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Chapter 6

# Progress in Second Generation Ethanol Production with Thermophilic Bacteria

Sean Michael Scully and Johann Orlygsson

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**Abstract**

Thermophilic bacteria have gained increased attention as prospective organisms for bioethanol production from lignocellulosic biomass due to their broad substrate spectra including many of the hexoses pentoses, and disaccharides found in biomass and biomass hydrolysates, fast growth rates, and high tolerance for extreme cultivation conditions. Apart from optimizing the ethanol production by varying physiological parameters, genetic engineering methods have been applied. This review focuses upon those thermophilic anaerobes recognized as being highly ethanologenic, their metabolism, and the importance of various culture parameters affecting ethanol yields, such as the partial pressure of hydrogen, pH, substrate inhibition, and ethanol tolerance. Also, recent developments in evolutionary adaptation and genetic engineering of thermophilic anaerobes are addressed.

**Keywords:** thermophilic bacteria, biofuel, bioethanol, lignocellulosic biomass, bioprocessing, genetic engineering

## 1. Introduction

The production of sustainable biofuels have increased in recent years because of a driving need for highly renewable and environmentally friendly energy carriers with bioethanol, biobutanol, biomethane, and biohydrogen being the most widely investigated. In order to meet international obligations to address climatic and geopolitical issues, many governments have set production targets as a response to meet these mandates. Ethanol production has been the main aim of many authorities as a suitable biofuel, for instance, a target set by the EU necessitates that 20% of energy production must be from renewable sources and energy

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efficiency must increase by 20% while greenhouse gases must decrease by 20% by 2020 [1]. This has led to a dramatic increase in the production of bioethanol from 48 billion liters in 2007 to 2.6 billion liters in 2017 [2]. Both the United States and Brazil are by far the largest producers of bioethanol although the vast majority of ethanol produced is from first generation biomasses such as sucrose-rich sugarcane and easily fermentable starch-rich crops such as corn. However, there is a growing concern over the use of these feedstocks because they are food and feed related and thus in a direct competition with food production [3–5]. In addition, increased concern has been regarding the negative impact on agricultural areas used for the production of this biomass.

Production of bioethanol by second-generation non-food (lignocellulosic) biomass, such as agricultural residues, addresses some of the above mentioned environmental concerns although poses several challenges as a raw material for bioprocessing. Second generation biomass requires extensive and costly chemical or physical pretreatment in addition to enzymatic treatment processes which negatively impacts its industrial feasibility. Lignocellulosic biomass is often difficult to degrade due to the lignin sheath and the highly crystalline nature of cellulose [6]. In addition to cellulose, lignocellulose is also composed of lignin and hemicelluloses of which the latter contains a plethora of monosaccharides with various connectivities and varying degrees of branching. Therefore, processing lignocellulosic biomass and subsequent fermentation of the liberated sugars to ethanol has proven to be a major complication for large-scale production [3–5].

To address the challenges posed by lignocellulosic biomass, fermentative organisms that can meet these process needs will help improve the feasibility of bioethanol production from lignocellulosic biomass. At present, the majority of bioethanol is produced using well-established mesophilic organisms despite some of the inherent advantages to the use of thermophilic microorganisms such as higher operating temperatures and utilizing a non-glucose hexoses and pentoses such as xylose and arabinose. This work focuses on the physiology of ethanol-producing thermophiles with an emphasis on their salient features relevant to the utilization of lignocellulosic biomass as well as the use of genetic engineering to improve their potential for bioethanol utilization.

## 2. Selected aspects of ethanol production from lignocellulosic biomass

For the fermentative production of ethanol from biomass to be commercially successful, several key processes and organisms need to be considered [3, 4, 7–9]. These process requirements needed to simultaneously consider two viewpoints: the physiological properties of the ethanologen used and process requirements. Concerning organism requirements, an ideal strains should be homoethanologenic, with high productivity (> 1/g/L/h), have broad substrate spectra and high tolerance of ethanol, inhibitory compounds and high initial substrate concentrations. Other key factors include high cellulolytic activity, simple nutritional needs, low biomass production and ease of genetic manipulation. Ideally, a single organism that

is both highly ethanologenic and cellulolytic would be ideal for consolidated bioprocessing (CBP) although co-cultures of organisms together fulfilling these requirements may also be considered in a simultaneous saccharification and fermentation (SSF) setup. To adequately meet the process requirements, ethanol yields should reach a minimum of 90% of theoretical, achieve high ethanol titers (> 5% v/v), have a minimum number of process steps, and require minimal or no process cooling. Additionally, cells should be robust enough to be recyclable and substrates co-fermented and pretreatment should be limited or excluded.

