Recent Advances in Genetic Engineering of Thermophilic Ethanol Producing Bacteria

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Abstract Thermophilic bacteria have gained increased interest as bioprocessing platforms for bioethanol production from second generation biomass with a particular emphasis on thermophilic clostridia species. Although thermophilic bacteria possess many advantages such as broad substrate spectra, fast growth rates, and high tolerance for environmental factors, they usually tolerate less ethanol than yeasts and produce various by-products apart from ethanol. These two factors have been addressed for several thermophilic bacteria through genetic engineering, to increase ethanol tolerance or cut off branching fermentation pathways and direct end product formation towards ethanol only. The best wild type ethanol producers belong to clostridia, particularly *Thermoanaerobacter*, *Thermoanaerobacterium*, and *Clostridium*. Additionally, non-native ethanol producers exhibiting cellulolytic properties have been genetically modified to insert genes for ethanol production pathways, as of *Caldicellulosiruptor bescii*. The scope of this chapter is on recent genetic engineering of thermophilic ethanol-producing bacteria.

Keywords Lignocellulose · Bioethanol · Extremophiles · Combined bioprocessing · Anaerobes

1 Introduction

The genetic modification of ethanol producing microorganisms is one strategy to meet increased global mandates for highly-renewable and environmentally benign liquid energy carriers. An ideal bioethanol producing organism would meet the demands of combined bioprocessing (CBP) in which the degradation of lignocellulose and the subsequent fermentation of the liberated sugars is carried out by the

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same organism in one reaction vessel (Jouzani and Taherzadeh 2015; Scully and Orlygsson 2014). As of yet, no single organism solidly meets the criteria of being a good CBP candidate although a number of thermophilic anaerobes show great potential as either being strongly ethanologenic and/or capable of degrading cellulose, starch, and hemicellulose such as xylan. While a number of cellulases are commercially available, studies have demonstrated that they do not function well under anaerobic conditions or at suboptimal temperatures (Herring et al. 2016). Genetic engineering approaches offer potential solutions to improve already ethanologenic and cellulolytic strains. While the introduction of novel genetic elements or the modification of existing elements is facile in principle, in practice, alterations of genes can disrupt other metabolic pathways having unintended results such as poor growth, low ethanol yield, or the production of unwanted side products. In this work, the genetic engineering of natively ethanologenic thermophilic anaerobes is reviewed.

Beyond the utilization of renewable substrates, achieving a high ethanol titer is critical. Historically, the wild type strains of many thermoanaerobes cannot directly compete with the ethanol titers achieved by yeasts due to poor ethanol tolerance, substrate inhibition, or lower yields because of mixed end product formation thus making these shortcomings attractive targets for genetic improvement. In order for a given fermentative organism to become economically and industrially feasible, ethanol production must be at least 40 g/L (Dien et al. 2003). Additionally, achieving a strong expression of hydrolytic enzymes is necessary.

The commercial production of bioethanol produced from biomass for many decades, has primarily been from plants rich in easily fermentable carbohydrates including starch from corn and sucrose from sugarcane. This production methodology is controversial due to the environmental issues associated with using arable land for fuel production and the fact this first generation biomass is directed to fuel production that directly competes with feed and food production. Thus, second generation bioethanol production from lignocellulosic biomass has been named as a future biomass for ethanol production. Lignocellulose originates mainly from four sources; (a) energy crops (e.g. switch grass, yellow poplar), (b) agricultural residues (e.g. corn stover, wheat straw, rice straw) as summarized in Fig. 1, (c) forest residues (woods, foliage, branches) and (d) waste (municipal solid waste, food waste) (Sánchez and Cardona 2008). However, lignocellulosic biomass is much

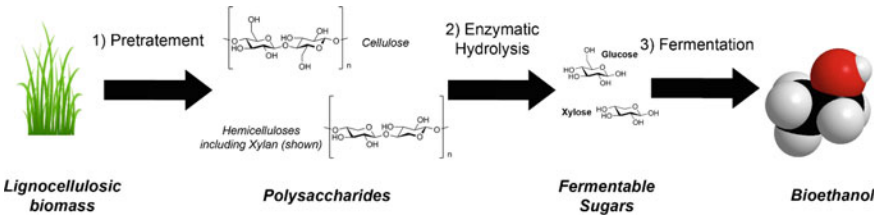

Fig. 1 Conversion of biomass to bioethanol

more complex than starch and sugars. It is composed of cellulose (glucose units linked with β-1,4-*O* glycosidic bonds), hemicelluloses (several heteropolymers composed of a variety of hexoses and pentoses including glucose, galactose, mannose, xylose, arabinose, glucuronic acid, galacturonic acid, and L-rhamnose) and lignin which is composed of randomly connected aromatic structural units (Hahn-Hägerdal et al. 2006; Sánchez and Cardona 2008). These structures are all embedded in the complex matrix of plant cells which needs to be disrupted to make polysaccharides more accessible to enzymatic degradation. Thus, for releasing the sugars present in lignocellulose and lignin removal, which cannot be used for ethanol production, the biomass needs to be pretreated and enzymatically hydrolyzed (Kumagai et al. 2014; Mosier et al. 2005). The costliest step in ethanol production from lignocellulose is the enzymatic hydrolysis (Lynd et al. 2002) making native cellulolytic ethanologens particularly attractive candidates for bioethanol production.

Ethanol production from simple substrates, such as starch and sucrose-rich crops, have traditionally been fermented by yeasts of which the best known is *Saccharomyces cerevisiae*. The main reason for using *S. cerevisiae* is that it is homoethanologenic, produces high ethanol yields (>95% of the theoretical yield) from sugars, possesses high ethanol tolerance, its cells can be recycled with ease and it has a long history in industrial production. The reader is directed to a number of recent reviews covering traditional mesophilic ethanologens (such as yeasts) and process considerations (Jouzani and Taherzadeh 2015).

Degradation of more complex biomass such as lignocellulose, however, requires microorganisms that have broader substrate spectrum due to the increased variety of sugars present. A number of thermophilic clostridia are noteworthy for their broad substrate spectra often degrading most of the hexoses, pentoses, and disaccharides liberated from complex lignocellulose biomass (Carreira et al. 1983; Herring et al. 2016; Jessen and Orlygsson 2012). The earliest applications of thermophilic anaerobes for bioethanol production date back to the late 1970s (Ben-Bassat et al. 1981; Lamed and Zeikus 1980a, b) including ethanol production directly from lignocellulosic biomass (Carreira et al. 1983; Lamed et al. 1988). High growth rates, and high ethanol titers make these bacteria attractive candidates for large-scale industrial bioethanol production. Furthermore, their broad substrate spectra make them of particular interest for the fermentation of lignocellulosic hydrolysates. However, thermophilic bacteria are not homoethanologenic and produce many other end products which lower ethanol yields, they tolerate only very low initial substrate concentrations and display relatively low ethanol tolerance. Recent work has focused on the genetic modification of ethanologenic thermophilic anaerobes to decrease carbon flow to side-products and improve the biological robustness to make these organisms powerful platforms for bioethanol production.

