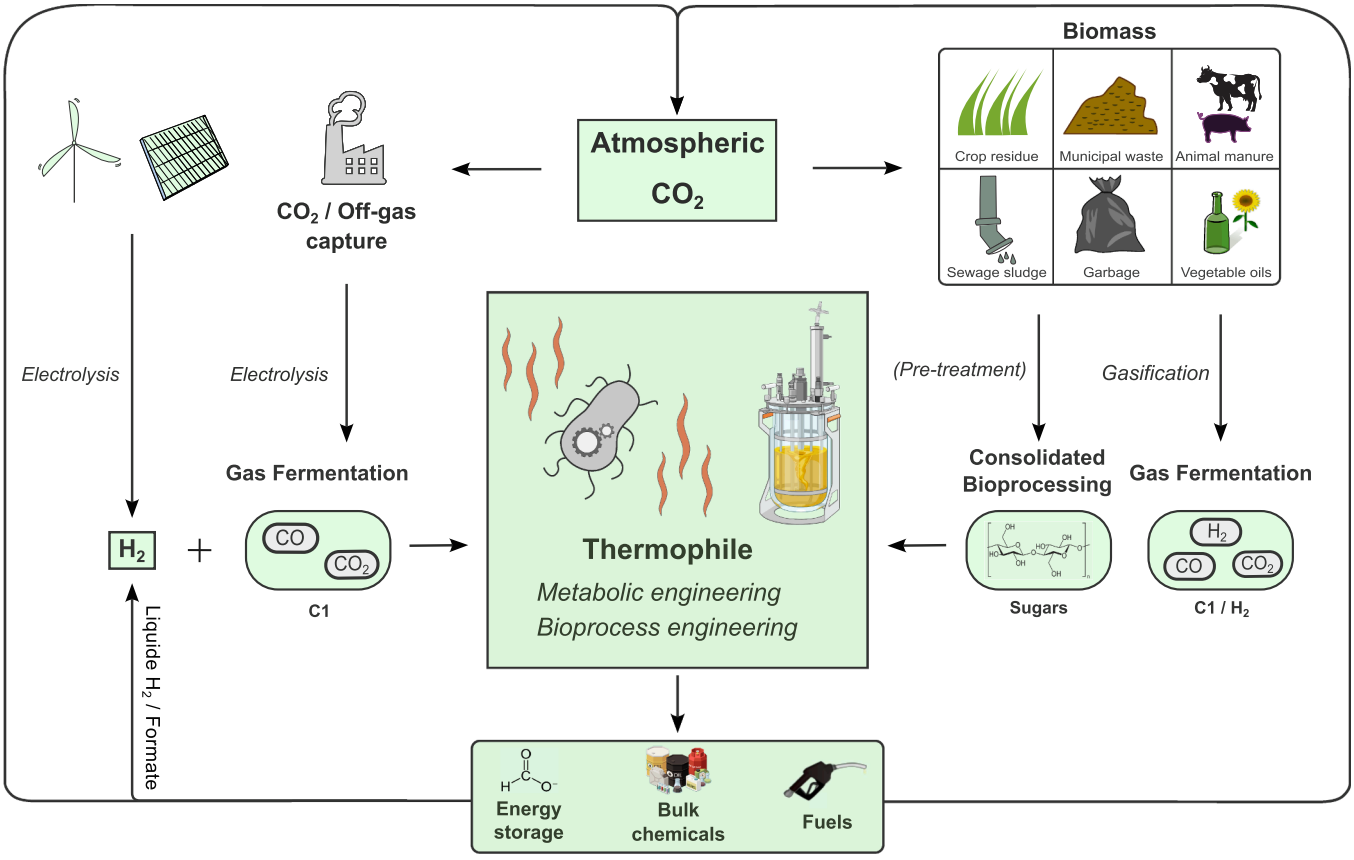


**Fig. 1.** Thermophilic bioprocessing in the circular carbon economy. Feedstocks are replenishable and recycled: $CO_2$ from direct air capture, biomass or renewable electricity. Feedstock processing includes water/$CO_2$ electrolysis, biomass gasification or hydrolysis. The generated feedstocks can be gases ($CO_2$, CO, $H_2$) or sugars (from biomass) which are used by a microbial catalyst in a selected bioprocess (gas fermentation or consolidated bioprocessing) for the generation of bioproducts (chemicals and fuels) of interest. Content from Biorender.com is included in the figure.

