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Ethanol production by engineered thermophiles☆

Daniel G Olson[1,3], Richard Sparling[2] and Lee R Lynd[1,3]

We compare a number of different strategies that have been pursued to engineer thermophilic microorganisms for increased ethanol production. Ethanol production from pyruvate can proceed via one of four pathways, which are named by the key pyruvate dissimilating enzyme: pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), pyruvate formate lyase (PFL), and pyruvate ferredoxin oxidoreductase (PFOR). For each of these pathways except PFL, we see examples where ethanol production has been engineered with a yield of >90% of the theoretical maximum. In each of these cases, this engineering was achieved mainly by modulating expression of native genes. We have not found an example where a thermophilic ethanol production pathway has been transferred to a non-ethanol-producing organism to produce ethanol at high yield. A key reason for the lack of transferability of ethanol production pathways is the current lack of understanding of the enzymes involved.

Addresses
[1] Thayer School of Engineering at Dartmouth College, Hanover, NH 03755, United States
[2] Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada R3T 5V6
[3] BioEnergy Science Center, Oak Ridge, TN 37830, United States

Corresponding author: Lynd, Lee R (Lee.R.Lynd@Dartmouth.edu)

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Introduction

There is broad consensus that biomass has an important role to play in a low-carbon energy future [1], and that transport fuels are among the highest priority uses for biomass [2]. Ethanol is the biofuel produced in the largest amounts today worldwide, with essentially all resulting from fermentation by the mesophilic yeast *Saccharomyces cerevisiae* or closely related species [3].

Ethanol production using thermophilic bacteria has been suggested based on several factors. The property of thermophiles for which there is the strongest case for economic impact is the ability of some microbes from this group to rapidly ferment cellulosic biomass without added enzymes [4,5]. In addition, processing at elevated temperatures reduces the extent of heat exchange, both following pretreatment and prior to distillation, and may reduce the risk of contamination [4].

Notwithstanding the interest in thermophiles, most organisms in this class do not naturally carry out a homoethanol fermentation, and do not naturally exhibit high product tolerance. Strain development, often involving metabolic engineering, is required in order to address these deficiencies. This in turn requires advances in understanding the underlying metabolism of thermophilic microbes. As we make progress in strain development, we are learning that the metabolism of thermophilic anaerobes is more complex and more distinctive than previously imagined.

In this report we review recent understanding of the metabolism of thermophilic microbes, focusing primarily but not exclusively on anaerobes that ferment cellulose or hemicellulose. Thereafter, we document recent progress toward engineering these microbes to produce ethanol at high yield, and in some cases titer.

Metabolic pathways of ethanol production in thermophiles

The native capabilities of thermophilic organisms to produce ethanol have been reviewed recently ([6–9], Ana Faria Tomás, PhD thesis, Technical University of Denmark, 2013). In this work we will focus only on those thermophilic organisms that have been engineered for increased ethanol production. This includes several obligate anaerobic bacteria: *Thermoanaerobacterium saccharolyticum*, *Thermoanaerobacter ethanolicus*, *Thermoanaerobacter mathranii*, *Clostridium thermocellum* and *Caldicellulosiruptor bescii*, the facultative anaerobic bacterium *Geobacillus thermoglucosidasius*, the anaerobic archaeon *Pyrococcus furiosus* and the methylotrophic yeast *Ogataea polymorpha*. All of the organisms described above have had their genomes sequenced, allowing basic metabolic reconstructions to be performed computationally [10••].

Compared to a decade ago, understanding of converting pyruvate and various electron carriers into fermentation products has deepened substantially. Moreover this

☆ Note on formatting — in order to clarify the differences between genes, proteins and enzymes, names of genes are presented in lower case italic, proteins are presented in title case roman and enzyme activities are presented in all caps.

conversion is now known to be more complicated than formerly thought, particularly with respect to redox reactions. As might be expected given these observations, results of efforts to enhance ethanol production in thermophilic microorganisms via targeted molecular modifications have often not yielded the results predicted or desired. There are two key problems:

1. Although we have a good general understanding of the individual steps in fermentation pathways that lead to ethanol production, we do not fully understand the complex interactions between these various pathways.
2. For a given reaction, we do not know which gene or genes are responsible due to inaccuracies in annotation and apparent functional redundancies within the genomes.

Most organisms, like the ones discussed above, live in complex microbial communities where readily metabolized organic matter is a rather scarce commodity that requires solubilization of complex substrates [11]. This has led to the development of multiple fermentation branches leading to different end-products. The thermodynamic efficiency of a given pathway can vary depending on a variety of factors, including concentrations of cofactors such as NAD^+, $NADP^+$, NADH, NADPH, Acetyl-CoA, HS-CoA, AMP, ADP, ATP, and PP_i, as well as the concentration of carbon intermediates and end-products. The thermodynamics of the reactions are further modulated by both the temperature and the pH of the medium [10••]. Figure 1 illustrates the various pathways of pyruvate dissimilation in the organisms discussed. It is hypothesized that the different pathways are necessary in order to provide metabolic flexibility.

One way to think about individual pathways within a metabolic network is by considering key elementary modes. The theoretical maximum ethanol yield of all of the organisms described here is one ethanol per pyruvate, and is described by equation 1, where the NADH is assumed to come from the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction of glycolysis.

$$\text{Pyruvate} + \text{NADH} \rightarrow \text{ethanol} + CO_2 \quad (1)$$

For the network shown in Figure 1, there are four elementary modes that allow ethanol production at the theoretical maximum, and are described by the various combinations of the following enzymes: pyruvate decarboxylase (PDC), pyruvate dehydrogenase (PDH), pyruvate formate lyase (PFL), pyruvate ferredoxin oxidoreductase (PFOR), nicotinamide ferredoxin oxidoreductase (NFO), formate dehydrogenase (FDH), aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH).

Of these enzymes, PDC performs non-oxidative decarboxylation of pyruvate whereas other enzymes (PDH, PFL and PFOR) perform oxidative decarboxylation. Although an important distinction, in each case there are additional enzymes which can transfer the electrons back to ethanol and thus reaction (1) is valid for all four modes.