No single wild type organism possesses all these features. Although genetic manipulation has yielded only modest improvements for ethanologens although transformants are not always stable [9–11]. Many thermophilic clostridia have much broader substrate spectrum as compared to standard ethanologens such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*. Additionally, cultivations of thermoanaerobes do not require extensive agitation or temperature control of the fermentation vessels and these often tolerate extremes of pH and salt concentrations during fermentation with minimal need for nutrient supplementation [4]. The mixing of reaction vessels and pumping of liquids are easier at elevated temperatures due to reduced viscosity as well increased substrate solubility [12].

## 3. Ethanol producing thermophilic anaerobes

While more than 300 species of thermophilic anaerobic bacteria have described as of 2008 from a wide range of environs with new species being continuously discovered. Thermophilic anaerobes have been isolated from a diverse range of environments [13] including deep-sea vents [14], geothermal areas [15–17], compost piles [18], municipal solid waste or sewage sludge [19], oil wells [20], and canned goods [21]. Most thermophilic microorganisms are either obligatory or facultative anaerobic, likely due to the limited availability of oxygen and highly reducing nature of geothermal features [22]. The majority of the those that are highly ethanologenic that have been described in the literature are often strict anaerobes within the genera of *Clostridium*, *Caloramator*, *Caldanaerobacter*, *Thermoanaerobacter*, or *Thermoanaerobacterium* [3, 23].

The highly polyphyletic genus *Clostridium* within the class *Clostridia* (family *Clostridiaceae*, order *Clostridiales*) currently has greater than 200 species with standing in nomenclature although only about 15 are strains within the genus are thermophilic [24, 25] usually with temperature optima for growth between 45 and 65°C although several strains reportedly grow at temperatures as high at 75°C. All species within the genus are strictly anaerobic and can typically be isolated from a broad range of nutrient-rich environments [26]. Many members within the genus can hydrolyze cellulose and produce ethanol, making them target of extensive research on biofuel production from complex [27, 28].

*C. thermocellum* is a thermophilic species that degrades crystalline cellulose using a cellulosome which is comprised of a complex arrangement of glycohyldrolases attached to a scaffold-like matrix [6]. Several other members of *Clostridium* have glycohydrolases including *C. acetobutylicum* [29, 30] and *C. cellulovorans* [31]. Ethanol yields by *Clostridium* species are often moderate and vary depending on environmental conditions with other organic acids,

including lactic acid, being common end-products. Examples of ethanol production from sugars by members of the genus include *Clostridium thermocellum* [32, 33] and *Clostridium* strain AK1 with 1.5 mol ethanol/mol glucose [34].

The genus *Thermoanaerobacterium* is comprised of thermophilic anaerobes which fall within Cluster V of *Clostridia* [35]. Currently, the genus currently consists of nine species and *T. thermosulfurigenes* is the genus type species [36]. Species within *Thermoanaerobacterium* are usually amylo- and xylanolytic with a $T_{opt}$ between 55 and 65°C and have been isolated from a diverse range of environments including geothermal features and from heat-treated canned foods [21, 37, 38]. They catabolize a broad range of hexoses, pentoses, and disaccharides to a mixture of ethanol, acetate, lactate, hydrogen, and $CO_2$. One challenge for these organisms is achieving good ethanol yields from high initial substrate concentrations which considerably lower ethanol yields. Examples of ethanol production from sugars by members of the genus include *Thermoanaerobacterium saccharolyticum* with 1.18 mol ethanol/mol glucose [37] and *Thermoanaerobacterium* strain AK17 with 1.50 and 1.33 mol ethanol/mol glucose and xylose, respectively [39].

*Thermoanaerobacter* species have similar physiological characteristics as *Thermoanaerobacterium* species; all species within the genus are highly saccharolytic and produce end-products which include ethanol, acetate, lactate, alanine, $CO_2$, and $H_2$. Nineteen species and five subspecies belong to the genus [24, 25]. The main difference between *Thermoanaerobacter* and *Thermoanaerobacterium*, is that the majority of *Thermoanaerobacter* species produce $H_2S$ from thiosulfate whereas *Thermoanaerobacterium* produces sulfur [37]. Additionally, the temperature optima for *Thermoanaerobacter* species (65–75°C) are higher as compared to *Thermoanaerobacterium* species (55–65°C). The type species, *Thermoanaerobacter ethanolicus* and several other species within the genus, have been extensively studied for ethanol production [40–43]. High ethanol yields have been observed by several members of the genus including *T. pseudoethanolicus*, *T. mathranii*, *T. pentosaceus*, *Thermoanaerobacter* strain AK5, and *Thermoanaerobacter* strain J1 [17, 38, 44–46]. The ethanol yields, however, vary extensively depending on culture conditions [17, 38]. Recently, *Thermoanaerobacter subterraneous* was moved to the genus *Caldanaerobacter* that currently comprises two species: *Caldanaerobacter subterraneous* (and its four subspecies) and *Caldanaerobacter uzonensis* [24, 25]. Other representative examples of thermophilic ethanologenic bacteria can be found within the genera of *Caldicellulosiruptor* [47], *Caloramator* [48], *Geobacillus* [49], *Caloramator boliviensis*, for example, produces 1.53 mol ethanol/mol xylose [50].