**Table 4**
Examples illustrate recent advancements made in thermophilic bioprocessing. Table is organized alphabetically by the product name in each category, and then by titer (for liquid products) and rate (for $H_2$).

| Organism | Feedstock | Product | Metabolic engineering | Bioprocessing | Titer | Rate | Yield | Reference |
|---|---|---|---|---|---|---|---|---|
| **Biomass saccharification and consolidated bioprocessing** | | | | | | | | |
| *Thermoanaerobacterium saccharolyticum* | Cellulose (Sigmacell-20, 100 g/L), acetate (10 g/L), xylose (35 g/L), glucose (20 g/L) | Ethanol, acetate | Δ*pta*, Δ*ack*, Δ*ldh*, Δ*tsac_0795*, Ct_*ureABCDEFG*, *metE*, ΔEPSoperon, ALE | SSCF, 1 L, 60 h, 55 °C | 61.4 g/L | 0.66 g/Lh$^{-1}$ | 0.48 g/g | (Herring et al., 2016) |
| *Thermoanaerobacter italicus* | Wheat straw (121 g/L sugar eq.), YE, CSL, raw glycerol (3 g L$^{-1}$) | Ethanol | Δ*pta* Δ*ack* Δ*ldh* | Continuous, 0.5 L, 60 days, 66 °C | 58.0 g/L | 1.5 g/Lh$^{-1}$ | 0.48 g/g | (Andersen et al., 2015) |
| *Thermoanaerobacterium saccharolyticum* | Pre-treated hardwood hydrolysate (118.1 g/L sugar eq.), YE | Ethanol, acetate | Δ*pta*, Δ*ack*, Δ*ldh*, Δ*tsac_0795*, Ct_*ureABCDEFG*, *metE*, ΔEPSoperon, Δ*perR*, *mgs*::*pta/ack*-KanR | SHF, 1 L, 60 h, 55 °C | 49.5 g/L | 0.83 g/Lh$^{-1}$ | 0.49 g/g | (Herring et al., 2016) |
| *Acetovibrio thermocellus* /*Thermoanaerobacterium saccharolyticum* | Cellulose (Avicel, 92.2 g/L), YE | Ethanol, acetate | *A.thermocellus* Δ*hpt*, Δ*pta*, Δ*ldh* / *T. saccharolyticum* Δ*pta* − *ack*, Δ*ldh* | Batch, 146 h, 55 °C | 38.1 g/L | 0.26 g/Lh$^{-1}$ | 0.41 g/g | (Argyros et al., 2011) |
| *Thermoanaerobacterium saccharolyticum* | Pre-treated hardwood (cellulose: 64.5 g/L, glucose: 1.2 g/L, xylose: 16.6 g/L), hemicellulose extract, YE | Ethanol, acetate | Δ*pta*, Δ*ack*, Δ*ldh*, Δ*tsac_0795*, Ct_*ureABCDEFG*, *metE*, ΔEPSoperon, Δ*perR*, *mgs*::*pta/ack*-KanR | SSCF, 100 L, 60 h, 55 °C | 30.8 g/L | 0.51 g/Lh$^{-1}$ | 0.44 g/g | (Herring et al., 2016) |
| *Acetovibrio thermocellus* | Cellulose (120 g/L) | Ethanol, isobutanol | Δ*hpt* Δ*ldh* Δ*pta*:: P*gapD–cat–hpt*, *AdhE*$^{D4949G}$, adapted by pH auxostat | Batch, 0.5 L, 55 °C | 29.9 g/L | 0.14 g/Lh$^{-1}$ | 0.29 g/g | (Holwerda et al., 2020) |
| *Thermoanaerobacterium aotearoense* SCUT27 | Wheat straw (17 g/L xylose eq.) | Ethanol, acetate | Δ*tdk*, Δ*ldh*, Δ*argR* | Batch, 36 h, 55 °C | 10.5 g/L | 0.29 g/Lh$^{-1}$ | 0.67 g/g | (Dai et al., 2022) |