The combinations of enzyme activities corresponding to the four elementary modes of high-yield ethanol production are described by Eqs. (2)–(5).

$$\text{PDC} + \text{ADH} \quad (2)$$

$$\text{PDH} + \text{ALDH} + \text{ADH} \quad (3)$$

$$\text{PFL} + \text{FDH} + \text{ALDH} + \text{ADH} \quad (4)$$

$$\text{PFOR} + \text{NFO} + \text{ALDH} + \text{ADH} \quad (5)$$

Modes of ethanol production from pyruvate

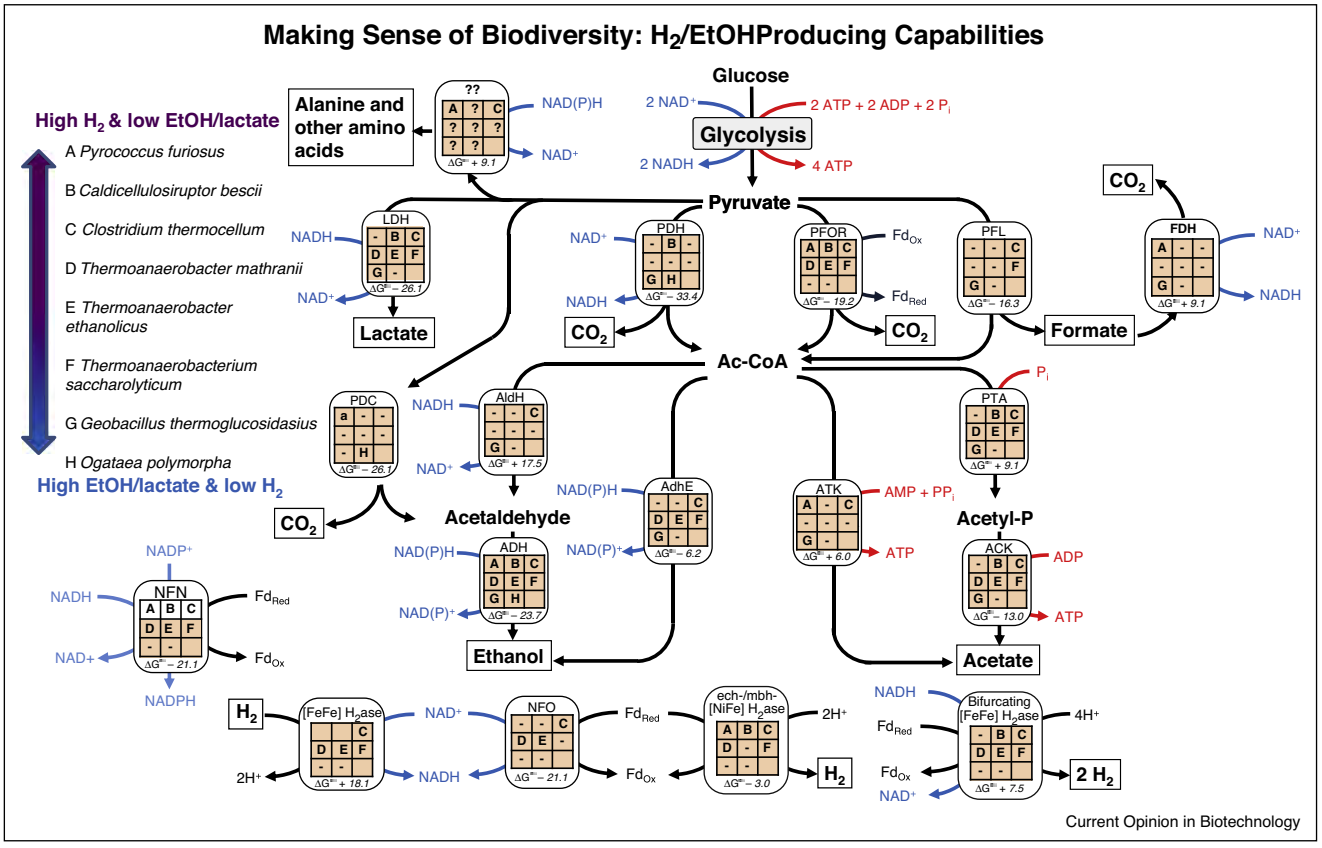
Since each mode can be identified by the enzyme used for pyruvate dissimilation (i.e. PDC, PDH, PFL or PFOR), we will use this enzyme name to refer to the whole mode. The PDC mode is frequently found in mesophilic organisms [12,13], however it is much less common in thermophiles (depending somewhat on the definition of a thermophile) and in this study it is only found in *O. polymorpha*. Although there are a variety of PDC enzymes with high thermostability [13], attempts to introduce this pathway into thermophilic bacteria have met with limited success [14,15]. It has been observed that the PFOR enzymes of some thermophilic archaea exhibit PDC activity [16,17], however none of these organisms have been shown to produce more than trace amounts of ethanol [10••].

The PFL mode is not found in any of the high-yielding organisms in this study. Although several organisms have a PFL enzyme, none of them have the formate dehydrogenase (FDH) enzyme needed to transfer electrons from formate to ethanol. This strategy has, however, been used for mesophilic ethanol production [18].

The PDH mode is found in *G. thermoglucosidasius*. Although PDH is found in *C. bescii* and *O. polymorpha* as well, both lack the ALDH enzyme necessary to allow ethanol production via this mode (Figure 1), and instead likely use it to generate acetyl-CoA for biosynthesis.

The PFOR mode is found in many obligate anaerobes including: *C. thermocellum*, *T. mathranii*, *T. ethanolicus* and *T. saccharolyticum*. Organisms using this mode generate reduced ferredoxin. In order to produce ethanol at high yield, electrons from the reduced ferredoxin need to be transferred to nicotinamide cofactors (NAD^+ or $NADP^+$). This can be accomplished by the $NAD(P)^+$-ferredoxin oxidoreductase (NFO) enzyme. Here we use NFO (alternatively FNO and FNOR) as a general term covering

Figure 1



A, B, C, and G: from Carere CR, Rydzak T, Verbeke TJ, Cicek N, Levin DB, Sparling R: Linking genome content to biofuel production yields: a meta-analysis of major catabolic pathways among select H2 and ethanol-producing bacteria. *BMC Microbiol* 2012, **12**:295. **D** and **E** are from Verbeke TJ, Zhang X, Henrissat B, Spicer V, Rydzak T, Krokhin OV, Fristensky B, Levin DB, Sparling R: Genomic Evaluation of *Thermoanaerobacter* spp. for the Construction of Designer Co-cultures to Improve Lignocellulosic Biofuel Production. *PLOS ONE* 2013, **8**: e593625758. **F**: while based on Shaw *et al.* (2008), was complemented by manual search and BLAST to confirm that the NFOR is related to *nfnAB*, that the *hyd* is related to the bifurcating hydrogenases. A further Fe–Fe hydrogenase was observed in the genome. The absence of other genes was confirmed both from the annotation as well as BLAST anchored in *C. thermocellum* and *T. thermohydrosulfuricum* WC1, including lack of membrane bound RNF-type NFO. ***nfnAB***: Presence/absence was based on Wang S, Huang H, Moll J, Thauer RK: $NADP^+$ reduction with reduced ferredoxin and $NADP^+$ reduction with NADH are coupled via an electron bifurcating enzyme complex in Clostridium kluyveri. *J Bacteril* 2010, **192**: 5115–23. For *C. thermocellum* it was through the analysis by Rydzak T, Grigoryan M, Cunningham ZJ, Krokhin OV, Ezzati P, Cicek N, Levin DB, Wilkins JA, Sparling R: Insights into electron flux through manipulation of fermentation conditions and assessment of protein expression profiles in *Clostridium thermocellum*. Appl Microbiol Biotechnol 2014, **98**: 6497–6510. With respect to **B** and **F** it was through BLAST and side-by-side location of both genes needed for *nfnAB*. The annotated genes in F corresponding to *nfnAB* were TheetDRAFT_0838 and 0839. **Lower case (a) for PDC**: based on Ma K, Hutchins A, Sung SJS, Adams MWW: Pyruvate ferredoxin oxidoreductase from the hyperthermophilic archaeon, *Pyrococcus furiosus*, functions as a CoA-dependent pyruvate decarboxylase. *Proc Natl Acad Sci U S A* 1997, **94**: 9608–13. ***O. polymorpha***: based on Ravin NV, Eldarov Ma, Kadnikov VV, Beletsky AV, Schneider J, Mardanova ES, Smekalova EM, Zvereva MI, Dontsova Oa, Mardanov AV, *et al.*: Genome sequence and analysis of methylotrophic yeast Hansenula polymorpha DL1. *BMC Genomics* 2013, **14**:837. Amino acid synthesis indicates organisms where this phenotype has been observed. Question marks indicate that amino acid production has not been reported in these organisms.