## 4. Culture parameters

Most saccharolytic thermophiles use the Embden-Meyerhof-Parnas (EMP) pathway [5, 51] but do not use pyruvate decarboxylase for converting pyruvate to acetaldehyde as do yeasts. The theoretical yields of ethanol from 1 mol of hexose and pentose are 2.0 and 1.66 mol, respectively [5]. There are several routes from pyruvate to other end-products than ethanol. The following equations show the most common end-products from glucose with anaerobic bacteria:

1. 1 Glucose → 2 Ethanol +2 $CO_2$
2. 1 Glucose → 2 Lactate
3. 1 Glucose → 2 Acetate +2 $CO_2$ + 4$H_2$
4. 1 Glucose → 1 Butyrate +2 $CO_2$ + 2 $H_2$
5. 1 Glucose +2 $NH_4^+$ → 2 Alanine

Butyrate is not a commonly observed end-product with thermophilic anaerobes and alanine is not commonly assayed. The distribution of end products are known to be influenced by various factors which can be of direct relevance for the production of ethanol; these conditions include the substrate types and concentrations, the partial pressure of hydrogen, pH, and temperature. Some of these factors are discussed in detail below.

### 4.1. Partial pressure of hydrogen

Early observations of the influence of hydrogen concentrations on the end-product formation of *Thermoanaerobacter ethanolicus* were reported in 1981 [15]. Higher partial pressure of hydrogen ($pH_2$) leads to increased ethanol production and less acetate production from glucose fermentations [15, 38, 46]. Strict anaerobes produce $H_2$ either via pyruvate ferredoxin oxidorecutase or NAD(P)-dependent oxidoreductase [52]. It has been well established that the high concentrations affects mesophilic bacteria more severely than thermophiles because the NADH ferredoxin oxidoreductase (FNOR) that converts NADH to $Fd_{red}$ is more strongly inhibited. The reduction potential is −400 mV for the $Fd_{red}/Fd_{ox}$ couple but −320 mV for the $NADH/NAD^+$ couple [52, 53]. Therefore, at low temperatures, elevated hydrogen concentrations inhibit $H_2$ evolution at much lower concentrations as compared to at high temperatures. Microorganisms respond to this by directing their reducing equivalents to other more favorable electron acceptors and consequently produce reduced products such as ethanol and lactate. In nature, hydrogen accumulation usually does not occur because of hydrogen-utilizing organism such as methanogens and sulfate-reducers, allowing for a complete catabolism of glucose to end-products. However, batch fermentation with monocultures allows hydrogen to accumulate leading to a change in end production profile in some *Thermoanaerobacter* species [15, 38, 41]. For instance, during degradation of glucose and xylose, the major end-product for *Thermoanaerobacter brockii* was ethanol [54]. Under hydrogen scavenging conditions, however, the flow of electrons from glucose degradation was directed away from ethanol but towards acetate with extra ATP produced. Several experiments using different liquid-to-gas ratios have revealed that changes in end-product formation occur during hydrogen accumulation among species of *Clostridium, Thermoanaerobacter,* and *Caloramator*. Hydrogen accumulation in these cultures can either change the carbon flow to more reduced end-products or inhibit substrate degradation. The inhibition observed can be either direct, inhibiting the hydrogenases, or indirect by productions of acids, lowering the pH in a closed system, and thus stopping further degradation of the substrates.

### 4.2. Substrate loadings

In natural environments of thermophilic bacteria, the concentration of sugars is relatively low. It is thus not surprising that most thermophilic bacteria are inhibited at relatively low (often between 10 and 30 mM) initial substrate concentrations as compared to yeasts and *Z. mobilis* [4, 38, 39, 46]. This inhibition may be caused by accumulated hydrogen or by acid accumulation and pH drop, or it could also be an intriguing factor for thermophiles. *Thermoanaerobacter*, strain J1, has been shown to be very tolerant towards high sugar concentrations [17]. This high ethanol producing thermophile produces up to 1.7 mol ethanol/mol glucose at 100 mM initial glucose concentration. Recent work on *Thermoanaerobacter pentosaceus* showed a complete removal of xylose at 13.3 mM initial concentrations but only about 30% removal at 10 times higher concentrations [55]. Additionally, the ratio of ethanol to acetate and lactate decreased by a factor of more than six resulting in dramatic decrease in ethanol yields.