2 Thermophilic Ethanol Producing Bacteria

Thermophiles are classified according to their optimum growth temperature with the "lower" thermophilic boundary being widely recognized as 50 °C (Sundaram 1986). Moderately thermophilic bacteria have temperature optima between 45 and 55 °C whereas "true" thermophiles have optima between 55 and 75 °C and extreme or ("hyper") thermophiles with optimum temperature above 80 °C (Burgess et al. 2007; Wagner and Wiegel 2008). While geothermal areas often offer stability in heat and are thus favorable habitats for thermophilic bacteria (Brock 1986), they are ubiquitous and have been isolated from a wide range of habitats in which a complex community of organisms are responsible for the solubilization, degradation, and fermentation of various carbon sources to many types of end products. While it is true that there seems to be less diversity amongst strict anaerobes in geothermal habitats (Wagner and Wiegel 2008), and that many ethanologenic thermoanaerobes have broad substrate spectra, some degree of specialization does occur. Generally, most known thermophilic species are obligate or facultative anaerobes since geothermal areas have low oxygen concentrations due to the limited solubility of oxygen at elevated temperatures.

Several genera of thermoanaerobes are notable for their highly ethanologenic species within of *Clostridium*, *Thermoanaerobacter*, and *Thermoanaerobacterium* although work with the cellulolytic genera of *Caldicellulosiruptor* has been recently reported. The amount of available information regarding biofuel production from individual species varies often with only basic physiological properties being available. Furthermore, studies on lignocellulosic biomass and hydrolysates and the effect of inhibitory compounds produced as a part of the pretreatment process has not been widely reported. Selected properties of the wild types of several well-known cellulolytic or ethanologenic thermoanaerobes with potential as platforms for CBP ethanol production that have been selected for genetic modification or are outstanding ethanologens in their own right are presented in Table 1.

One of the barriers to the adoption of thermoanaerobes is the limited availability of phenotypic and physiological data. Below is a description of the best known thermophilic ethanol producing bacteria.

2.1 Clostridium

The genus *Clostridium* is a large, polyphyletic genus currently containing 211 species according to list of prokaryotes names with standing in nomenclature (LPSN) (Parte 2014). Cellulolytic and ethanologenic *Clostridium* species have been extensively investigated, particularly those which produce cellulosomes due to their potential to degrade cellulose and hemicellulose (Canganella and Wiegel 1993; Carreira et al. 1983; Demain et al. 2005). Cellulosomes as well as the functionality of specific glycohydrolase genes is reviewed in Doi and Kosugi (2004). Most

Table 1 Comparison of potential saccharolytic and ethanol-producing thermoanaerobes

Characteristic	*Caldicellulosiruptor bescii*	*Clostridium* strain AK1	*Clostridium thermocellum*	*Thermoanaerobacterium saccharolyticum*	*Thermoanaerobacter pseudoethanolicus*	*Thermoanaerobacter ethanolicus*
$T_{min}/T_{opt}/T_{max}$	40/78–80/90	45/55/60	X/65/X	45/60/70	ND/65/ND	37/69/78
Substrate spectra	Broad	Broad	Medium	Broad	Broad	Broad
Cellulose	+	–	+	–	–	–
Pectin	+	+	NR	NR	NR	NR
Starch	+	+	NR	+	+	+
Xylan	+	+	+	+	+	NR
Ethanol production	–	+	+	+	+	+
Ethanol tolerance	NR	NR	Low	NR	NR	NR
Inhibitory compound resistance	NR	NR	Low	NR	NR	NR
Commercial acceptance	No	No	No	No	No	No
Whole genome sequenced	Yes	IP	Yes	Yes	Yes	Yes
Ease of genetic modification	High	NR	Poor	High	NR	NR
Cellulase genes	+	NR	+	–	NR	–
Xylanase genes	+	+	+	+	+	+
References	Yang et al. (2010)	Orlygsson (2012)	Freier et al. (1988)	Lee et al. (1993)	Lamed and Zeikus (1980a)	Wiegel and Ljungdahl (1981)

NR not reported

Clostridium species are mesophilic and only about 15 thermophilic strains have been isolated and characterized of which most are moderately thermophilic. The most widely studied ethanologenic and thermophilic member of *Clostridium* has been *Cl. thermocellum* (Ben-Bassat et al. 1981; Lamed and Zeikus 1980a, b). Major works on *Cl. thermocellum* are reviewed in Akinosho et al. (2014). Recent studies on ethanol production from cellulose and lignocellulosic biomass were done with *Cl. thermocellum* SS21 and SS22 with yields ranging from 4.60 (corn stubs) to 8.10 mM/g hexose equivalent (sorghum stover) (Sudha Rani et al. 1998). The carbon balances for *C. thermocellum* strains are often low due to the production of other end products such as pyruvate and free amino acids (Ellis et al. 2012). Strain of *Clostridium* strain AK1, a moderate thermophile, has demonstrated ethanol yields up to 1.5 mol ethanol/mol hexose (Orlygsson 2012).

2.2 Thermoanaerobacterium *and* Caldanaerobium

Thermoanaerobacterium was first described as a new genus in 1993 when two thermophilic, xylan degrading strains were isolated from Frying Pan Springs in Yellowstone National Park in Wyoming (Cann et al. 2001). Today, the genus of *Thermoanaerobacterium* consists of 7 species while *Caldanaerobium* contains 2 species isolated from various environments (Parte 2014). These bacteria degrade a variety of sugars present in lignocellulosic biomass and produce a variety of end-products. Some species have shown promising ethanol and hydrogen production capacity, but production of mixed end products limit their use (Ren et al. 2008, 2009; Romano et al. 2010; Sveinsdottir et al. 2009). *Thermoanaerobacterium saccharolyticum* has, however, been genetically engineered and both acetate and lactate formation has been knocked out (Shaw et al. 2008). *Thermoanaerobacterium* AK_{17}, isolated from Icelandic hot spring, has been extensively studied for ethanol production (Almarsdottir et al. 2012; Koskinen et al. 2008; Sveinsdottir et al. 2009). This strain produces 1.5 and 1.1 mol ethanol from one mole of glucose and xylose, respectively. The strain has been genetically modified to knock out acetate and lactate formation (unpublished results).