any reaction that transfers electrons from ferredoxin to a nicotinamide cofactor. Because of the difference in expected electronegativity between ferredoxin and NAD^+, or $NADP^+$, cells can take advantage of the $\Delta G^{\circ\prime}$ associated with this reaction to drive a thermodynamically unfavorable reaction. There are several classes of coupled NFO enzymes: RNF (rhodobacter nitrogen-fixation) couples the NFO reaction (NAD^+ specific) to transport of a Na^+ or H^+ ion across the membrane [19], MBX (membrane-bound oxidoreductase) also couples the NFO reaction ($NADP^+$ specific) to transport of H^+ across the membrane (note that this has not been confirmed experimentally, but is suggested by protein sequence analysis) [20], NFN (NADH-dependent reduced ferredoxin:$NADP^+$ oxidoreductase) couples the NFO activity with transhydrogenation (i.e. interconversion of NADH and NADPH) [21•]. Typically NFO activity is measured by enzyme assay with viologen dye.

Unfortunately this is a somewhat crude measurement, since viologen dyes are known to react with a variety of compounds in addition to ferredoxin. Determining the exact nature of the coupling often requires intricate biochemical experiments. Therefore the presence of NFO activity is often determined by sequence similarity. *C. thermocellum* contains an *rnf* gene cluster, *P. furiosus* contains an *mbx* gene cluster [20]. *C. thermocellum*, *T. mathranii*, *T. ethanolicus* and *T. saccharolyticum each* contain an *nfnAB* gene cluster [22,23].

The bifunctional AdhE protein

In organisms using the PFL, PDH or PFOR modes for ethanol production, the ALDH and ADH reactions are commonly mediated by a single protein, AdhE. In fact, the presence of the *adhE* gene is correlated with ethanol production in many organisms [10••]. Furthermore, deletion of *adhE* has been shown to eliminate anaerobic ethanol production in all organisms where this deletion has been created: *T. mathranii* [24], *Thermoanaerobacterium thermosaccharolyticum* [25], *C. thermocellum* [26], *T. saccharolyticum* [26] and *G. thermoglucosidasius* (personal communication with Michael Danson). Indeed, the weak link appears to be the production of acetaldehyde. Numerous fermentative thermophiles have alcohol dehydrogenases, as is the case in *Thermococcus guaymasensis*, *C. bescii* and most other extreme thermophiles, yet they do not have *adhE* and do not produce ethanol. Thus it appears that the lack of conversion of acetyl-CoA to acetaldehyde (i.e. ALDH activity) is preventing ethanol production in those strains.

The *adhE* gene is a frequent target for spontaneous mutations in ethanol producing strains. Mutations have been observed in *adhE* in several *Clostridium thermocellum* strains adapted for increased ethanol tolerance [27•,28•], as well as one strain engineered for increased ethanol production by deletion of hydrogenase genes [29]. Mutations in *adhE* have also been observed in strains of *T. saccharolyticum* engineered for increased ethanol production (Zheng *et al.*, unpublished data).

AdhE is clearly an interesting target for metabolic engineering. Recently the crystal structure of the ADH domain of the AdhE protein from *G. thermoglucosidasius* was determined to 2.5 Å resolution [30]. This detailed structure will allow for better predictions of targeted mutations.

Aldehyde ferredoxin oxidoreductase (AOR) pathway

Of the organisms that produce ethanol from acetyl-CoA, the most common pathway for subsequent ethanol production involves the ALDH reaction, which converts acetyl-CoA to acetaldehyde. There is an alternative pathway, however, where acetyl-CoA is converted to acetate by acetyl-CoA synthetase (ACS, Eq. (6)) and then acetate is converted to acetaldehyde by aldehyde ferredoxin oxidoreductase (AOR, Eq. (7)).

$$\text{acetyl-CoA} + \text{ADP} \rightarrow \text{Acetate} + \text{CoA} + \text{ATP} \quad (6)$$

$$\text{acetate} + \text{Fd(red)} \rightarrow \text{acetaldehyde} + \text{Fd(ox)} \quad (7)$$

This pathway was first suggested by White *et al.* in 1989 [31], and later mentioned as a theoretical possibility in *Clostridium ljungdahlii* [32], however direct evidence of the functioning pathway has only recently been presented [33•].

Acetate and ATP production

We have previously discussed elementary modes that allow for theoretical yield of ethanol production. There are a number of alternative modes for other fermentation products. Eq. (8) describes a mode for the production of acetate:

$$\text{Pyruvate} + \text{NADH} \rightarrow \text{acetate} + \text{ATP} + \text{CO}_2 + 2\text{H}_2 \quad (8)$$

Comparing Eqs. (1) and (8) shows the tradeoff between ethanol and acetate or H_2 production, if electrons are diverted to hydrogen production, additional ATP can be generated by acetate kinase. Further energy can be conserved in the form of a proton motive force through the transfer of electrons from reduced ferredoxin to protons generating H_2 via a membrane-integral energy-conserving proton-translocating NiFe-hydrogenase. The generation of H_2 from NAD(P)H via a hydrogenase is not thermodynamically favorable, so organisms that produce exclusively acetate, CO_2 and H_2 (Eq. (8)), couple the transfer of electrons from ferredoxin to H_2 with the transfer of electrons from NADH to H_2 via an electron-bifurcating hydrogenase [34••].