### 4.3. Ethanol tolerance

One of the most important traits for good ethanol producers is their ethanol tolerance. For an economic ethanol recovery to occur, using classical downstream processes, the microorganism should grow and produce ethanol in the presence of at least 4% (v/v) ethanol [56]. It is well known that growth rates of many organisms decrease markedly with increasing ethanol concentrations because of leaky membranes resulting in loss of energy during cellular metabolism and finally cell lysis. Yeasts and *Z. mobilis* tolerate much higher ethanol concentrations as compared to thermophiles mainly due to their composition of fatty acid in their cell membrane.

Studies on ethanol tolerance of wild-type species of thermophiles show tolerance between 0.5 and 3.0% (v/v) [4, 46, 57, 58]. Substantial efforts to increase ethanol tolerance of wild type thermophiles, have been done. The highest ethanol tolerance observed for a thermophile has been with a mutant strain of *Thermoanaerobacter ethanolicus* (12.7% v/v) [28]. However, later studies with one of its mutant derivatives, JW200 Fe 4, showed much less tolerance [59]. *Thermoanaerobacter* BG1L1 showed 8.3% (v/v) tolerance in continuous culture studies [43] on xylose. Increased ethanol tolerance was also observed with *Thermoanaerobacter thermohydrosulfuricus* 39E by successively sub-culturing the strain to higher ethanol concentrations [57]. The resulting mutant strain 39EA tolerated 10.1% (v/v ethanol) at 45°C but only 2.6% (v/v) at 68°C. Additionally, the ethanol yields dropped considerably.

### 4.4. Other culture parameters

Other environmental factors of importance for thermophilic bacteria is their pH and temperature growth optimum, their tolerance towards inhibitory compounds like furfuraldehyde and 5-hydroxymethyl-furfuraldehyde (5-HMF) and their need for trace elements and vitamins often originating from complex medium supplements like yeast extract.

## 5. Production of ethanol from lignocellulose

Production of bioethanol from lignocellulosic biomass by wild type thermophilic bacteria has been widely reported in the literature where the focus has been mostly on *Clostridium*

*thermocellum* and species within the genera *Thermoanaerobacterium* and *Thermoanaerobacter*. However, there is a large variation in the type and concentration of biomass used, fermentation processes (batch, semi-batch, continuous), pretreatment methods as well as whether pure or mixed cultures are used.

The theoretical maximum yield of ethanol obtained from glucose fermentation is 0.51 g ethanol/ g glucose (2 mol ethanol/mol glucose or 11.1 mM/g). Unsurprisingly, considering the complex structure of lignocellulosic biomass, ethanol yields are usually considerably lower from such substrates as seen in **Table 1**.

| Organisms | Substrate | Fermentation mode | Pre-treatment | Ethanol yields (mM/g) | Temp (°C) | References |
|---|---|---|---|---|---|---|
| *Clostridium thermocellum* | Avicel (2.5 g/L) | Batch | A | 5.00 | 60 | [60] |
| *Clostridium thermocellum* | Whatman paper (8.0 g/L) | Batch | None | 7.20–8.00 | 60 | [61] |
| *Clostridium thermocellum* | Paddy straw (8.0 g/L) | Batch | None | 6.10–8.00 | 60 | [61] |
| *Clostridium* strain DBT-IOC-C19 | Avicel (10.0 g/L) | Batch | None | 3.26 | 60 | [62] |
| *Clostridium* strain AK1 | Hemp (5.0 g/L) | Batch | A/Alk | 3.5 | 50 | [34] |
| *Thermoanaerobacter pentosaceus* | Rapeseed straw (5 0 g/L) | Con | Alk | 1.40 | 70 | [55] |
| *Thermoanaerobacter mathranii* | Wheat straw (6.7 g/L) | Batch | WO/E | 2.61 | 70 | [63] |
| *Thermoanaerobacter ethanolicus* | Beet molasses (30.0 g/L) | Batch | None | 4.81 | 65 | [64] |
| *Thermoanaerobacter* BG1L1 | Corn stover, wheat straw (25.0–150.0 g/L) | Batch | WO/E | 8.50–9.20 | 70 | [42] |
| *Thermoanaerobacter* BG1L1 | Wheat straw (30.0–120.0 g/L) | Batch | WO/E | 8.50–9.20 | 70 | [65] |
| *T. ethanolicus* | Wood HL (8.0 g/L) | Batch | E | 3.30–4.50 | 70 | [66] |
| *Thermoanaerobacter* strain AK5 | Grass (4.5 g/L) | Batch | A/E | 4.31 | 65 | [38] |
| *Thermoanaerobacter* strain J1 | Hemp (4.5 g/L) | Batch | A/E | 4.3 | 65 | [17] |
| *T. saccharoylticum* | Xylan (10.0 g/L) | Batch | WO | 6.30 | 60 | [67] |
| *Thermoanaerobacterium* strain AK17 | Grass (2.5 g/L) | Batch | A/Alk/E | 5.5 | 60 | [39] |