| *Caldicellulosiruptor bescii* | Cellulose (Avicel, 20 g/L) | Ethanol, acetoin, acetate | Δ*pyrE*, Δ*ldh*, P*slp* Cthe-*adhE* PBF-hydrogenase *rnfCDGEAB* | Batch, pH-stat, 200 h, 60 °C | 3.5 g/L | 17.5 mg/Lh$^{-1}$ | 0.30 g/g | (Williams-Rhaesa et al., 2018) |
| *Moorella thermoacetica* | Rice straw hydrolysate (9.9 g/L sugars eq.), YE | Ethanol | Mt-Δ*pduL*1Δ*pduL2*::*aldh* | Batch, 125 ml, 168 h, 55 °C | 1.25 g/L | 7 mg L/h | 0.40 g/g | (Rahayu et al., 2020) |
| *Thermotoga maritima* | Pre-treated date waste juice (60 mmol/L hexose eq.) | $H_2$ | | Continuous, MBR, 2 L, 80 °C | | 70.2 mmol/Lh$^{-1}$ | 2.2 mol mol$^{-1}_{hexose}$ | (Saidi et al., 2022) |
| *Thermotoga maritima* | Untreated rice straw 1 % (w/v) | $H_2$ | | Batch, 50 ml, 5 days, 75 °C | | 0.16 mmol/Lh$^{-1}$ | 2.7 mmol g straw$^{-1}$ | (Nguyen et al., 2010) |
| *Caldicellulosiruptor saccharolyticus* | LCB (switchgrass) without pre-treatment | $H_2$ | | Consolidated bioprocessing, 6 days, 65 °C | | 0.1 mmol/Lh$^{-1}$ | 11.2 mmol/g | (Talluri et al., 2013) |
| *Caldicellulosiruptor* sp. DIB 104C | Cellulose (Avicel, 200 g/L), YE | Lactate | ALE | Batch, 3 L, 1.3–1.5 bar, 70 °C | 70 g/L | 1 g/Lh$^{-1}$ | 96 % | (Svetlitchnyi et al., 2022) |
| **Conversion of one carbon feedstocks into liquid products** | | | | | | | | |
| *Thermoanaerobacter kivui* | $CO_2$, electricity | Acetate | | Microbial electrosynthesis, 65 °C | 23.4 g/L | 0.76 g/Lh$^{-1}$ | | (Deutzmann and Spormann, 2024) |
| *Thermoanaerobacter kivui* | $CO_2$, $H_2$ (3:1) | Acetate, formate | | CSTR, 8 bar, 1 L, 66 °C, R13 | 15.8 g/L | 1.1 g/Lh$^{-1}$ | 0.56 mol mol$^{-1}_{CO2}$ 0.20 mol mol$^{-1}_{H2}$ | (Regis et al., 2024) |
| *Moorella thermoacetica* | Syngas (CO:$H_2$, 1:1) | Isopropanol, acetate | pduL2::*sadh*, pduL2::IPA | Batch, 125 mL, 55 °C | 0.12 g/L | 0.40 mg/Lh$^{-1}$ | | (Kato et al., 2024) |
| **$H_2$ – formate interconversion** | | | | | | | | |
| *Thermoanaerobacter kivui* | $H_2$, $CO_2$/bicarbonate | Formate | | Batch, 66 °C | 2.3 g/L | 1.7 mmol/Lh$^{-1}$ | | (Schwarz and Müller, 2020) |
| *Thermococcus onnurineus* NA1 | Formate (10 g/L), YE, vitamins | $H_2$ | | Batch, pH-stat, 3 L, 18 h, 80 °C | | 2,820 mmol/Lh$^{-1}$ | 1 mol/mol, 100 % | (Lim et al., 2012) |
| *Thermoanaerobacter kivui* | Formate (600 mM) | $H_2$, acetate | | Batch, stirred-tank bioreactor, 60 °C | | 80 mmol/Lh$^{-1}$ | 0.70 mol/mol | (Burger et al., 2022) |
| *Pyrococcus furiosus* | Formate (50 mM), tryptone, vitamins | $H_2$ | Δ*pyrF* P*gdh_pyrF* P*mbh1 (TON1563-TON1580)* | Batch, 56 h, 80 °C | | 0.52 mmol/Lh$^{-1}$ | | (Lipscomb et al., 2014) |
| **$H_2$ production from CO** | | | | | | | | |