2.3 Thermoanaerobacter *and* Caldanaerobacter

The genus *Thermoanaerobacter* and *Caldanaerobacter* are closely related to *Thermoanaerobacterium*. The genus was first described in 1981 when Wiegel and Ljungdahl described *T. ethanolicus*, the type species of the genus. The genus *Thermoanaerobacter* contains 15 species and 5 subspecies while *Caldanaerobacter* contains 2 species and 4 subspecies (Parte 2014). Most species within these genera have a very broad substrate spectrum but produce a mixture of end products. Earlier investigations on their ethanol production property include work on *T. brockii* and

T. thermohydrosulfuricus (Ben-Bassat et al. 1981; Lamed and Zeikus 1980a, b; Lovitt et al. 1984). Ethanol yields by *T. brockii* were only moderate or between 0.38 (Lamed and Zeikus 1980b) and 0.44 mol ethanol/mol glucose equivalents (Ben-Bassat et al. 1981). Higher yields were later observed by *T. thermohydrosulfuricus*, or 0.9–1.9 mol ethanol/mol glucose (Lovitt et al. 1984, 1988). *T. ethanolicus* showed yields ranging from 1.1 to 1.9 mol ethanol/mol glucose (Wiegel and Ljungdahl 1981). Later this strain has been extensively studied by Lacis and Lawford more than 20 years ago (Lacis and Lawford 1988a, b; 1989; Lacis et al. 1991). *T. ethanolicus* JW200 showed also very good ethanol yields from xylose and glucose at low (10 g/L) substrate concentrations, or 1.45 and 1.95 mol/mol, respectively (Carreira et al. 1982). A mutant strain was later developed [JW200Fe(4)] that showed similar yields but at higher (30 g/L) substrate concentrations (Carreira et al. 1983). Other investigations on this species on sucrose showed between 1.76 and 3.60 mol ethanol/mol sucrose with high substrate concentrations (15–30 g/L) (Avci and Dönmez 2006). A study of bacteria isolated from Icelandic hot spring shows that a *Thermoanaerobacter* strain AK33 showed good ethanol yields on monosugars (Sveinsdottir et al. 2009). Glucose and xylose fermentations resulted in 1.5 and 0.8 mol ethanol from one mole of glucose and xylose, respectively. Later studies on *Thermoanaerobacter* species isolated from Icelandic hot springs show that *Thermoanaerobacter* strain J1, was a good ethanol producer, producing 1.7 and 1.5 mol ethanol from one mole of glucose and xylose, respectively (Jessen and Orlygsson 2012). Recent studies of *T. pentosaceus* showed ethanol yields of 1.36 mol ethanol/mol consumed sugars in batch study. Immobilization in different support material in continuous culture gave better yields or 1.5 mol ethanol/mol glucose (Sittijunda et al. 2013).

2.4 Caldicellulosiruptor

The genus *Caldicellulosiruptor* contains nine species and are mostly known for their high hydrogen production capacity (De Vrije et al. 2007; Kádár et al. 2004; Parte 2014; Zeidan and van Niel 2010). All species have been isolated from geothermal environments and have relatively broad substrate spectrum capable of utilizing cellulose, cellobiose, xylan, and xylose among other substrates (Rainey et al. 1994; Yang et al. 2010). Members of the genus have emerged as candidates for CBP since they are capable of growing on biomass without conventional pretreatment (Ivanova et al. 2009). A comparative analysis of the genus revealed that there is a high degree of variability in the ability of type strain to degrade cellulose due to a diversity of glycohydrolase genes present (Zurawski et al. 2015). Ethanol production from wild type *Caldicellulosiruptor* is generally very low but *C. bescii* has been genetically modified to produce high ethanol yields (Chung et al. 2015a). Additionally, all nine of the type strains have been whole genome sequenced.

2.5 Other

Other well know thermophilic bacteria that have been investigated for ethanol production are e.g. *Caloramator boliviensis* (Crespo et al. 2012a, b) and *Geobacillus thermoglucosidasius* (Barnard et al. 2010) who has also been genetically manipulated to enhance ethanol production (Cripps et al. 2009).

3 Systems Biology for Better Understanding the Physiology of Ethanol Production

While the fermentative pathways for the degradation of carbohydrates and their subsequent fermentation to end products such as ethanol are well known, the complex interactions between other pathways are not well understood. The use of a systems biology approach including genomics, metabolomics, proteomics, and transcriptomics have been applied in recent years for understanding solvent production by thermoanaerobes. While these approaches are useful, understanding the substrate and cofactors preferences of specific gene products is dependent upon the use of old-fashioned enzymological techniques. Over the past few years, a large number of whole genome sequences for thermophilic clostridia species have become available which greatly aids our understanding of the interactions of specific pathways leading to the formation of different end product ratios. Early studies of the systems biology of anaerobes have been restricted to mesophilic bacteria, particularly *Clostridium acetobutylicum* (Durre 2009 and references therein) although several more recent studies on thermophilic anaerobes are highlighted here in the context of the physiology of thermoanaerobes.

A simple model of the production of ethanol involves the degradation of polysaccharides leading to the formation of oligo-, di-, and monosaccharides, the import of carbon into the cell, followed by the fermentation of sugars to end products as summarized in Fig. 2.

3.1 Degradation of Polysaccharides

Among thermophilic clostridia, the degradation of biopolymers such as cellulose, xylan, pectin, and starch is performed by systems of extracellular enzymes. The degradation of biopolymers by clostridia species is reviewed in Leschine (2005). Among the best-studied system is the cellulosome produced by *Cl. thermocellum* (Demain et al. 2005; Doi and Kosugi 2004). The degradation of crystalline cellulose by *Caldicellulosiruptor* species is recently reviewed in Zurawski et al. (2015). Work on *Cl. thermocellum* has revealed the presence of many glucosidases (Lagaert et al. 2014; Sizova et al. 2011). Much work has been done on the production of