Cultivation were either in batch or continuous (con). Ethanol yields given in mM/g substrate degraded as well as substrate concentrations and incubation temperature are also shown. A—acid; Alk—alkaline; E—enzymatic; and WO—wet oxidation.

**Table 1.** Examples of ethanol production from lignocellulosic biomass by thermophilic bacteria.

Early experiments on ethanol production from lignocellulose included as the ethanol-producing organisms *Thermoanaerobacter ethanolicus* and *Clostridium thermocellum* with hemicellulose from birch- and beechwood as a feedstock [66]. *Clostridium thermocellum* produced between 7.2 mM ethanol /g and 8.0 mM/g from avicel and Whatman paper, respectively. Studies of ethanol production from paddy straw, sorghum stover and corn stubs, pretreated with alkali showed similar results [68]. However, these results were obtained with relatively low substrate loadings (8.0 g/L) but later studies showed that increased substrate loadings lowered the ethanol yields considerable [69]. The highest ethanol yields reported from lignocellulose are by *Thermoanaerobacter* BG1L1 grown on corn stover and wheat straw [42, 43] that were pretreated with acid or wet oxidation, or 9.2 mM/g for biomass hydrolysates. *Thermoanaerobacterium* strain AK17 showed ethanol yields of 2.0 (paper) mM/g, 2.9 (grass) mM/g and 5.8 (cellulose) mM/g biomass [23]. Optimization experiments showed an increase in ethanol yields on grass and cellulose up to 4.0 and 8.6 $mM \cdot g^{-1}$, respectively. The main culture factors increasing ethanol yields was obtained by lowering of the substrate concentration from 7.5 to 2.5 g/L [39]. Recent investigations on two *Thermoanaerobacter* strains, AK5 and J1, showed promising results from various types of hydrolysates made from chemically and enzymatically pretreated lignocellulosic biomass [17, 38] (**Table 1**).

## 6. Evolutionary adaptation and genetic engineering of thermophiles

The thermophilic anaerobes mentioned in the previous sections make logical targets for genetic improvement due to their ability to produce ethanol from a wide range of substrates as evidenced by acceptable yields on lignocellulosic biomass. There are two general strategies for enhancing characteristics for ethanol production by wild type microorganisms: evolutionary adaptation or genetically modify the organisms. Early work often used classical methods such as the selection of clones and nonspecific mutagenesis to improve ethanol production [70]. These methods are time-consuming, and genetic modification is not without drawbacks as modified strains can exhibit poor growth and unexpected shifts in end-product formation. More recent work has focused more on modern techniques in molecular biology discussed herein.

### 6.1. Evolutionary adaptation

One of the major drawbacks of using thermophiles for the production of ethanol is their low substrate and ethanol tolerance. The use of classical evolutionary adaptation methods, such as non-specific mutagenesis and artificial selection, to enhance specific traits of microorganisms for industrial bioethanol production have been applied to thermophilic anaerobes on a limited basis. Examples of adaptation methods on three new mutant strains of *Thermoanaerobacter ethanolicus* were obtained by selection of pyruvate and iron deprivation [51] leading to enhanced ethanol tolerance (10% v/v) at substrate concentrations above 10 g/L. *Clostridium thermocellum* showed increased ethanol tolerance (up to 5% v/v) by stepwise increasing and transferring cultures to increased ethanol concentrations [71]. *Thermoanaerobacter pentosaceus* has been gradually adapted

to higher substrate concentrations and demonstrated higher ethanol tolerance and substrate utilization [72]. Thus, evolutionary adaptation, may still be used for evolving of wild type strains and further improving GM strains to meet requirements for tolerance to high ethanol titers, improve substrate utilization, and potentially resistance to inhibitory compounds generated during biomass pretreatment such as 5-HMF and fufuraldehyde.