(*continued on next page*)

**Table 4** (*continued*)

| Organism | Feedstock | Product | Metabolic engineering | Bioprocessing | Titer | Rate | Yield | Reference |
|---|---|---|---|---|---|---|---|---|
| *Thermococcus onnurineus* NA1 | CO, YE | $H_2$ | | Continuous, 3 L, increasing pressure to 9 bars, 15 h, 80 °C | | 577 mmol/ $Lh^{-1}$ | 1.0 mol/ mol, 0.07 g/g | (Kim et al., 2020) |
| *Thermococcus onnurineus* NA1 | CO, YE, vitamins | $H_2$ | Strain 156 T: ALE to CO | CSTR, 1 L, 500 h, 80 °C | | 472 mmol/ $Lh^{-1}$ | | (Lee et al., 2022) |
| *Thermococcus onnurineus* NA1 | CO, YE, vitamins | $H_2$ | | CSTR bubble column, pH-stat, 7 bar, 14 L, 10 h, 80 °C | | 450 mmol/ $Lh^{-1}$ | | (Park et al., 2022) |
| *Carboxydothermus hydrogenoformans* | CO, YE, vitamins | $H_2$ | | Continuous, Hollow fiber MBR, 0.16 L, 126 days, 70 °C | | | 0.91 ± 0.03 mol/mol | (Zhao et al., 2013) |

### 4.1. Biomass saccharification and consolidated bioprocessing

For LCB valorization, thermophilic (hemi-)cellulolytic strains have arguably the most to offer in a CBP scenario. Indeed, LCB conversion involves several critical steps, and their combination in a minimal number of unit operations should be energetically and economically favorable, and ideally should accommodate different LCB feedstocks. Temperature ranges compatible with thermophilic growth facilitate LCB liquefaction and simultaneous conversion. With that in mind, a few promising bioprocesses featuring cellulolytic and hemi-cellulolytic thermophiles have already been successfully pioneered (Lynd et al., 2022).

Maximizing fermentative performances via strain engineering has already proven efficient, both with targeted and random genetic engineering approaches. Rational metabolic engineering has notably been undertaken as an initial approach to increase product specificity and yield by knocking out competing metabolic pathways in processes targeting ethanol, lactate and $H_2$ production from LCB in various thermophiles (Table 4) (Andersen et al., 2015; Cha et al., 2013; Herring et al., 2016; Holwerda et al., 2020; Rahayu et al., 2017; Williams-Rhaesa et al., 2018). Moreover, ALE has efficiently been used to complement rational metabolic engineering, thereby significantly boosting strain performance (feedstock/product conversion and tolerance) (Herring et al., 2016; Holwerda et al., 2020; Svetlitchnyi et al., 2022). Coupled with the development of bioprocesses, target values for T-R-Y could be reached for LCB to ethanol and lactic acid processes starring selected thermoanaerobes (Andersen et al., 2015; Herring et al., 2016; Holwerda et al., 2020; Svetlitchnyi et al., 2022).