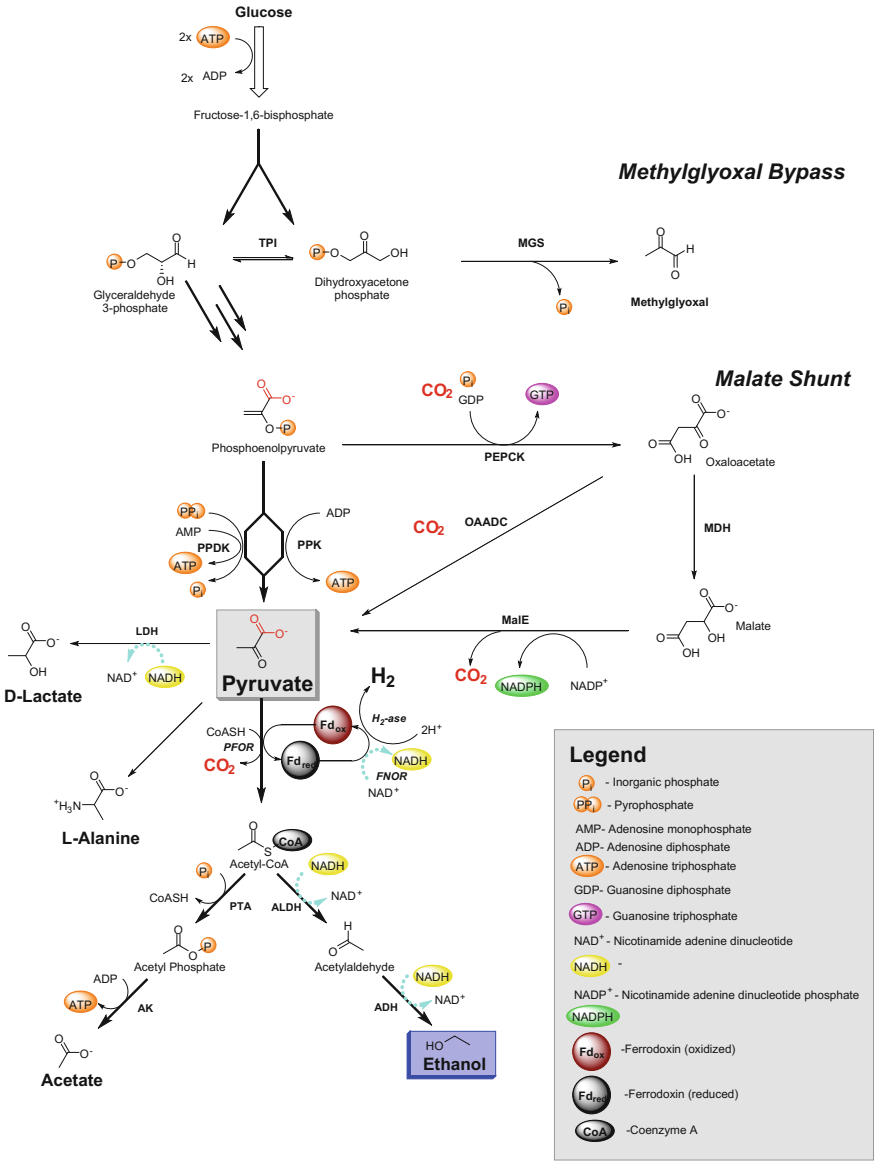

Fig. 2 Embden-Meyerhof and related pathways involved in fermentation of carbohydrates to ethanol and other end products. *TPI* triose phosphate isomerase; *MGS* methylglyoxal synthase; *PEPCK* Phosphoenolpyruvate carboxykinase; *OAADC* oxaloacetate decarboxylase; *MDH* malate dehydrogenase; *MalE* malic enzyme; *LDH* lactate dehydrogenase; *PFOR* pyruvate-ferredoxin oxidoreductase; *H_2-ase* hydrogenase; *FNOR* formate NADH ferredoxin oxidoreductase; *PTA* phosphate acetyltransferase; *ALDH* aldehyde dehydrogenase; *ADH* alcohol dehydrogenase; *AK* acetate kinase

designer cellulosomes which is beyond the scope of this review but is covered in the following references (Barak et al. 2012; Doi and Kosugi 2004; Stern et al. 2015, 2016; Vazana et al. 2013). Interestingly, a study revealed that *Cl. thermocellum* directly imports small oligosaccharide products of cellulose hydrolysis thus making *Cl. thermocellum* highly efficient at cellulose degradation despite the need to produce specialized enzymes (Zhang and Lynd 2005). While it is known that *Caldicellulosiruptor* and *Thermoanaerobacter* species often grow on cellobiose, their ability to import oligosaccharides has not been explored.

3.2 Fermentative Pathways

As mentioned above thermophilic bacteria can degrade many carbohydrates and produce various end products, among them ethanol. There is tremendous metabolic diversity among thermoanaerobes, particularly thermophilic clostridia. Central to bioethanol production is the degradation of glucose to end products. Figure 2 shows the carbon flow from glucose by fermentation by the use of Embden-Meyerhof pathway (EMP). The degradation of glucose via EMP generates two moles of NADH and pyruvate, the key intermediate in most organisms.

Several alternatives to the flow of carbon through glycolysis have been described that are relevance to ethanol production. The methylglyoxal bypass of glycolysis provides an alternative route for carbon through glycolysis; the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate is a bottleneck particularly when glucose is rapidly catabolized or phosphate becomes limiting. Thus the conversion of DHAP to methylglyoxal enables the recovery of inorganic phosphate although generates highly cytotoxic methylglyoxal. The malate shunt can generate reducing potential as NADPH via transhydrogenation which can influence the flow of electrons to end products (Carere et al. 2012). *Clostridium thermocellum* has some atypical features in glycolysis particularly the flow of carbon through the malate shunt (Zhou et al. 2013).

Pyruvate can be converted to a variety of volatile fatty acids, predominately acetate with traces of propionate and butyrate occasionally reported, and reduced end products including hydrogen, ethanol, lactate, and alanine (Fig. 2). The distribution of end products is highly dependent upon the microorganism and the carbon source as well as the cultivation conditions. It should be noted that while alanine production has been reported among some *Thermoanaerobacter* and *Thermoanaerobacterium* species, its has not been universally reported. The presence of other reduced end products, such as 1,2-propanediol, have also been reported for *Thermoanaerobacterium saccharolyticum* (Lee et al. 1993) but specific end product concentrations were not described.

The conversion of acetyl CoA to ethanol is facilitated by alcohol dehydrogenase (ADH) or the bi-functional alcohol and aldehyde dehydrogenase (ADHE). The consumption of one mole of NADH for the reduction of pyruvate regenerates the NAD^+ required for the first steps of glycolysis thus highlighting the critical nature

of intracellular redox balance. Pyruvate can also be reduced to lactate by lactate dehydrogenase (LDH).

Understanding the correlation between the presence or absence of specific genes and observed end product distributions is critical. A meta-genomic analysis of selected Firmicutes including several thermoanaerobes, found that organisms that are regarded as good hydrogen producers (*Caldicellulosiruptor*, *Thermococcus*, *Thermotoga*, and *Pyrococcus*) lack genes coding for acetaldehyde dehydrogenases and bifunctional acetaldehyde/alcohol dehydrogenases while organisms lacking hydrogenases have higher lactate production (Carere et al. 2012).

3.3 Importance of Hydrogen Synthesis to Ethanol Production

The most thermodynamically favorable pathway for anaerobic bacteria is to oxidize pyruvate to acetyl-CoA and CO_2 by using pyruvate:ferredoxin oxidoreductase (PFOR); acetyl-CoA is then converted to acetate with the production of ATP from the acetyl-phosphate intermediate. The primary advantage for an organism to produce acetate is the formation of ATP. The electrons are transported to reduced ferredoxin which acts as an electron donor for hydrogenases and H_2 is produced as the reduced product. There are mainly two types of hydrogenases: NiFe hydrogenases and the FeFe hydrogenases. Recent overview articles have been published on the subject (Chou et al. 2008; Kengen et al. 2009). Acetyl coenzyme A can also be converted to acetaldehyde by acetaldehyde dehydrogenase (ACDH) and further to ethanol by alcohol dehydrogenase.