### 6.2. Genetic engineering

Despite other promising features, one of the main drawback of most wild type thermophiles is their production of mixed end-products resulting in lower ethanol yields and the fact that highly ethanologenic organisms are not natively cellulolytic and *vice versa*. Two main strategies have been used to metabolically engineer thermophilic organisms for consolidated bioprocessing (CBP). The first strategy is to increase the ethanol yields of cellulase-producing organisms while the other is to express enzymes for biomass deconstruction in highly ethanologenic microorganisms [73, 74]. The first approach involves increasing ethanol yields by redirecting the flow of carbon and electrons which involves eliminating other potential fermentation products. Obvious targets include knocking out acetate and lactate pathways. The second approach involves addition of cellulolytic genes to the genome of a good ethanol producing bacterium.

The first thermophilic bacterium to be genetically modified to increase ethanol yields was *Thermoanaerobacterium saccharolyticum* in 2004 [75]. Since then, several other ethanologenic thermophiles have been genetically modified to increase ethanol titers and minimize the formation of other end-products (**Table 2**).

Deletion of genes involved to the production of various end-products to increase ethanol production capacity is the most obvious way to increase ethanol titers. This has been done by knocking out lactate dehydrogenase in *Thermoanaerobacterium saccharolyticum* [73, 82], *Thermoanaerobacter mathranii* [79], *Clostridium thermocellum* [83] and *Geobacillus thermoglucosidasius* [78].

Wild type *Clostridium thermocellum* produces a mixture of ethanol, acetate, lactate, hydrogen, and carbon dioxide [84] from cellulose and other substrates. The first successful transformation of the species was performed in 2006 [85], later on leading to the development of genetic systems to knock out the *pta* gene and thus acetate formation [85]. However, growth of the resultant strain was abnormal although cellulase active remained intact. Later work on *C. thermocellum* showed improved ethanol yields in an adapted strain (Δ*hpt*, Δ*ldh*, Δ*pta*) lacking acetate and lactate pathways and was successfully used in co-culture with *Thermoanaerobacterium saccharolyticum* [85].

Early work on *Thermoanaerobacterium saccharolyticum* were performed by using electroporation and shuttle vectors [86], but later on this strain has been further modified by inserting a cellobiohydrolase gene from *Clostridium thermocellum* into its genome [77]. Also a *ldh* gene knock out was done using *Thermoanaerobacterium saccharolyticum* [75] and then a double knock out of both *ldh* and *ak* [73]. The knocking out of acetate production led to less available energy,

| Strain | Genotype | Substrate | Mode | Ethanol yields (mol/mol) | References |
|---|---|---|---|---|---|
| *C. thermocellum* | Δ*pyrF*, Δ*pta*::gapDHp-cat | Cellobiose (5.0 g/L) | Batch | 0.59 | [76] |
| *C. thermocellum* | Δ*pyr*F, Δ*pta*::gapDHp-cat | Avicel (5.0 g/L) | Batch | 0.71 | [76] |
| *C. thermocellum* adhE*(EA) Δldh | Δ*hpt*, Δ*ldh* | Cellobiose (5.0 g/L) | Batch | 0.37 | [77] |
| *C. thermocellum* | Δ*hpt*, Δ*ldh*, Δ*pta* (evolved) | Avicel (19.5 g/L) | Batch | 1.08 | [77] |
| *C. thermocellum*/*T. saccharolyticum* | Δ*hpt*, Δ*ldh*, Δ*pta* (evolved) and Δ*pta*, Δ*ack*, Δ*ldh* | Avicel (19.5 g/L) | Batch | 1.26 | [77] |
| *T. saccharolyticum* TD1 | Δ*ldh* | Xylolse (5.0 g/L) | Batch | 0.98 | [77] |
| *T. saccharolyticum* ALK2 | Δ*pta*, Δ*ack*, Δ*ldh* | Cellobiose (70.0 g/L) | Con | ND | [73] |
| *T. saccharolyticium* HK07 | Δ*ldh* , Δ*hfs* | Cellobiose (1.8 g/L) | Batch | 0.86 | [74] |
| *T. saccharolyticium* M0355 | Δ*ldh* , Δ*ack*, Δ*pta* | Cellobiose (50.0 g/L) | Batch | 1.73 | [74] |
| *T. saccharolyticum* M1051 | Δ*ldh* , Δ*ack* Δ*pta*, *ure* | Cellobiose (27.5 g/L) | Batch | 1.73 | [74] |
| *G. thermoglucosidasius* TM242 | Δ*ldh*-, *pdh* up, *pfl*B- | Glucose (34.0 g/L) | Batch | 1.73 | [78] |
| *G. thermoglucosidasius* TM242 | Δ*ldh*-, *pdh* up, Δ*pflB*- | Glucose (34.0 g/L) | Batch | 1.84 | [78] |
| *G. thermoglucosidasius* TM242 | Δ*ldh*-, Δ*pdh* up, Δ*pflB*- | Xylose (29.0 g/L) | Batch | 1.37 | [78] |
| *T. mathranii* BG1L1 | Δ*ldh* | Wheat straw (30-120 g/L) | Con | 1.53–1.67 | [65] |
| *T. mathranii* BG1G1 | Δ*ldh*, *GldA* | Glucose + glycerol (5.0 g/L) | Batch | 1.68 | [79] |
| *T. mathranii* BG1G1 | Δ*ldh*, *GldA* | Xylose + glycerol (5.0 g/L) | Batch | 1.57 | [79] |
| *T. mathranii* BG1G1 | Δ*ldh*, *GldA* | Xylose + glycerol (12.8 and 7.2 g/L) | Con | 1.53 | [79] |
| *Thermoanarobacter* Pentocrobe 411 | Δ*ldh*, Δ*ack*, Δ*pta* | Wheat straw (65 g/L) | Con | 1.84 | [80] |
| *C. bescii* JWCB018 | Δ*ldh*- | Celo (10 g/L) | Batch | 0 | [81] |
| *C. bescii* JWCB032 | Δ*ldh*-, *adhE*+ | Celo (10 g/L) | Batch | 0.66 | [81] |
| *C. bescii* JWCB049 | Δ*pyrFA*, Δ*ldh*- | Celo (10 g/L) | Batch | 0.54 | [81] |
| *C. bescii* JWCB054 | Δ*pyrFA*, Δ*ldh*- | Celo (10 g/L) | Batch | 0.28 | [81] |