Amino acid production

Amino acids are an often-overlooked fermentation end-product. From a redox and carbon balance, the amino acids alanine and valine are equivalent to lactate (i.e. they consume pyruvate and NAD(P)H in a 1:1 ratio). Indeed alanine is a major end-product in wild type *Pyrococcus furiosus* [35], as well as a range of thermophilic Archaea and Bacteria [36]. Amino acids, including alanine and valine, as significant end-products have also been observed in *C. thermocellum* under certain growth conditions [37]. As we use molecular techniques to direct flux to a single end product, we need to be mindful of these alternative possibilities.

Metabolic engineering

For many years, the availability of genetic tools was a major limitation to engineering of thermophilic organisms. Over the past several years, genetic tools have been developed for the engineering of a variety of thermophilic

organisms, including examples from the genera *Clostridium*, *Thermoanaerobacterium*, *Thermoanaerobacter*, *Geobacillus*, *Caldicellulosiruptor* [7,38–40], *Pyrococcus* [41], and *Ogataea* [42]. Technologies are being developed to further broaden the suite of organisms for which molecular engineering is available.

Thermoanaerobacterium saccharolyticum

T. saccharolyticum is a gram-positive anaerobic thermophile that was originally isolated for its ability to grow on xylan at pH < 4.5 [43]. It natively produces ethanol, acetate, lactate, H_2 and CO_2.

Initial metabolic engineering of *T. saccharolyticum* JW/YS-485 involved the elimination of lactate production by deletion of the lactate dehydrogenase (*ldh*) gene, resulting in a 5% increase in ethanol yield [44,45•], Subsequent elimination of acetate production by deletion of phosphotransacetylase (*pta*) and acetate kinase (*ack*) resulted in a strain (ALK1) that produced only ethanol with yields of 90–100% of theoretical. This strain was cultivated in a continuous culture for 3000 h with increasing feed concentrations to produce strain ALK2, which achieved an ethanol yield of 92%, titer of 33 g/l and productivity of 2.2 g/l/h (Table 1) [45•]. In strain ALK2, both of the genetic modifications resulted in the chromosomal incorporation of an antibiotic resistance marker. Since only two antibiotic markers were available for *T. saccharolyticum* at the time, no further modifications could be performed with that strain. To overcome this problem, the *ldh* and *pta–ack* deletion strain was reconstructed with a maker recycling strategy [46••]. This new strain, M0355, had similar performance to ALK2, with an ethanol yield of 94%, titer of 25 g/l and productivity of 1.13 g/l/h (Table 1).

It has been shown that salt accumulation from pH control is a major factor limiting the growth of *Thermoanaerobacterium* strains [47]. Although strain M0355 did not produce significant quantities of organic acids, ethanol production resulted in the acidification of the medium due to uptake of ammonium. To reduce the need for pH control, the strain was engineered to use urea as a source of nitrogen [48]. This (along with adaptation and mutagenesis of the parent strain, M0863), enabled strain M1051 to achieve an ethanol titer of 54 g/l, while maintaining a yield of 88% of theoretical (Table 1). Further engineering was performed on this strain, including repair of a broken methionine gene and elimination of genes involved in the production of polysaccharide. The resulting strain, M1442, produced ethanol with a yield of 90% of theoretical, a titer of 61 g/l and a productivity of 2.13 g/l/h (Table 1). It is unclear whether these additional improvements were due to the genetic modifications or changes in fermentation conditions [49].

Another approach to engineering *T. saccharolyticum* was the deletion of hydrogenases to constrain electron flux. Conversion of pyruvate to acetyl-CoA via PFOR produces reduced ferredoxin (Figure 1). The electrons from ferredoxin can either be used for hydrogen production or ethanol production, and theoretically a decrease in hydrogen production should result in an equivalent increase in ethanol production. Deletion of the *hfs* hydrogenase resulted in 96% reduction in hydrogen production and 95% reduction in acetate production, but no change in ethanol production [50]. The organism instead distributed carbon and electron flux to lactate production. Subsequent elimination of lactate production by deleting the *ldh* gene increased ethanol yield, but it was still only 67% of theoretical (Table 1).

Thermoanaerobacter mathranii

Although it is a different genus, *T. mathranii* BG1 (wild type) is an anaerobic thermophile similar in physiology to *T. saccharolyticum*. It has a strong native ability to produce ethanol, with yields of 62–90% of the theoretical maximum [24,51,52]. The ethanol yield depends on the carbon source, with mannitol giving the highest yield, followed by xylose and then glucose. Several engineering strategies have been pursued to further increase the ethanol yield. In the wild type strain, the carbon flux not directed to ethanol production is directed to lactate and acetate production. The first strategy was deletion of lactate dehydrogenase in strain BG1 to generate strain BG1L1, which resulted in an improvement in ethanol yield of 3% [24] to 35% (Table 1) [52]. This improvement was seen on several different substrates, including glucose, xylose and mannitol.

A second engineering strategy attempted to increase the availability of reducing equivalents by expressing glycerol dehydrogenase (*gldA*) and feeding the strain glycerol in addition to either glucose or xylose [52]. Expression of *gldA* under control of a constitutive promoter led to a 55% decrease in ethanol production. Expression of *gldA* under control of a xylose-inducible promoter, in combination with a deletion of *ldh*, led to a 20% increase in ethanol production (compared to the *ldh* deletion alone). It is difficult to know the relative effect of the difference in promoter (constitutive versus xylose-inducible) compared with the difference in genetic background (wild type versus *ldh* deletion), since the factors were not tested separately. A further confounding factor is that the strains expressing *gldA* were found to consume at least some of the glycerol added to the media, but in many cases this was not factored into the ethanol yield calculations. This strain produced ethanol at a yield of 94% of theoretical (Table 1) [52].

A third engineering strategy was overexpression of the bifunctional alcohol and aldehyde dehydrogenase, *adhE*, which increased ethanol production by 10% compared with the parent strain (*ldh* deletion), during growth on xylose. Because the *adhE* gene was under the control of a

Table 1

Theoretical yield calculations assume that one glucose (or equivalent) molecule can be converted into two ethanol molecules, and one xylose molecule can be converted into 5/3 ethanol molecules. In cases where the amount of substrate consumed was not reported, it was assumed that the substrate was completely consumed