Strictly anaerobic bacteria generate hydrogen using either pyruvate ferredoxin oxidoreductase or NADH ferredoxin oxidoreductase while facultative anaerobes also rely upon pyruvate-formate lyase (PFL). Firstly, from a NAD(P)H by GAPDH and from pyruvate ferredoxin oxidoreductase (PFOR) (Jones 2008). The principal H_2 production pathway is through PFOR because of thermodynamics hindrance of re-oxidizing NADH (Jones 2008). It is a well-known phenomenon that the low hydrogen yields observed by mesophilic and moderate thermophilic bacteria are due to the fact that hydrogen production from either ferredoxin or NAD(P)H are thermodynamically unfavorable (Hallenbeck 2009; Jones 2008). The redox potential of Fd_{red}/Fe_{ox} couple depends on the microorganism and temperature involved; the redox potentials for relevant reactions involved in fermentations are shown in Table 2.

In nature, high partial pressures of H_2 are relatively uncommon because of the activity of H_2 scavenging microbes, e.g. methanogens or sulfate reducing bacteria (Cord-Ruwisch et al. 1988). This results in a low partial pressure of H_2 which is favorable for a complete oxidation of glucose to acetate and CO_2. At high temperatures, the influence of the partial pressure of H_2 is less on the key enzymes

Table 2 Selected redox potentials for some electron transfer reactions

Redox couple	Enzyme system	$E^{0'}$ (mV)
CO_2/formate	Pyruvate-formate lyase	−432
H^+/H_2	Hydrogenase	−414
Fd_{ox}/Fd_{red}	Ferredoxin oxidoreductase	−398
$NAD(P)^+$/NAD(P)H	Hydrogen:NAD^+ oxidoreductase	−320
FAD/$FADH_2$	Hydrogen:FAD^+ oxidoreductase	−220

Modified from Plugge and Stams (2005)

responsible for hydrogen production. This is the main reason why extremophilic bacteria have been reported to produce up to 4 mol of H_2 together with 2 mol of acetate in pure cultures and also for the fact that microorganisms growing at lower temperatures direct their end product formation to other reduced products. At lower temperatures, the oxidoreductases that convert NADH to Fd_{red} is strongly inhibited by hydrogen. The E° is −398 mV for Fd_{red}/Fd_{ox} couple but −320 mV for the NADH/NAD^+ couple (Hallenbeck 2009; Jones 2008). Therefore, at low temperatures, elevated H_2 concentrations inhibits H_2 evolution at much lower concentrations as compared to extreme temperatures.

3.4 Other Aspects of Ethanol Production

Detoxification of hydrolysates prior to fermentation is an obstacle for some organisms that can potentially be overcome with genetic engineering. 2-Furfuraldehyde (2-FF) and 5-hydroxymethylfurfuraldyde (5-HMF), produced from xylose and glucose under acidic conditions and high temperatures, respectively, are highly inhibitory for bacterial growth. A recent proteomic analysis of *Thermoanaerobacter pseudoethanolicus* 39E revealed that the addition of 2-FF and 5-HMF to the growth medium upregulated enzymes that catalyzed the reduction to their corresponding alcohols (Clarkson et al. 2014). It is worth noting that the identified gene products with this aldehyde reducing activity also showed activity towards acetaldehyde, butyraldehyde, and isobutyraldehyde.

Other factors of importance for thermophilic ethanologenic bacteria is their pH and temperature growth optimum, and their need for trace elements and vitamins often originating from complex medium supplements like yeast extract. Also, initial substrate concentrations are of importance, in general yeasts tolerate much higher (often more than 50 times) initial substrate concentration compared with thermophilic bacteria. Some thermophiles, such as *Thermoanaerobacter* strain J1 and *Thermoanaerobacterium saccharolyticum* have though been shown to tolerate quite a high substrate concentration (Altaras et al. 2001; Jessen and Orlygsson 2012). Finally, ethanol tolerance is of great importance, from an economic point of view

since low ethanol titers obtained will lead to more water usage and more power need for distillation processes. In general, thermophiles have low ethanol tolerance (often below 3% v/v). Several attempts have been made to improve ethanol tolerance in thermophilic bacteria and can be addressed in Scully and Orlygsson (2014).

4 Evolutionary Adaptation and Genetic Engineering in Thermophilic Bacteria for Ethanol Production

Recent reviews have covered the genetic engineering of thermoanaerobes for bioethanol production (Olson et al. 2015). An overview of the use of systems biology tools is covered in Mielenz and Hogsett (2010). While the authors are not aware of any specific overviews on the modification of thermoanaerobes, several relevant overviews on *Clostridium* might be of relevance (Bradshaw and Johnson 2010; Davia et al. 2005).

4.1 Evolutionary Adaptation

Classical evolutionary adaptation methods, such as non-specific mutagenesis and artificial selection, have been applied to thermophilic anaerobes on a limited basis to improve their ethanol production capacity. A major drawback of these approaches is a lack of control over which genetic changes take place. One of the major drawbacks associated with thermophilic anaerobes that must be overcome is their low substrate and ethanol tolerance compared to their mesophilic counterparts for which one solution is to adapt the strains by slowly increasing ethanol concentrations over the course of multiple fermentations or in continuous culture.

Three novel *Thermoanaerobacter ethanolicus* mutants were obtained by pyruvate and iron deprivation (He et al. 2009) leading to enhanced ethanol tolerance (10% v/v) at substrate concentrations above 10 g/L. *Clostridium thermocellum* also showed increased ethanol tolerance (up to 5% v/v) by transferring cultures stepwise to increased ethanol concentrations (Shao et al. 2011) as has *Thermoanaerobacter pentosaceus* which has been gradually adapted to higher substrate concentrations and demonstrated higher ethanol tolerance and substrate utilization (Sittijunda et al. 2013). Thus, evolutionary adaptation may still be used for evolving wild type strains with enhanced ethanol, substrate and inhibiting compound tolerance of 5-HMF and 2-furfuraldehyde. In general, less work has been done on thermophiles compared with mesophiles in this aspect.

4.2 Genetic Engineering

Genome sequencing technology and gene transfer systems have recently resulted in a new generation of engineered thermophilic ethanologens (Shaw et al. 2010, 2012). Traditional tools for genetic modification rely extensively on the use of plasmids to facilitate the introduction of genes, antibiotic markers or other reporter genes for the selection of transformants, or the natural competence of target strains.

Genetic engineering of thermophiles has mainly focused on two aspects. Firstly, on knocking out metabolic pathways leading to undesired end products (primarily lactate and hydrogen), and secondly on either inserting cellulolytic genes into non-cellulolytic microorganisms, or inserting genes for increased ethanol titers (Shaw et al. 2010). The majority of work appearing in the literature focuses on the latter approach. The first approach involves increasing ethanol yields by eliminating other fermentation products and improving ethanol tolerance whereas the second approach involves the addition of cellulolytic genes to the genome of a good ethanol producing bacterium.