*ack*—acetate kinase; *GldA*—glycerol dehydrogenase A; *hfs*—hydrogenase; *hpt*—hypoxanthine phosphoribosyl transferase; *pdh*—pyruvate decarboxylase; *pyrF*—orotidine-5-phoshate decarboxylase; *pfl*—pyruvate formate lyase; and *ure*—urease.

**Table 2.** Ethanol yields of genetically engineered thermophilic bacteria from different substrates and fermentation conditions.

thus less cell biomass and increased ethanol yields, both from glucose and xylose. Another double knock out of *Thermoanaerobacterium saccharolyticum* focused on the electron transfer system of the bacterium [74]. The *hfs* gene cluster, which codes for hydrogenase, and the *ldh* gene were knocked out resulting in a considerable increase in ethanol (44%) production as compared with the wild type.

*Thermoanaerobacter mathranii* has been modified and used in several investigations. The first mutant generated was BG1L1 by knocking out *ldh* resulting in a more than two-fold increase in ethanol production as compared with the wild type [87]. This strain showed good ethanol yields from undetoxified pretreated corn stover and wheat straw [42, 43]. Further manipulation of this strain involves overexpression of NAD(P)H-dependent alcohol dehydrogenase, resulting in the strain BG1E1. Clearly, this enzyme is of great importance for ethanol production and its overexpression resulted in higher ethanol yields [79]. The electron balance for sugar degradation was additionally focused upon with this strain when mannitol, which is more reduced than glucose and xylose, was used as a substrate [87] and this resulted in higher ethanol yields. The BG1G1 strain of *Thermoanaerobacter mathranii* was developed which included the insertion of a $NAD^+$-dependent glycerol dehydrogenase which increased ethanol yields by 40% greater than the type strain. Additionally, the strain utilized the highly reduced glycerol and co-metabolism of glycerol and sugars.

Recently, the highly ethanologenic strain *Thermoanaerobacter* BG1 "Pentocrobe 411" was genetically engineered by knocking out lactate dehydrogenase, phosphotransacetylase, and acetate kinase [80]. Pentocrobe 411 achieved very high ethanol titers (1.84 to 1.92 mol ethanol/mol hexose equivalent) nearing the maximum theoretical yield from hexoses and pentoses on various pretreated biomass in continuous culture.