Organism	Strain	Description	Yield	Titer	Productivity	Temperature	Reference	Notes
			% theoretical maximum	g/l	g/l/h	°C		
PDC mode								
Ogataea polymorpha	DL-1 356	wild type	34%	13.0	-	48°C	Grabek-Lejko et al 2011	glucose, table 1
Ogataea polymorpha	DL-1 356 mcHpGSH2	overexpressing gamma glutamylcysteine synthetase	88%	45.0	-	48°C	Grabek-Lejko et al 2011	glucose, table 1
Ogataea polymorpha	DL-1 356 mcHpGSH2	overexpressing gamma glutamylcysteine synthetase	96%	19.6	0.99	48°C	Grabek-Lejko et al 2011	glucose, figure 1
Geobacillus thermoglucosidasius	TM89	ldh deletion	51%	-	-	45°C	Van zyl et al. 2013	assuming maximum theoretical ethanol yield of 0.51 g ethanol per g glucose
Geobacillus thermoglucosidasius	TM89 pGO111	ldh deletion expression wild type PDC from Gluconobacter oxydans	47%	-	-	45°C	Van zyl et al. 2013	assuming maximum theoretical ethanol yield of 0.51 g ethanol per g glucose
Geobacillus thermoglucosidasius	TM89 pGOF111	ldh deletion expression codon harmonized PDC from Gluconobacter oxydans	69%	-	-	45°C	Van zyl et al. 2013	assuming maximum theoretical ethanol yield of 0.51 g ethanol per g glucose
PDH mode								
Geobacillus thermoglucosidasius	NCIMB 11955	wild type	22%	3.5	0.54	60°C	Cripps et al. 2009	corrected for ethanol in gas phase
Geobacillus thermoglucosidasius	TM89	ldh deletion	52%	8.5	0.70	60°C	Cripps et al. 2009	corrected for ethanol in gas phase
Geobacillus thermoglucosidasius	TM236	ldh and pfl deletions	60%	6.4	0.86	60°C	Cripps et al. 2009	corrected for ethanol in gas phase
Geobacillus thermoglucosidasius	TM180	ldh deletion, pdh upregulatd	84%	14.5	2.23	60°C	Cripps et al. 2009	corrected for ethanol in gas phase
Geobacillus thermoglucosidasius	TM242	ldh and pfl deletions, pdh upregulated	90%	15.9	2.12	60°C	Cripps et al. 2009	corrected for ethanol in gas phase
PFOR mode								
Thermoanaerobacterium saccharolyticum	wt	wild type	66%	1.7	0.29	50°C	Desai, Guerinot and Lynd 2004	glucose, table 1, rate calculated from figure 4
Thermoanaerobacterium saccharolyticum	TD1	ldh deletion	69%	1.8	0.32	50°C	Desai, Guerinot and Lynd 2004	glucose, table 1, rate calculated from figure 4
Thermoanaerobacterium saccharolyticum	WT	wild type	75%	1.6	-	55°C	Shaw et al. 2008	xylose, table S1
Thermoanaerobacterium saccharolyticum	L-ldh	ldh deletion	79%	1.7	-	55°C	Shaw et al. 2008	xylose, table S1
Thermoanaerobacterium saccharolyticum	pta/ack	pta-ack deletion	100%	2.2	-	55°C	Shaw et al. 2008	xylose, table S1
Thermoanaerobacterium saccharolyticum	ALK1	ldh, pta-ack deletion	104%	2.2	-	55°C	Shaw et al. 2008	xylose, table S1
Thermoanaerobacterium saccharolyticum	WT	wild type	60%	3.4	-	55°C	Shaw et al. 2008	xylose, table S2
Thermoanaerobacterium saccharolyticum	ALK2	ldh, pta-ack deletion, evolved in chemostat	90%	5.5	-	55°C	Shaw et al. 2008	xylose, table S2
Thermoanaerobacterium saccharolyticum	ALK2	ldh, pta-ack deletion, evolved in chemostat	92%	33.0	2.20	55°C	Shaw et al. 2008	xylose, grown in chemostat, figure S1
Thermoanaerobacterium saccharolyticum	Wild type	wild type	58%	1.4	-	55°C	Shaw et al. 2011	cellobiose, table 1
Thermoanaerobacterium saccharolyticum	M0350	pyrF, pta-ack deletion	56%	0.6	-	55°C	Shaw et al. 2011	cellobiose, table 1
Thermoanaerobacterium saccharolyticum	M0350(pMU424)	pyrF, ldh deletion, pta-ack deletion repaired	70%	1.7	-	55°C	Shaw et al. 2011	cellobiose, table 1
Thermoanaerobacterium saccharolyticum	M0353	pyrF, pta-ack, ldh deletion	77%	1.0	-	55°C	Shaw et al. 2011	cellobiose, table 1
Thermoanaerobacterium saccharolyticum	M0355	pta-ack, ldh deletion	59%	0.8	-	55°C	Shaw et al. 2011	cellobiose, table 1
Thermoanaerobacterium saccharolyticum	M0355	pta-ack, ldh deletion	94%	25.3	1.13	55°C	Shaw et al. 2011	cellobiose, figure 2
Thermoanaerobacterium saccharolyticum	YS485	wild type	47%	1.2	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO1	hyd deletion	52%	1.3	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO2	ech deletion	46%	1.2	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO3	hfs deletion	41%	0.9	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO4	ech, hyd deletion	60%	1.5	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO5	ech, hfs deletion	39%	0.8	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO6	hfs, hydA deletion	32%	0.5	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	HKO7	hfs, ldh deletion	67%	0.8	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	ALK2	pta-ack, ldh deletion	101%	2.5	-	55°C	Shaw et al. 2009	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	M0863	pta-ack, ldh deletion, evolved for ethanol tolerance	79%	3.8	-	55°C	Shaw et al. 2012	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	M1051	pta-ack, ldh deletion, expressing ureABCDEFG at ldh locus	85%	12.6	-	55°C	Shaw et al. 2012	cellobiose, table 2
Thermoanaerobacterium saccharolyticum	M1051	pta-ack, ldh deletion, expressing ureABCDEFG at ldh locus	88%	54.3	-	55°C	Shaw et al. 2012	cellobiose, figure 3
Thermoanaerobacterium saccharolyticum	M1051	pta-ack, ldh deletion, expressing ureABCDEFG at ldh locus	83%	12.6	0.72	55°C	Shaw et al. 2012	cellobiose, figure 2