It should be noted that Shaw et al. (2010) demonstrated that several strains of *Thermoanaerobacter* and *Thermoanaerobacterium* are naturally competent for genetic alteration. *Caldicellulosiruptor*, on the other hand, extensively methylates its genome making and using a system of restriction enzymes making it resistant to transformation (Chung et al. 2013a, b and references therein). This requires an improved understanding of its restriction-modification system which seems to differentiate between species (Pawar 2014).

4.3 Case Studies

Among thermoanaerobes, relatively few have been targeted for genetic modification/strain improvement. All of the strains that have been selected for genetic modification are all good ethanol producers with the exception of *Caldicellulosiruptor bescii* for which the wild type is a cellulose degrading acetate and hydrogen producer. Recent advances made with modified *Thermoanaerobacterium* and *Thermoanaerobacter strains* highlight the potential improvements that can be made to thermophilic anaerobes that are highly ethanologenic while attempts to modify *Caldicellulosiruptor bescii* provide some promise for engineering cellulolytic bacteria to produce higher ethanol titers. A summary of the ethanol yields for genetically engineered thermoanaerobes discussed below is presented in Table 3.

Table 3 Ethanol yields of genetically engineered thermophilic bacteria from different substrates and fermentation conditions

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
Cl. thermocellum	ΔpyrF, Δpta:: gapDHp-cat	Celo	5.0	Batch	0.59	Tripathi et al. (2010)
Cl. thermocellum	ΔpyrF, Δpta:: gapDHp-cat	Av	5.0	Batch	0.71	Tripathi et al. (2010)
Cl. thermocellum	adhE*(EA), Δhpt, Δldh	Celo	5.0	Batch	0.37	Biswas et al. (2014)
Cl. thermocellum	Δhpt, Δldh, Δpta (evolved)	Av	19.5	Batch	1.08	Biswas et al. (2014)
Cl. thermocellum/*T. saccharolyticum*	Δhpt, Δldh, Δpta (evolved) and Δpta Δack Δldh	Av	19.5	Batch	1.26	Biswas et al. (2014)
T. saccharolyticum TD1	Δldh	Xyl	5.0	Batch	0.98	Biswas et al. (2014)
T. saccharolyticum ALK2	Δpta, Δack, Δldh	Celo	70.0	Cont	ND	Shaw et al. (2008)
T. saccharolyticum HK07	Δldh, Δhfs	Celo	1.8	Batch	0.86	Shaw et al. (2009)
T. saccharolyticum M0355	Δldh, Δack Δpta	Celo	50	Batch	0.44 g/g glu eq	Shaw et al. (2011)
T. saccharolyticum M0355	Δldh, Δack Δpta	Celo	50.0	Batch	1.73	Argyros et al. (2011)
T. saccharolyticum M1051	Δldh, Δack Δpta, ure	Celo	27.5	Batch	1.73	Shaw et al. (2009)
T. saccharolyticum ALK2	Δpta, Δack, Δldh	Mock HL	16.5[s1]	SSCF	1.81 glu eq	Herring et al. (2016)
T. saccharolyticum M1442	Δpta, Δack, Δldh; ΔTsac_0795, +ure, +metE	Mock HL	16.5[s1]	SSCF	1.83 glu eq	Herring et al. (2016)
T. saccharolyticum M1442	Δpta, Δack, Δldh	Mock HL	16.5[s1]	SSCF	1.72 glu eq	Herring et al. (2016)

(continued)

Table 3 (continued)

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
T. saccharolyticum ALK2	Δpta, Δack, Δldh	PT Hardwood	12.0[s2]	SSCF	1.52 glu eq	Herring et al. (2016)
T. saccharolyticum M2886	+pta/ach-KanR into mgs gene	PT Hardwood	12.0[s2]	SSCF	1.62 glu eq	Herring et al. (2016)
T. saccharolyticum M2886	+pta/ach-KanR into mgs gene	PT Hardwood	12.0[s3]	SSCF	1.57 glu eq	Herring et al. (2016)
T. saccharolyticum M2886	+pta/ach-KanR into mgs gene	Hardwood HL	118.1[s4]	SHF	1.80 glu eq	Herring et al. (2016)
G. thermoglucosidasius TM242	Δldh^{-}, pdh up, $pflB^{-}$	Glu	34.0	Batch	1.73	Cripps et al. (2009)
G. thermoglucosidasius TM242	Δldh^{-}, pdh up, $\Delta pflB^{-}$	Glu	34.0	Batch	1.84	Cripps et al. (2009)
G. thermoglucosidasius TM242	Δldh^{-}, Δpdh up, $\Delta pflB^{-}$	Xyl	29.0	Batch	1.37	Cripps et al. (2009)
T. mathranii BG1L1	Δldh	Wheat straw	30–120	Cont.	1.53–1.67	Georgieva et al. (2008)
T. mathranii BG1G1	Δldh, GldA	Glu + gly	5.0	Batch	1.68	Yao and Mikkelsen (2010b)
T. mathranii BG1G1	Δldh, GldA	Xyl + gly	5.0	Batch	1.57	Yao and Mikkelsen (2010b)
T. mathranii BG1G1	Δldh, GldA	Xyl + gly	12.8 and 7.2	Cont.	1.53	Yao and Mikkelsen (2010b)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Wheat straw	65[x]	Cont.	1.84 sug eq	Andersen et al. (2015)

(continued)

Table 3 (continued)

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
Thermoanaerobacterobacter Pentocrobe 411	Δldh, Δack Δpta	Wheat Straw	137[x]	Cont	1.88 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Wheat straw	77[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Birch	86[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Corn cob	134[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cane bagasse	436[x]	Cont	1.88 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cardboard	56[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Biowaste	75[x]	Cont	1.88 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Oil palm EFB	190[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Oil palm frond	130[x]	Cont	1.92 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cane syrup	868[x]	Cont	1.84 sug eq	Andersen et al. (2015)
Thermoanaerobacter Pentocrobe 411	Δldh, Δack Δpta	Cane molasses	878[x]	Cont	1.88 sug eq	Andersen et al. (2015)

(continued)

Table 3 (continued)

Strain	Genotype	Substrate	Substrate concentration (g/L)	Culture mode	Ethanol yields (mol EtOH/mol substrate)	Reference
C. bescii JWCB018	Δldh^-	Celo	10	Batch	0	Chung et al. (2014)
C. bescii JWCB032	Δldh^-, $adhE^+$	Celo	10	Batch	0.66	Chung et al. (2014)
C. bescii JWCB049	ΔpyrFA, Δldh^-	Celo	10	Batch	0.54[a]	Chung et al. (2015a)
C. bescii JWCB054	ΔpyrFA, Δldh^-	Celo	10	Batch	0.28	Chung et al. (2015a)

ack acetate kinase; *GldA* glycerol dehydrogenase A; *hfs* hydrogenase; *hpt* hypoxanthine phosphoribosyl transferase; *ldh* lactate dehydrogenase; *pdh* pyruvate decarboxylase; *pyrF* orotidine-5-phoshate decarboxylase; *pta* phosphotransacetylase; *pfl* pyruvate formate lyase; *ure* urease; *ΔTsac_0795* possible helicase or protein kinase, *metE* methionine synthase; *mgs* methylglyoxal synthase; *celo* cellobiose; *Av* avicel; *Xyl* xylose; *Glu* glucose; *Gly* glycerol; *SSCF* simultaneous saccharification and co-fermentation; *SHF* simultaneous saccharification and fermenation; *Batch* batch culture; *Cont* continuous culture