In *T. saccharolyticum*, multiple targeted gene modifications aiming at improving ethanol production were completed by several rounds of ALE to overcome substrate toxicity and maximize growth rate, yielding a strain able to produce 31 g/L ethanol (>90 % maximum theoretical yield, 1 g/$Lh^{-1}$) from pre-treated hardwood in a SSF bioprocess (Herring et al., 2016). A similar approach in various engineered *A. thermocellus* strains improved overall cellulolytic and ethanologenic properties with up to 29.9 g/L ethanol produced from high-loadings of cellulose (>100 g/L glucose equivalent) (Holwerda et al., 2020).

ALE is interestingly not limited to laboratory strains or genetically tractable microbes but has also been readily applied to strains isolated directly from nature. The cellulolytic *Caldicellulosiruptor* sp. DIB 104C was, for instance, evolved for increased lactic acid tolerance and production capability, with T-R-Y reaching 70 g/L, 1 g/$Lh^{-1}$ and 85 % of maximum yield, respectively, from cellulose in a consolidated bioprocessing scenario (Svetlitchnyi et al., 2022).

Continuous bioprocesses are interesting for the production of low-value products, as these typically increase operating time while limiting cleaning and sterilization costs. Using a genetically engineered *Thermoanaerobacter italicus* ethanol-producing strain (devoid of lactic acid and acetate production pathways), Andersen and coworkers established a flexible continuous process capable of accommodating various LCB feedstocks (Andersen et al., 2015). In this configuration, fermentation could be run for up to 60 days, with T-R-Y reaching as high as 58 g/L, 1.5 g/$Lh^{-1}$ and 0.48 g/g, respectively, illustrating the potential of thermoanaerobes for LCB conversion into ethanol. Recovery of ethanol as a volatile product (boiling point of 78 °C) is largely facilitated via gas stripping into the off-gas (≈50 % of the product). Such a strategy could be further advanced by using fermentation temperatures exceeding the ethanol boiling point. In the archeon *Pyrococcus furiosus*, the proof-of-concept of ethanol production at 95 °C was shown from maltose and CO, in a so-called "bioreactive distillation" process, in which ethanol production and distillation are combined in a single unit operation (Lipscomb et al., 2023). Although the ethanol titer was relatively low (0.55 g/L), the approach is promising and could render an LCB-to-ethanol (or another alcohol/ketone) conversion process highly efficient.

Finally, process engineering has also been applied to alleviate growth inhibition from feedstocks and products. Indeed, LCB pretreatment can yield considerable amounts of inhibitory compounds, which can be removed with activated carbon, lime treatment and nanofiltration before fermentation, increasing fermentation performance (Herring et al., 2016; Lee et al., 2011). For LCB-to-$H_2$ conversion, *Caldicellulosiruptor saccharolyticus* ferments switchgrass to $H_2$ with T-R-Y reaching 14.3 mmol/L, 0.1 mmol/$Lh^{-1}$ and 11.2 mmol/g, which can be further improved by 13 % in yield and 18 % in productivity, when using chemically defined medium (Talluri et al., 2013; Willquist and Van Niel, 2012). While *C. saccharolyticus* can ferment high amounts of LCB (up to 30 g/L) without detrimental effects on productivity, product inhibition is observed in $H_2$ concentrations above 2.2 mmol/L, which can be alleviated by increasing mass transfer and gas stripping (Ljunggren et al., 2011).

### 4.2. Upgrading of gaseous one carbon feedstocks and $H_2$

#### 4.2.1. Conversion of one carbon feedstocks into liquid products

Research has for the most part been focused on using *Moorella thermoacetica* and *T. kivui*, two thermophilic acetogens, which ferment $H_2$ and $CO_2$ to acetate, a relatively low-value chemical (Deutzmann and Spormann, 2024; Regis et al., 2024). Both acetogens utilize formate, with *T. kivui* being able to grow with no media additives, while *M. thermoacetica* can additionally utilize methanol.