Thermoanaerobacterium saccharolyticum	M1442	Δpta-ack Δldh Δor796 urease metE Δeps	90%	61.0	2.13	55°C	Herring et al. 2012	cellulose with added enzyme, data from text
Thermoanaerobacter mathranii	BG1	wild type	62%	1.6	-	70°C	Yao and Mikkelsen 2010a	growth on xylose, calculated from data from figure 3
Thermoanaerobacter mathranii	BG1G1	ldh deletion, gldA from T. maritima, xylose inducible promoter	83%	1.8	-	70°C	Yao and Mikkelsen 2010a	growth on xylose, calculated from data from figure 3
Thermoanaerobacter mathranii	BG1	wild type	68%	1.6	-	70°C	Yao and Mikkelsen 2010a	growth on xylose, figure 4
Thermoanaerobacter mathranii	BG1L1	ldh deletion	80%	1.8	-	70°C	Yao and Mikkelsen 2010a	growth on xylose, figure 4
Thermoanaerobacter mathranii	BG1G1	ldh deletion, gldA from T. maritima, xylose inducible promoter	94%	2.3	-	70°C	Yao and Mikkelsen 2010a	growth on xylose, figure 4
Thermoanaerobacter mathranii	BG1L1	ldh deletion	83%	4.6	-	70°C	Georgieva et al. 2008	glucose and xylose, from table 1
Thermoanaerobacter mathranii	BG1	wild type	84%	2.3	-	70°C	Yao and Mikkelsen 2010b	xylose, from table 4
Thermoanaerobacter mathranii	BG1L1	ldh deletion	87%	2.4	-	70°C	Yao and Mikkelsen 2010b	xylose, from table 4
Thermoanaerobacter mathranii	BG1E1	ldh deletion, adhE expression with xylose inducible promoter	95%	2.6	-	70°C	Yao and Mikkelsen 2010b	xylose, from table 4
Thermoanaerobacter ethanolicus JW200	wt	wild type	89%	3.6	-	72°C	Wiegel and Ljungdahl 1981	glucose, table 2
Thermoanaerobacter ethanolicus JW200	wt	wild type	15%	0.3	-	45°C	Peng, Wu and Shao 2008	glucose, table 1
Thermoanaerobacter ethanolicus JW200	adhE	adhE overexpression	21%	0.4	-	45°C	Peng, Wu and Shao 2008	glucose, table 1
Clostridium thermocellum	wt	wild type	24%	0.7	-	55°C	Tripathi et al. 2010	cellobiose, figure 4
Clostridium thermocellum	ΔpyrF	pyrF deletion	25%	0.7	-	55°C	Tripathi et al. 2010	cellobiose, figure 4
Clostridium thermocellum	Δpta:gapDHp-cat	pyrF and pta deletion	30%	0.8	-	55°C	Tripathi et al. 2010	cellobiose, figure 4
Clostridium thermocellum	M0003	wt	12%	1.4	-	55°C	Argyros et al. 2011	cellulose, figure 3
Clostridium thermocellum	M1354	hpt	15%	1.6	-	55°C	Argyros et al. 2011	cellulose, figure 3
Clostridium thermocellum	M1375	hpt ldh	18%	2.0	-	55°C	Argyros et al. 2011	cellulose, figure 3
Clostridium thermocellum	M1448	hpt pta	18%	2.0	-	55°C	Argyros et al. 2011	cellulose, figure 3
Clostridium thermocellum	M1434	hpt ldh pta	23%	2.6	-	55°C	Argyros et al. 2011	cellulose, figure 3
Clostridium thermocellum	M1570	hpt ldh pta evolved	51%	5.6	-	55°C	Argyros et al. 2011	cellulose, figure 3
Clostridium thermocellum	M1726	Δhpt Δspo0A	17%	0.4	-	55°C	van der Veen et al 2013	cellobiose, table 2
Clostridium thermocellum	M1629	Δhpt Δspo0A Δldh	19%	0.5	-	55°C	van der Veen et al 2013	cellobiose, table 2
Clostridium thermocellum	M1630	Δhpt Δspo0A Δpta	29%	0.8	-	55°C	van der Veen et al 2013	cellobiose, table 2
Clostridium thermocellum	M1725	Δhpt Δspo0A Δldh Δpta, evolved	21%	0.5	-	55°C	van der Veen et al 2013	cellobiose, table 2
Clostridium thermocellum	WT 1313	wild type	29%	0.8	-	55°C	Biswas et al. 2014	cellobiose, figure 5
Clostridium thermocellum	adhE*(EA)	mutated adhE	2%	0.0	-	55°C	Biswas et al. 2014	cellobiose, figure 5
Clostridium thermocellum	adhE*(EA)Δldh	mutated adhE and ldh deletion	38%	1.0	-	55°C	Biswas et al. 2014	cellobiose, figure 5
Clostridium thermocellum	WT 1313	wild type	34%	0.9	-	55°C	Biswas et al. (unpublished)	cellobiose
Clostridium thermocellum	ΔhydG	deletion of all hydrogenases except ech	53%	1.4	-	55°C	Biswas et al. (unpublished)	cellobiose
Clostridium thermocellum	ΔhydGΔech	deletion of all hydrogenases except ech	61%	1.6	-	55°C	Biswas et al. (unpublished)	cellobiose
Clostridium thermocellum	WT	wild type	34%	0.9	-	55°C	Deng et al. 2013	cellobiose, figure 3 from corrigendum
Clostridium thermocellum	LL345	hpt deletion	36%	0.9	-	55°C	Deng et al. 2013	cellobiose, figure 3 from corrigendum
Clostridium thermocellum	DS8	LL345 with pyruvate kinase inserted at ldh locus	39%	1.0	-	55°C	Deng et al. 2013	cellobiose, figure 3 from corrigendum
Clostridium thermocellum	LL1113	DS8 with malic enzyme deletion	47%	1.2	-	55°C	Deng et al. 2013	cellobiose, figure 3 from corrigendum
Caldicellulosiruptor bescii	JWCB001	wild type	0%	0.0	-	65°C	Chung et al. 2014	cellobiose, table s2, rate data from figure 4
Caldicellulosiruptor bescii	JWCB018	ldh deletion	0%	0.0	-	65°C	Chung et al. 2014	cellobiose, table s2, rate data from figure 4
Caldicellulosiruptor bescii	JWCB032	ldh deletion and adhE overexpression	33%	0.7	-	65°C	Chung et al. 2014	cellobiose, table s2, rate data from figure 4
Pyrococcus furiosus	COM1	deletion of pyrF, for genetic manipulation	2%	0.05	-	72°C	Basen et al. 2014	maltose, figure 2C, residual substrate not reported
Pyrococcus furiosus	E	COM1 expressing adhE (Teth514_0627)	4%	0.10	-	72°C	Basen et al. 2014	maltose, figure 2C, residual substrate not reported
Pyrococcus furiosus	EA	COM1 expressing adhE (Teth514_0627) and adhA (Teth_0564)	17%	0.45	-	72°C	Basen et al. 2014	maltose, figure 2C, residual substrate not reported
Pyrococcus furiosus	A	COM1 expressing adhA (Teth514_0564)	35%	0.97	0.02	72°C	Basen et al. 2014	cellobiose, figure 2D, residual substrate not reported