[a]Calculated per mole cellobiose; [s1]on a % solids basis (100 g/L cellulose, 20 g/L glucose, 35 g/L xylose); [s2]on a # solids basis (65.5 g/L cellulose, 1.0 g/L glucose, 13.9 g/L xylose, 3.8 g/L other sugars); [s3]on a # solids basis (65.5 g/L cellulose, 1.0 g/L glucose, 13.9 g/L xylose, 3.8 g/L other sugars; [s4]total sugar basis (88.6 g/L glu, 24.3 g/L xyl, 5.2 g/L other sugars); [x]on a total sugar basis

4.3.1 *Thermoanaerobacterium saccharolyticum*

The first thermophilic bacterium to be genetically engineered to enhance ethanol production was *Thermoanaerobacterium saccharolyticum* in 2004 (Desai et al. 2004). The species has a very broad substrate spectrum, degrading for example starch, xylan, glucose, cellobiose, xylose, arabinose, mannose and galactose. As for other members of *Thermoanaerobacterium* they cannot degrade cellulose (Lee et al. 1993). The sugars are degraded to a wide array of end products, including ethanol, acetate, lactate, carbon dioxide, and hydrogen.

From the early work on *T. saccharolyticum* using electroporation and shuttle vectors (Rachek et al. 1997), this strain has been further modified by inserting a cellobiohydrolase gene from *Clostridium thermocellum* into its genome (Biswas et al. 2014). Later work with *T. saccharolyticum* involved an *ldh* gene knock out (Desai et al. 2004; Shaw et al. 2008). Elimination of acetate production was then established by knocking out genes responsible for phosphotransacetylase (PTA) and acetate kinase (AK) and the resulting strain (ALK1) produced ethanol with 90–100% of the theoretical yield. Knocking out acetate formation leads to less available energy and thus less cell biomass and increased ethanol yields, both from glucose and xylose. Strain ALK2 was obtained by cultivating strain ALK1 in continuous culture for almost four months with increasing substrate loadings. This strain produced up to 33 g/L of ethanol corresponding to 92% of the theoretical yields and ethanol productivity rate of 2.2 g/L/h. Another double knock-out of *T. saccharolyticum* focused on the electron transfer system of the bacterium (Mai and Wiegel 2000). The authors deleted the *hfs* gene cluster and *ldh* gene which encode for a hydrogenase and LDH, respectively. Again, a considerable increase in ethanol (44%) production was obtained as compared with the wild type.

Shaw et al. (2011) reported the development of a markerless strategy using haloacetic acid as the selective agent to generate strain M0355 which lacked *ldh*, *pta*, and *ack* genes and produced high ethanol titers on cellobiose, up to 1.72 mol ethanol/mol glucose. The use of a markerless strategy is advantageous because it does not rely upon antibiotic resistance as a selective agent and enables multiple modifications for further strain improvement.

Finally, in a recent study of *T. saccharolyticum* strain M2886, the genes for exopolysaccharide synthesis were removed, the regulatory gene *perR*, and re-introducing phosphotransacetylase and acetate kinase into the methylglyoxal synthase gene (Herring et al. 2016). Also, this strain was subjected to multiple rounds of adaptation and selection, resulting in mutations later identified by resequencing. This strain produced 70 g/L of ethanol in batch on cellobiose and maltodextrin. These are the highest titers obtained by a thermophilic bacterium. Ethanol concentration on hardwood obtained by this strain was 26 g/L.

4.3.2 *Clostridium thermocellum*

Clostridium thermocellum is a cellulolytic bacterium producing a mixture of ethanol, acetate, lactate, hydrogen and carbon dioxide (Lynd et al. 1989). Its capability of hydrolyzing cellulose and producing ethanol has led to intensive investigations of this bacterium. The first successful transformation of the species was performed in 2006 (Tyurin et al. 2006), later on leading to the development of genetic systems to knock out the *pta* gene and thus acetate formation (Argyros et al. 2011). This strain, however, grew abnormally but retained its cellulase activity. At first, this strain did not produce more ethanol compared with the wild type but subsequent serial transfer resulted in 5.6 g/L of ethanol (52% of theoretical yields). Later work on *Cl. thermocellum* showed improved ethanol yields in a Δ*hpt*, Δ*ldh*, Δ*pta* evolved strain as well as the successful use of co-culture of this strain with *Thermoanaerobacterium saccharolyticum* (Argyros et al. 2011). Another approach to increase ethanol production was made by disrupting the sporulation pathway gene (*spo0A*) and then deletion of both *ldh* and *pta* genes (van der Veen et al. 2013). Thereafter the strain was adapted in continuous culture resulting in 29% of the theoretical yield.

4.3.3 *Thermoanaerobacter mathranii*

Thermoanaerobacter mathranii was isolated from an Icelandic hot spring (Larsen et al. 1997) and has later been modified and used in several investigations. The wild type produces from 62 to 90% of ethanol theoretical maximum (Georgieva et al. 2008; Yao and Mikkelsen 2010a, b) depending on the carbon source given. The first mutant generated was BG1L1 by knocking the LDH gene out of the wild type. This strain showed more than two-fold increase in ethanol production as compared to the wild type, up to 1.52 mol ethanol/mol xylose (Yao and Mikkelsen 2010b). The strains have also been shown to have similar ethanol yields from undetoxified pretreated corn stover and wheat straw (Georgieva and Ahring 2007; Georgieva et al. 2007, 2008). Further manipulation of this strain involves overexpression of NAD(P)H-dependent bi-functional aldehyde/ADH, resulting in the strain BG1E1. Clearly, this enzyme is of great importance for ethanol production and its over-expression resulted in higher ethanol yields (Crespo et al. 2012a, b). The electron balance for sugar degradation was also examined using mannitol, which is more reduced than glucose and xylose, as a substrate (Yao and Mikkelsen 2010a) leading to more ethanol production. Another *Thermoanaerobacter mathranii* strain was developed, BG1G1 where the gene encoding for NAD^+-dependent glycerol dehydrogenase was inserted. This increased ethanol production by 40% as compared to the wild type. Additionally, the strain utilized the highly reduced glycerol and co-metabolism of glycerol and sugars.