Although both acetogens are genetically tractable, acetogenic metabolism from gas yields very low amounts of ATP per acetate (Schuchmann and Müller, 2014), which in turn significantly hampers efforts to redirect the carbon flow towards more ATP-intensive products. Using energetically dense C1 feedstocks is a common approach that can circumvent this issue. CO in particular has a much higher ATP yield than $H_2$ and $CO_2$ and has been used to enable ethanol, acetone and isopropanol production from gas (Kato et al., 2024, 2021; Takemura et al., 2021). Methanol is another energetically favorable C1 feedstock that could be used in a similar fashion (Kremp and Müller, 2021), but it has

yet to be attempted in acetogenic thermophiles.

Nevertheless, metabolic engineering efforts have successfully diverted the product pattern to higher-value chemicals, such as lactate (Iwasaki et al., 2017), ethanol (Takemura et al., 2021, *T. kivui*: personal communication, 2023), acetone (Kato et al., 2021; Takemura et al., 2023), and isopropanol (Kato et al., 2024), albeit at relatively low titers from gas, autotrophic growth from $H_2$ and $CO_2$ being even abolished in some cases.

#### 4.2.2. $H_2$ – Formate interconversion

Although a promising energy source, $H_2$ is difficult to store and deliver safely, a potentially significant drawback that could be alleviated by transiently converting $H_2$ to liquid energy carriers, such as formate (Enthaler et al., 2010). Thermophilic biotechnological approaches to tackle gas interconversion to liquids have mainly focused on formate-to-$H_2$ potential processes. *Thermococcus onnurineus* naturally converts formate to $H_2$ (Lim et al., 2012). The reaction is thermodynamically favored at high temperatures (in this case, 80 °C), and a maximum hydrogen evolution rate (HER) of ≈ 0.3 mol $L^{-1}h^{-1}$ could be achieved in a pH-stat continuous process with a wild-type strain of *T. onnurineus* (Lim et al., 2012), showcasing the potential of this species for $H_2$ formation from formate. Alternatively, *Thermoanaerobacter kivui*, a thermophilic acetogen, expresses a soluble $H_2$-dependent $CO_2$ reductase (HDCR), which catalyzes the reversible conversion of $H_2$ and $CO_2$ to formate with high turnover frequencies (TOF) at mild conditions (~9.5 and 9.8 million $h^{-1}$ for formate and $H_2$ formation, respectively) (Schwarz et al., 2018). These values are considerably higher compared to TOFs for chemical catalysis generating formate (3,400–150,000 $h^{-1}$) requiring harsh conditions (high temperature and/or pressure) (Beller and Bornscheuer, 2014). Wild-type *T. kivui* was further used as a whole-cell catalyst in both a formate-to-$H_2$ and an $H_2$-to-formate scenario, with a HER and formate production rate of up to ≈ 1 mol $L^{-1}h^{-1}$ and 270 mmol/$Lh^{-1}$, respectively (Burger et al., 2022; Schwarz and Müller, 2020).

Another interesting application of gas-fermenting thermophiles could be electricity production. *Aquifex aeolicus*, a hyperthermophilic bacterium, can generate $H_2O$ by $H_2$ and $O_2$ oxidation, the so-called "Knallgas" reaction. *A. aeolicus* uses a thermophilic hydrogenase which can be used in a microbial fuel cell without being inhibited by $O_2$, unlike platinum catalysts. Recent progress on the immobilization of the hydrogenase from *A. aeolicus* on an electrode has enabled the development of an $H_2/O_2$ enzymatic fuel cell, that powered a wireless device for 7 h (Monsalve et al., 2015). In theory, this system could be coupled to the HDCR of *T. kivui* to generate electricity from $H_2$ stored in liquid form as formate.

