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Redirecting carbon flux through exogenous pyruvate kinase to achieve high ethanol yields in *Clostridium thermocellum*

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ABSTRACT

In *Clostridium thermocellum*, a thermophilic anaerobic bacterium able to rapidly ferment cellulose to ethanol, pyruvate kinase (EC 2.7.1.40) is absent based on both the genome sequence and enzymatic assays. Instead, a new pathway converting phosphoenolpyruvate to pyruvate via a three-step pathway involving phosphoenolpyruvate carboxykinase, NADH-linked malate dehydrogenase, and NADP-dependent malic enzyme has been found. We examined the impact of targeted modification of enzymes associated with this pathway, termed the "malate shunt", including expression of the pyruvate kinase gene from *Thermoanaerobacterium saccharolyticum*, mutation of the phosphoenolpyruvate carboxykinase and deletion of malic enzyme gene. Strain YD01 with exogenous pyruvate kinase, in which phosphoenolpyruvate carboxykinase expression was diminished by modifying the start codon from ATG to GTG, exhibited 3.25-fold higher ethanol yield than the wild-type strain. A second strain, YD02 with exogenous pyruvate kinase, in which the gene for malic enzyme and part of malate dehydrogenase were deleted, had over 3-fold higher ethanol yield than the wild-type strain.

1. Introduction

Cellulosic biomass has many attractive features as a raw material for production of fuels and chemicals (Lynd et al., 2008), but is not widely used for this purpose today because of its recalcitrant character (Himmel et al., 2007; Lynd et al., 1999). Consolidated bioprocessing (CBP), featuring the production of saccharolytic enzymes, plant cell wall solubilization, and fermentation of resulting in sugars a single, integrated step, is a promising strategy for reducing processing costs (Olson et al., 2011).

Clostridium thermocellum is a thermophilic gram-positive bacterium able to ferment cellulose and products of cellulose solubilization to ethanol, acetic acid, lactic acid, formic acid, hydrogen, and CO_2. *C. thermocellum* appears to be a cellulose-utilizing specialist (Demain et al., 2005; Lynd et al., 2002), and is of interest for development for CBP.

Several microbes that produce a mix of fermentation products similar to *C. thermocellum*, including the thermophile *Thermoanaerobacterium saccharolyticum*, have been engineered to produce ethanol at high yield (Shaw et al., 2008). Achieving this objective in *C. thermocellum* has, however, met with limited success to date. In particular, Argyros et al. (2011) found that targeted deletion of enzymes associated with organic acid formation decreased acetate and lactate formation, but did not appreciably increase ethanol yields.

Apparently in *C. thermocellum*, the biochemistry of conversion of sugars to ethanol and acetate is different from that in *T. saccharolyticum* (Shaw et al., 2008). It was therefore decided to investigate one of the major differences between the two organisms namely the nature of the conversion of phosphoenolpyruvate (PEP) to pyruvate. Lamed and Zeikus (1980) proposed a pyridine nucleotide transhydrogenation pathway based on detection of malate dehydrogenase and malic enzyme activities and their cofactor dependence. Pyruvate kinase (EC:2.7.1.40), which mediates the conversion of phosphoenolpyruvate (PEP) to pyruvate is normally the final step in the Embden–Meyerhof–Parnas (EMP) pathway but has not been identified in the genome sequence of *C. thermocellum* DSM 1313(Feinberg et al., 2011). Based on these observations, it appears that conversion of PEP to pyruvate may occur via a "malate shunt" consisting of reaction of PEP to oxaloacetate (OAA) catalyzed by phosphoenolpyruvate carboxykinase (PEPCK, EC 4.1.1.32, Clo1313_0415), reaction of OAA to malate catalyzed by malate dehydrogenase (MDH, EC 1.1.1.37, Clo1313_1878),