4.3.4 *Thermoanaerobacter italicus*

A recent paper using a lactate dehydrogenase, phosphotransacetylase and acetate kinase knockout strain of *Thermoanaerobacter* BG1, dubbed Pentocrobe 411 (DSM 23015), was recently described by Andersen et al. (2015). Pentocrobe 411 achieved impressive ethanol titers (1.84–1.92 mol ethanol/mol sugar equivalent) nearing the maximum theoretical yield from hexoses and pentoses on various pretreated biomass in continuous culture.

4.3.5 *Geobacillus thermoglucosidasius*

Thermophilic bacteria within the genus of *Geobacillus* have also attracted increased interest due to their ethanol production capacity recently. These bacteria are facultative anaerobes and can ferment various sugars to pyruvate by pyruvate dehydrogenase to acetyl-Coenzyme A (Cripps et al. 2009). Under aerobic conditions, however, pyruvate formate lyase is used and a variety of end products are formed. Cripps et al. (2009) manipulated *Geobacillus thermoglucosidasius*, obtaining an upregulated expression of pyruvate dehydrogenase under anaerobic conditions in lactate dehydrogenase-inactivated strain. Several mutants were developed (TM89; *ldh* knockout; TM180; *ldh* knockout and upregulated *pdh*; TM242; *ldh*, *pdh* up, and *pfl*). The TM180 strain produced 1.45 mol ethanol/mol hexose (the wild type produced 0.39 mol ethanol/mol hexose and TM89 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/mol) and good productivity rates. *Geobacillus thermoglucosidasius* has recently be genetically modified by expressing pyruvate decarboxylase from *Gluconobacter oxydans* (Van Zyl et al. 2014). Ethanol yields obtained were as high as 1.37 mol ethanol/mol glucose.

4.3.6 *Caldicellulosiruptor bescii*

New genetic tools have been used recently for insertion, deletion and heterologous/homologous expression of genes in *C. bescii* with considerable success (Chung et al. 2013a, b, 2014, 2015a, b).

One species, *C. bescii* has been genetically engineered to shift end product formation towards ethanol production (Cha et al. 2013). The wild type produces mainly lactate, acetate and hydrogen as main end products. Some members within the genus produce small amounts of ethanol but *C. bescii* does not. Recently, the species was targeted for gene deletion when the gene coding for lactate dehydrogenase (LDH) was deleted (Cha et al. 2013). The *ldh* gene was deleted by constructing a non-replicating plasmid which was introduced into the *C. bescii* chromosome by marker replacement. The resulting strain did not produce any lactate but instead increased acetate and hydrogen by 21–34% when cultivated on sugars (cellobiose and lactose) and on switch grass. Also, the authors noticed

increase in biomass formation because of the extra ATP produced via acetate production. Later work on this strain was performed to introduce ethanol production pathways (Chung et al. 2014). The authors introduced the NADH-dependent *adhE* gene from *Clostridium thermocellum* to the *ldh* mutant (JWCB018) resulting in strain *C. bescii* JWCB032. The wild type of *C. bescii* is very tolerant for ethanol concentrations and shows no inhibition up to 13.8 g/L. The wild type produced lactate (3.1 mM) and acetate (5.4 mM) from the three tested substrates (cellobiose, avicel, switchgrass). Not surprisingly, the *ldh* mutant produced only acetate and hydrogen from these substrates, but acetate concentrations increased. The ldh^{-} $adhE^{+}$ mutant produced lower yields of acetate (4.3 mM) and redirected the carbon flux to ethanol (14.8 mM from 29.2 mM cellobiose). This strain did however only use 4.4 mM of cellobiose, but ethanol yields were high, or 1.67 mol ethanol/mol glucose. This strain did however not produced ethanol above 65 °C, most likely since the alcohol dehydrogenase genes originate from a moderate thermophile. More recent efforts have therefore been to introduce *adhB* and *adhE* genes from a true thermophile, *Thermoanaerobacter pseudoethanolicus*, to the genome of the *ldh* mutant (Chung et al. 2015a). The two mutant strains obtained produced ethanol at higher temperatures, although yields were considerably lower as compared with the JWCB032 (the ldh^{-} $adhE^{+}$ mutant). The *adhB* mutant produced 1.4 mM ethanol on avicel, and 0.4 mM on switch grass as well as acetate (13.0 mM and 15.7 mM on avicel and switch grass, respectively). The *adhE* mutant produced 2.3 and 1.6 mm of ethanol on avicel and switch grass, respectively and 12.3 and 15.1 mM of acetate on avicel and switchgrass, respectively. The authors speculate that the main limiting factor for ethanol production is the availability of cofactors. The *C. thermocellum* mutant containing *adhE* utilizes NADH as an electron donor for both acetyl-CoA reduction to acetaldehyde (ALDH) and acetaldehyde reduction (ADH) to ethanol. *T. pseodoethanolicus adhB* and *adhE* however both utilize NADPH for ADH activity and adhB also uses NADPH for acetyl-CoA thioesterase activity. The authors hypothesize that low electron flux through NADPH could be the limiting factor for ethanol production since the AdhE pathway needs one NADPH per mole ethanol produced, while the AdhB pathway requires one NADPH. This difference then explains the different ethanol yields between the two *adh* mutants tested.

5 Challenges and Future Directions

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. The relatively low tolerance of thermoanaerobes to inhibitory compounds formed under high temperatures and acidic conditions remains a major challenge for ethanol production from lignocellulosic biomass. While some work indicates that some strains can

convert inhibitory aldehydes to other less toxic compounds, the inclusion of genes responsible for these enzymes could make a promising tool to improve the already impressive ethanologenic bacteria.

The main reason for the increased interest of using thermophiles for ethanol production is their ability to degrade broad array of substrates present in lignocellulosic biomass. Some of these bacteria, e.g. *Clostridium* and *Caldicellulosiruptor* are cellulolytic whereas others like *Thermoanaerobacter* and *Thermoanaerobacterium* are not. *Clostridium thermocellum* has been investigated and genetically manipulated to increase ethanol titer by eliminating production of acetate. The wild type of *Caldicellulosiruptor bescii* does not produce ethanol, but the insertion of ethanol producing genes has shown promising results. Most work on genetic engineering of thermophilic ethanol producing bacteria has been towards eliminating by-product formation in naturally good ethanol producers like *Thermoanaerobacter* and *Thermoanaerobacterium* that are not cellulolytic. Less work has been on inserting genes to make these bacteria cellulolytic. Often, manipulating fermentative pathways results in undesired consequences, e.g. when cutting off acetate formation the bacteria will get less energy and become less stable. Interestingly, many thermophiles that have been regarded as moderate ethanol producers and can be manipulated to produce more ethanol by simply increasing the partial pressure of hydrogen that will direct the flow of electrons to more reduced end products like ethanol and lactate. This type of microorganism would be ideal by simply cutting out the lactate formation pathway.

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