Thermophilic bacteria within the genus of *Geobacillus* have also attracted increased interest due to their ethanol production capacity. *Geobacillus* strains are facultative anaerobes and can ferment various sugars to pyruvate by pyruvate dehydrogenase to acetyl-Coenzyme A) [78]. Under aerobic conditions, however, pyruvate formate lyase is used and a variety of end-products are formed. A research group led by Cripps manipulated *Geobacillus thermoglucosidasius*, producing variant with upregulated pyruvate dehydrogenase expression under anaerobic conditions in a strain lacking lactate dehydrogenase activity [78]. Several mutants were developed (TM89; *ldh* knockout; TM180; *ldh* knockout and upregulated *pdh*; TM242; *ldh*, upregulated *pdh* and *pfl*). The TM180 strain produced 1.45 mol ethanol/mol hexose (the wild type produced 0.39 mol ethanol/mol hexose and TM89 produced 0.94 mol ethanol/mol hexose). The triple mutant TM242 produced 1.65 mol ethanol/mol hexose. This mutant also showed good yields on xylose (1.33 mol ethanol/mol xylose) and good productivity rates. *Geobacillus thermoglucosidasius* has recently been genetically modified by expressing pyruvate decarboxylase from *Gluconobacter oxydans* [88]. Ethanol yields obtained were as high as 1.37 mol ethanol/mol glucose.

A natural target for the strategy of converting a cellulolytic organism into a good ethanol producer would be members of the genus of *Caldicellulosiruptor* which has several cellulolytic members although none are good ethanol producers. Recent work with *Caldicellulosiruptor bescii*, a naturally cellulolytic organism, has produced ethanol producing strains [89–93].

The type strains of *C. bescii* typically yield a mixture of lactic and acetic acid in addition to hydrogen and $CO_2$ as end-products although other strains within the genus of *Caldicellulosiruptor* have been noted to produce low ethanol titers. Work by Cha [89] deleted the gene coding for lactate dehydrogenase by introducing a non-replicating plasmid via marker replacement. The resultant knockout strain did demonstrate increased biomass yield as well as acetate and hydrogen production with no lactate production when grown on cellobiose and lactose as well as switch grass hydrolysates. Subsequent work by Chung [81] inserted a NADH-dependent *adhE* gene (from *Clostridium thermocellum*) into the *ldh* mutant (JWCB018) resulting in strain *C. bescii* JWCB032. The resultant $ldh^-$ $adhE^+$ strain yielded less acetate (4.3 mM) but produced 14.8 mM of ethanol from 29.2 mM cellobiose or 12.7% of the theoretical yield. It should be noted that this strain only used a small portion (4.4 mM of 29.2 mM cellobiose) provided and not produce ethanol above 65°C. Work by Cha [89] and Chung [93] introduced the alcohol dehydrogenase genes (*adhB* and *adhE*) from *Thermoanaerobacter pseudoethanolicus* into the *ldh* deficient strain. The two resultant strains yielded ethanol at temperatures greater than 65°C although titers were lower than the aforementioned strain JWCB032 ($ldh^-$ $adhE^+$). The *C. thermocellum* strain with *adhB* only produced 1.4 mM ethanol on avicel and 0.4 mM on switch grass while a strain with *adhE* gave 2.3 and 1.6 mM of ethanol on avicel and switch grass, respectively. One of the reasons for suggested for the low ethanol titers is the availability of cofactors and it should be noted that *T. pseudoethanolicus* ADHs utilize NADPH while the gene products from *C. thermocellum* use NADH as a source of reducing potential. Additional work is therefore needed to more carefully mimic the complex NAD(P)H system of multiple ADHs in *Thermoanaerobacter pseduoethanolicus*.

Overall, efforts to engineer thermophilic anaerobes to increase ethanol titers has resulted in modest gains in yields while minimizing or eliminating the formation of unwanted end products. Future targets for genetic manipulation might include the inclusion of the cellulolytic machinery of *C. thermocellum* into highly ethanologenic *Thermoanaerobacter* and *Thermoanaerobacterium* strains.

## 7. Conclusions

Bioethanol production from lignocellulosic biomass with thermophilic bacteria needs robust microbes regarding several aspects. One of the main advantages of thermophilic bacteria is their broad substrate spectra with many strains capable of simultaneous pentose and hexose degradations. Additionally, some thermophiles degrade complex carbohydrates like cellulose and hemicellulose although many of these strains are not highly ethanologenic. Recent advantages in genetic engineering have improved ethanol yields, mostly by knocking out pathways of undesired end-products. On the back side is the fact that yields and ethanol tolerance as well as low tolerance for high initial substrate concentrations still limits the use of thermophiles for large scale operations. The use of stable co-cultures where on microbe hydrolyses the sugar polymers and the other one ferments the sugars released to ethanol is an attractive way to go forward but warrants further investigations.

## Author details

Sean Michael Scully and Johann Orlygsson*

*Address all correspondence to: jorlygs@unak.is

Faculty of Natural Resource Sciences, University of Akureyri, Akureyri, Iceland

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