Rate was calculated only when time-course data was available.

xylose-inducible promoter, the effect of *adhE* expression during growth on glucose was not measured. The resulting strain produced ethanol from xylose at a yield of 95% of theoretical [24].

Thermoanaerobacter ethanolicus

Wild type *T. ethanolicus* JW200 (ATCC 31550) is an anaerobic thermophile similar to both *T. mathranii* and *T. saccharolyticum*. A key feature distinguishing *T. ethanolicus* from *T. mathranii* is the lack of the *ech* hydrogenase, which may account for its higher native ethanol yield [53]. *T. ethanolicus* produces ethanol at 88% of theoretical yield under certain conditions, although this value has been reported to vary between 55% and 95% depending on growth conditions [54]. One engineering strategy used to increase ethanol production in this organism was overexpression of *adhE*. Transformation of plasmid pTE16 (with *adhE* cloned into it) into *T. ethanolicus* resulted in a 3-fold increase in ALDH activity and a corresponding 40% increase in ethanol production (Table 1) [55]. The ethanol and acetate production data reported for this strain only account for 18% (wild type) and 25% (*adhE* overexpression strain) of the glucose carbon initially present, so it is difficult to make any strong conclusions about the effect of this metabolic engineering strategy.

Clostridium thermocellum

C. thermocellum is an anaerobic thermophile. It is similar to the previously described *Thermoanaerobacter* and *Thermoanaerobacterium* species in that it produces ethanol, acetate, lactate, CO_2 and H_2 as its major fermentation products, however it is capable of solubilizing crystalline cellulose (which *Thermoanaerobacter* and *Thermoanaerobacterium* cannot) and does not consume pentose sugars (which *Thermoanaerobacter* and *Thermoanaerobacterium* do). It is this cellulose-solubilizing ability that has generated interest in metabolic engineering of *C. thermocellum* for ethanol production.

Initial attempts to engineer *C. thermocellum* focused on eliminating lactate and acetate production [56]. Elimination of acetate production by deletion of the *pta* gene had very little effect on ethanol production, although lactate production increased. Development of additional genetic tools allowed the deletion of both *ldh* and *pta* simultaneously [57]. Initially there was no increase in ethanol production, but subsequent serial transfer to improve growth resulted in a strain that produced ethanol at 51% of theoretical yield and a titer of 5.6 g/l (Table 1). Another approach for engineering *C. thermocellum* started with a disruption of the sporulation pathway gene *spo0A*, followed by deletions of *ldh* and *pta* and adaptation for rapid growth in a chemostat [37]. The best strain from this strategy produced ethanol at a yield of 29% of theoretical. It was discovered that the engineered strains were also producing large quantities of amino acids, in some cases 17% of carbon flux.

Another metabolic engineering strategy involved adapting *C. thermocellum* for growth in the presence of high concentrations of exogenous ethanol [58]. This strain produced less ethanol and more lactate, acetate and ethanol. Subsequent deletion of *ldh* eliminated lactate production and increased ethanol production. The resulting strain had a yield of 38% of theoretical (Table 1) [59].

One interesting feature of *C. thermocellum* is its lack of pyruvate kinase, a common enzyme in glycolysis. *C. thermocellum* is thought to convert phosphoenolpyruvate to pyruvate via oxaloacetate and malate (known as the 'malate shunt'). To test the hypothesis that this pathway was responsible for low yield, a pyruvate kinase gene from *T. saccharolyticum* was expressed in *C. thermocellum*. This did not have a dramatic effect on ethanol production, however subsequent deletion of malic enzyme increased the ethanol yield to 47% of theoretical (Table 1) [60].

Another metabolic engineering strategy that was attempted was the deletion of hydrogenases. Although *C. thermocellum* has a several different hydrogenases, many of them can be disabled by deleting a key hydrogenase maturation protein, *hydG*. Deletion of *hydG* increased ethanol yield from 34% to 53% of theoretical. Further deletion of the *ech* hydrogenase completely eliminated hydrogen production and further increased the ethanol yield to 61% of theoretical (Table 1,[29]).

Caldicellulosiruptor bescii

C. bescii is an extremely thermophilic anaerobic bacterium with an optimal growth temperature of 75 °C. It consumes both simple and complex polysaccharides (including cellulose) and produces lactate, acetate, carbon dioxide and hydrogen, but does not produce ethanol. To allow for ethanol production, first lactate production was eliminated by deletion of the *ldh* gene. Then a bifunctional aldehyde and alcohol dehydrogenase, *adhE* from *C. thermocellum*, was introduced. The resulting strain produced ethanol at 33% of the maximum theoretical yield (Table 1) [61•]. This strain converts a significant amount of cellobiose to glucose, which suggests that metabolic bottlenecks remain in upper glycolysis.

Pyrococcus furiosus

P. furiosus is a hyperthermophilic archaeon with an optimum growth temperature of 100 °C. It can consume maltose, cellobiose, beta-glucan, starch and protein. It produces carbon dioxide and hydrogen, but does not produce ethanol. This organism uses the AOR pathway (described above, Eqs. (6) and (7)) for production of acetaldehyde. Recently it was engineered for ethanol production by the expression of *adhA* from *Thermoanaerobacter* sp. X514, which allows the acetaldehyde from the AOR pathway to be converted to ethanol [33•]. The resulting strain produced ethanol with a yield of 35% of theoretical (Table 1).

Geobacillus thermoglucosidasius

G. thermoglucosidasius is a thermophilic, facultative-anaerobic bacillus that can ferment glucose, xylose and arabinose and can tolerate up to 10% ethanol (v/v) [6]. The wild type organism makes predominantly lactate, with small amounts of ethanol, acetate and formate also produced. The first metabolic engineering strategy was elimination of lactate production by deletion of the *ldh* gene, improving ethanol yield from 22% to 52% of theoretical (Table 1) [62••]. The next engineering strategy was the deletion of the pyruvate-formate lyase (*pfl*) gene and overexpression of the pyruvate dehydrogenase (*pdh*) gene. Flux through PFL results in electron transfer to formate. Those electrons can be conserved by formate dehydrogenase (FDH) activity (which is annotated to be present in *G. thermoglucosidasius*, Figure 1). In strains of *G. thermoglucosidasius* with PFL present, very little formate accumulates (less than 10% of carbon flux on a C3 basis). Unfortunately, since no *pdh* deletion has been described in this strain, we cannot say anything about the relative flux distribution between PFL and PDH, only that FDH flux is slightly lower than PFL flux. Of the three changes, both the *ldh* deletion and *pdh* overexpression substantially increased ethanol production. Deletion of *pfl* had a mixed effect on ethanol production depending on the strain background. Combining all three modifications (*ldh* deletion, *pfl* deletion and *pdh* overexpression) resulted in strain TM242 [62••]. This strain produced ethanol at a yield of 90% of theoretical, titer of 15.9 g/l and productivity of 2.12 g/l/h (Table 1).

Another strategy for increasing ethanol yield is introduction of the PDC pathway (Eq. (2)). A PDC enzyme from *Zymomonas mobilis* was expressed in *G. thermoglucosidasius* and found to function at 52 °C, although there was no change in ethanol production [14]. A *pdc* gene from *Gluconobacter oxydans* was expressed in the *G. thermoglucosidasius ldh* deletion strain and showed increased ethanol production at 45 °C, but not 52 °C [15]. The resulting strain, TM89 pGOF111, produced ethanol at 69% of theoretical yield (Table 1). Overall this strategy was not as successful as the strategy of overexpressing a native *pdh* gene.