#### 4.2.3. $H_2$ production from CO

The conversion of CO, a major component of syngas and industrial off-gases, into $H_2$, has been pioneered in *T. onnurineus*. In this scenario, strain engineering via ALE has proven to be particularly efficient, yielding an evolved thermophile eventually shown to display high HER (up to ≈ 0.47 mol $L^{-1}h^{-1}$) over extended periods (500 h) in a stirred reactor (Lee et al., 2016, 2022).

Jeong *et al.* identified the optimum dissolved CO concentration ($C_L$) and the maximum specific CO consumption rate ($q_{CO}^{max}$) in *T. onnurineus*, which combined with kinetic modeling demonstrated that high kLa is needed to efficiently operate the CO-to-$H_2$ conversion in *T. onnurineus*, while maintaining high cell densities in the reactor (Jeong et al., 2016). Stirring a reactor is the simplest way to improve gas–liquid mass transfer (kLa) — which, as mentioned in chapter 2.2.3, is frequently the main limiting factor in gas fermentation. For large-scale production, the high volumetric power input of stirred tank reactors is cost-prohibitive. In turn, this significantly limits the industrial applicability of a CO-to-$H_2$ bioprocess.

To address this challenge, a bubble column reactor with increased pressure has been used which resulted in a HER (≈ 0.45 mol $L^{-1}h^{-1}$) comparable to (Park et al., 2022), or even higher (≈ 0.58 mol $L^{-1}h^{-1}$), than that of a stirred tank bioreactor (Kim et al., 2020). However, pressurizing the reactor vessel again increases the power input.

Another approach to increase mass transfer is the use of additives supplied to the cultivation medium. For example, $H_2$ production from CO with *T. onnurineus* was significantly enhanced (+61 %) by adding a chitosan/oleamide nanofluid to the medium (Kang et al., 2022). The nanofluid produces suspended nanoparticles, which are proposed to enhance mass transfer in multiple ways, i.e., by increasing the volumetric mass transfer coefficient, decreasing surface tension and potentially by directly increasing mass transfer at the cell membrane. However, using such medium additives could also increase costs and may prove problematic in downstream processing.

## 5. Perspectives

Most implemented industrial applications related to the proposed scenarios have been showcased in mesophilic microbial hosts (Lynd et al., 2017; Vees et al., 2020). Nevertheless, industrial feasibility and economic advantages of thermophilic bioprocessing have recently been described in a techno-economic analysis, where the conversion of LCB into acetone and $H_2$ was used as a consolidated bioprocessing case study with *C. bescii* (Bing et al., 2022).

Despite their potential, very few thermophilic bioprocesses are heading towards commercialization today: LCB to ethanol (*A. thermocellus*): Terragia biofuels (https://www.terragia.com) (Lynd et al., 2022), sucrose from sugarcane to PLA (*Bacillus smithii*): Totalenergies Corbion (commercialized) (Jem and Tan, 2020; Mougiakos et al., 2017), C5 and C6 to ethanol (*Thermoanaerobacter italicus*): BioGasol (Andersen et al., 2015), LCB to lactic acid (*Caldicellulosiruptor* sp. DIB 104C): BluCon Biotech GmbH (Svetlitchnyi et al., 2022).

The performance metrics T-R-Y of a bioprocess, whether it is operated with a mesophilic or thermophilic host, ultimately dictates its commercial success. To meet these performance criteria, interweaving metabolic and bioprocess engineering is necessary in most cases. Despite significant progress, strain engineering of thermoanaerobes remains challenging as, relatively to classic mesophilic workhorses, their metabolism and physiology are less understood, and their genetic toolbox is far more constrained. For bioprocess engineering, consideration of inherent traits of thermoanaerobes (such as lower biomass yields compared to mesophiles) is crucial to establish efficient bioproduction routes (Gorter de Vries et al., 2024). In this context, a potential strategy is process intensification (for example, the application of cell retention systems) to boost substrate turnover rates and productivity. For 2G or 3G bioprocessing, meeting the T-R-Y criteria is additionally complexified by the nature of the feedstocks with, e.g., low solubilization rates in the case of LCB or low gas–liquid mass transfer for gaseous feedstocks (Prasad et al., 2022; Yasin et al., 2019). Since the envisioned products for thermophilic bioprocesses are typically of low economic value and therefore need to be produced at high volumes to achieve favorable process economics, specialized bioreactors with low operational expenditures need to be employed.