Ogataea polymorpha

Ogataea (formerly *Hansenula*) *polymorpha* is a thermotolerant yeast that can produce ethanol from glucose at 48 °C. Although it has both pyruvate dehydrogenase (PDH) and pyruvate decarboxylase (PDC) enzymes, it seems likely that PDC is the primary pathway for ethanol production for the following reasons. In *O. polymorpha*, ethanol production under anaerobic conditions can be improved by overexpression of PDC [63]. In all eukaryotes that do not contain the bifunctional alcohol and aldehyde dehydrogenase gene, *adhE* (i.e. including *O. polymorpha*), ethanol is produced via PDC [64]. Finally, there are no reports of enzymes which can convert acetyl-CoA to acetaldehyde in yeast [65].

Another metabolic engineering strategy involved overexpression of gamma glutamylcysteine synthetase, which is thought to help the cell tolerate ethanol stress. The resulting strain, DL-1 356 mcHpGSH2, was able to produce ethanol with a yield of 96% of theoretical, titer up to 45 g/l and productivity of 1 g/l/h (Table 1) [66••].

Improving ethanol titer

As reviewed elsewhere [4], growth of thermophilic saccharolytic bacteria not previously exposed to ethanol is generally inhibited by modest (e.g. $\leq$20 g/l) concentrations of added ethanol. Growth in the presence of ethanol added at concentrations $\geq$50 g/l has been demonstrated for many organisms in this category following selection in the presence of ethanol. Maximum concentrations of ethanol produced by thermophilic bacteria are in general lower — typically by a factor of two or more — than the maximum concentrations of added ethanol that permit growth. We have previously termed this phenomenon the 'titer gap' [5]. Economical recovery of ethanol requires concentrations of >40 g/l [67]. Thus it appears that production of ethanol at these concentrations is not limited per se by tolerance, but by some other factor.

Closing the titer gap in order to produce commercially recoverable ethanol concentrations will likely require understanding the mechanisms of ethanol inhibition and taking steps to ameliorate them. Results reported to date point to imbalances in the concentrations of reduced and oxidized nicotinamide cofactors as being particularly important reasons for the cessation of growth and fermentation due to ethanol concentration. In both *C. thermocellum* and *T. pseudethanolicus*, it has been shown that mutations which reduce NADH-linked alcohol dehydrogenase activity are associated with increased ethanol tolerance [27•,68]. For *T. pseudethanolicus*, it has been suggested that elevated levels of NADH in the presence of ethanol inhibit glycolysis at the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) step [69]. In *O. polymorpha*, ethanol titer was improved from 13 to 45 g/l by overexpression of the GSH1 gene to increase intracellular levels of glutathione (Table 1) [66••]. Glutathione is a major cellular redox buffer, so modulating glutathione levels to increase ethanol production suggests that ethanol was causing a redox imbalance.

Exposure to ethanol has been correlated with changes in membrane composition in both *C. thermocellum* [70] and *T. ethanolicus* [68], and it has been suggested that these changes compensate for changes in membrane fluidity due to the presence of ethanol. Commenting on ethanol tolerance of *T. pseudethanolicus*, Lovitt *et al.* [69] suggest that inhibition by moderate ethanol concentrations (e.g. 4%) can be rectified by changes in redox metabolism, but

that cell membrane properties are responsible for tolerance and inhibition at 8% ethanol. In our view, redox imbalances are a more likely explanation than membrane effects for the titer gap exhibited by thermophiles in studies to date, but membrane effects may become important as redox imbalances are rectified.

Producing high concentrations of ethanol requires that high concentrations of substrate be fermented. Cessation of growth and/or fermentation at high substrate concentrations can occur for many reasons, and care must be taken to avoid mistakenly attributing such cessation to ethanol. Working with continuous cultures of *T. thermosaccharolyticum*, Baskaran *et al.* [47] attributed cessation of growth and fermentation at feed xylose concentrations exceeding 70 g/l to salt resulting from neutralizing organic acid production rather than to ethanol. Similarly, inhibition of *T. saccharolyticum* grown at high substrate concentrations was attributed to salt accumulation due to neutralization of acid produced in conjunction with ammonia uptake. Salt inhibition was avoided by introduction of the urease operon, resulting in the maximum ethanol titer increasing from 25 to 50 g/l [48]. The urea-utilizing strain of *T. saccharolyticum* was subsequently shown to produce ethanol at 61 g/l [49], believed to be the highest level reported for a thermophilic bacterium to date and sufficient for industrial application [4,67].

Improving understanding of metabolic pathways

Knowledge of metabolic network stoichiometry is required for the rational metabolic engineering. Genome analysis and automated annotation can provide a basic framework of metabolism. Functional genomics (proteomics, transcriptomics) can complement and refine our interpretation by allowing us to observe which metabolic pathways are operating under different growth conditions in both wild type and mutant strains [71,72]. However direct biochemical assay remains the gold standard for deepening our understanding of metabolism, particularly for subtle effects such as cofactor preference. For example, the role of GTP as an important energy currency in *C. thermocellum* has been recently described thanks to the observation that its glucokinase is GTP rather that ATP-dependent [73•]. Another example is the discovery of the widespread role of flavin-based electron bifurcation in microbial metabolism [34••].

In addition to studying a metabolic pathway in the context of its native host, pathways can be studied by transfer to an exogenous host. Attempts to transfer a pathway often result in the discovery of a previously overlooked component. For example, transferring the *pdc* gene from *Z. mobilis to E. coli* resulted in modest ethanol production which revealed the necessity of additionally transferring the *Z. mobilis adhB* gene [74]. To truly understand a pathway it is often necessary to transfer it into several different exogenous hosts.

Conclusion

To allow for inexpensive production of ethanol, an organism needs to achieve a yield of >90% of theoretical, titer of >40 g/l and productivity of >1 g/l/h [67]. There are many examples of metabolic engineering for increased yield, and it is possible to observe some similarities among approaches. Titer and productivity have only begun to be studied, and our understanding of the factors underlying these properties is still piecemeal.

Of the four elementary modes for ethanol production from pyruvate, we have found thermophilic examples for three of them: the PDH mode from *G. thermoglucosidasius*, the PFOR mode from *T. saccharolyticum* (and several others) and the PDC mode from *O. polymorpha*. In most of the cases presented, a native pathway has been modified to allow ethanol production near theoretical maximum yield. In *C. bescii* and *P. furiosus*, ethanol was not produced in the wild type strains (or produced at trace levels), and addition of an exogenous gene dramatically increased ethanol production, however in both of these strains the ethanol yield is far below the theoretical maximum.

There are no examples of transferring a high-yielding thermophilic ethanol production pathway to a strain with low ethanol production that result in ethanol production at near theoretical yield. By contrast, the mesophilic pET operon, consisting of pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*, reliably produces ethanol at high yield in a wide range of mesophilic organisms [75–77]. It is hoped that by better understanding the genes and pathways involved in the thermophilic ethanol production pathways, we will one day be able to transfer these pathways to new organisms as readily as the pET pathway in mesophiles.

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