Transitioning away from fossil feedstocks is a complex, multilayered task, that should rely on complementary strategies efficiently exploiting local resources. Depending on the geographical location, industrial sites can establish bioprocesses for off-gas capture and conversion, whereas agricultural and rural sites could invest in biomass gasification or consolidated bioprocessing. With further advances, other food and feed-land-independent waste products (e.g., sewage sludge, plastics) could be used as feedstocks in a circular bioeconomic scenario (Haberzettl et al., 2021; Yan et al., 2021).

For each feedstock, suitable biocatalysts could be selected to tailor specific applications. For this, genome-scale metabolic models (Zhang et al., 2021), cell-free systems (Cui et al., 2020), and *in vitro* kinetic models (Loder et al., 2016) can aid in identifying the key metabolic engineering targets and optimal enzyme expression to minimize

metabolic burden. Furthermore, finding or designing thermostable variants of mesophilic enzymes, identifying proteins with unknown functions, and addressing the knowledge gap for ferredoxin-linked enzymes which play a crucial role in the central carbon and redox metabolism of many thermoanaerobes will be a crucial step toward the implementation of bioproduction pathways (Poudel et al., 2021; Rigoldi et al., 2018).

Finally, when the desired feedstock and product do not match the capabilities of one individual host, synthetic co-cultures could be designed to mix and match desirable traits, as showcased by *A. thermocellus* (cellulose degradation) and *T. saccharolyticum* or *Thermoanaerobacter* sp. X514 (ethanol production) (Argyros et al., 2011). Another example is CO sequestration demonstrated by a co-culture of *C. hydrogenoformans* and other carboxydotrophs and methanotrophs (Diender et al., 2018; Parshina et al., 2005). In this configuration, $H_2$ produced from CO by *C. hydrogenoformans* can be used as an electron donor by its co-culture partner, providing a way to circumvent problems related to CO-rich gases (as CO partially or fully inhibits the fermentative growth of many microbes). Besides higher substrate turnover rates through synergy, co-cultures bring many benefits, including cross-feeding of nutrients (Diender et al., 2021; McCarty and Ledesma-Amaro, 2019). The challenges that remain to be solved include controlling the composition especially when the bioprocess moves to a large-scale bioreactor, where monitoring the presence of each member of the co-culture can prove crucial (Bäumler et al., 2022).

## 6. Conclusion

Considering non-model microorganisms such as thermoanaerobes as platform hosts is of great interest in the context of resource-efficient bioproduction as bioprocesses operated at high temperatures offer advantages that can be beneficial for the overall process economics. This includes high substrate turnover rates which in turn allows for high productivity as well as low energy consumption for preparation (no sterilization) and operation (no/low cooling) of bioreactors at scale. Recent progress in the development of genetic tools, and advancements in the understanding of the physiology and metabolism of thermoanaerobes are now enabling the development of bioprocessing scenarios close to industrial reality.

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## CRediT authorship contribution statement

**Angeliki Sitara:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Rémi Hocq:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Josef Horvath:** Writing – original draft, Investigation. **Stefan Pflügl:** Writing – review & editing, Project administration, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rémi Hocq is affiliated with CIRCE Biotechnologie GmbH, a company developing industrial gas fermentation processes. The remaining authors declare no competing interest.

## Data availability

Data will be made available on request.

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