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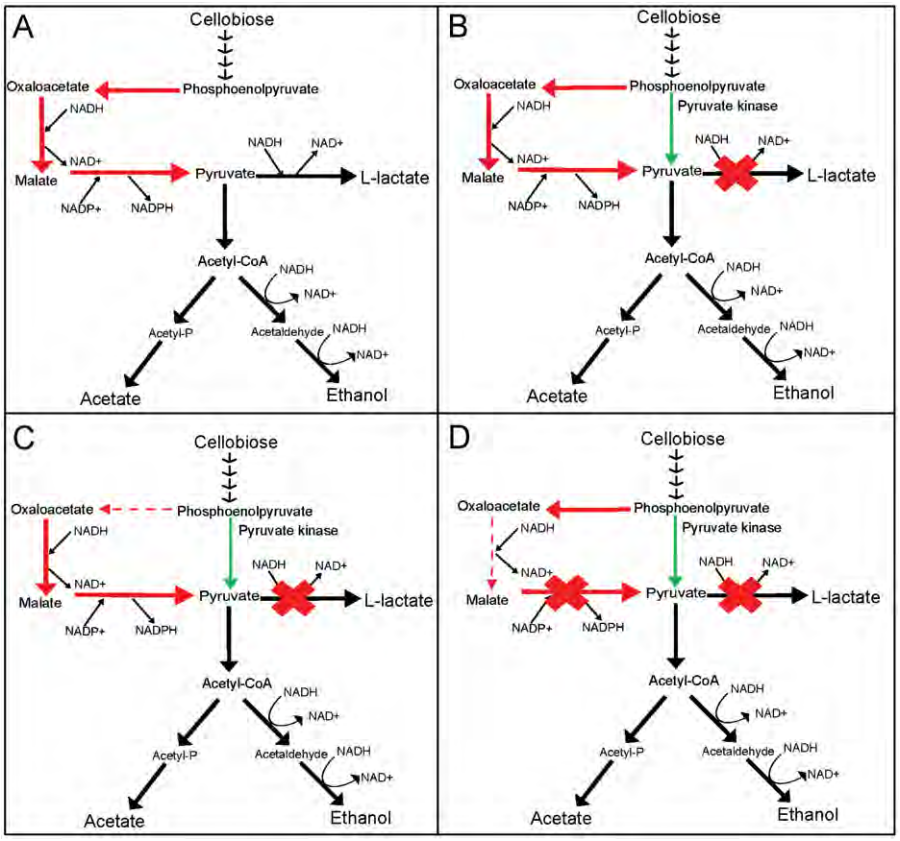

Fig. 1. The genetic modifications made in *C. thermocellum* to increase ethanol yields. Red arrows: the genes involving malate shunt; red broken arrows: the gene was down-regulated or partially deleted; red crosses: the genes which have been deleted; Green arrow: expression of pyruvate kinase from *T. saccharolyticum*. (A) glycolysis of wild-type strain; (B) DS8: Δ*hpt*, Δ*ldh*::*Peno-pyk*; (C) YD01: Δ*hpt*, Δ*ldh*::*Peno-pyk*, *pepck* (down-regulate); (D) YD02: Δ*hpt*, Δ*ldh*::*Peno-pyk*, Δ*me*, Δ*mdh* (partial deletion). *Peno*: promoter for enolase; *pyk*: pyruvate kinase from *T. saccharolyticum*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
The strains and plasmids used in this study.

Strain/plasmid	Relevant genotype/comments	Source
E. coli Top10	mcr*A*, Δ(mrr-hsd*RMS*-mcr*BC*), ΔlacX74, deo*R*, rec*A1*, ara*D*139Δ(ara-leu)7697, gal*K*, rps*L*, end*A1*, nup*G*	Invitrogen
E.coli BL21	*fhuA2 [lon],ompT, gal (λ DE3), dcm, ΔhsdS, λ DE3 = λ sBamHIo, ΔEcoRI-B, int::(lacI::PlacUV5::T7 gene1) i21, Δnin5*	NEB
C. thermocellum DSM1313	Wild-type	DSMZ
C. thermocellum DS8	*Δldh::pyk, Δhpt*	This study
C. thermocellum YD01	*Δldh::pyk, Δhpt*	This study
C. thermocellum YD02	*Δldh::pyk, Δhpt, Δme, Δmdh*	This study
T. saccharolyticum JW/SL-YS485	Wild-type	Mascoma Corporation
Plasmid		
pDGO-05	Insertion of pyruvate kinase vector	This study
pYD01	Replacing ATG by GTG on PEPCK vector	This study
pYD03	Deletion of malic enzyme and partial deletion of malate dehydrogenase vector	This study

and reaction of malate to pyruvate by malic enzyme (ME, EC 1.1.1.40, Clo1313_1879) (Lamed and Zeikus, 1980). This pathway is termed the ''malate shunt''. The overall result of this shunt is transhydrogenation of NADH to NADPH (Fig. 1A).

Argyros et al. (2011) deleted the pathways for organic acids production but the ethanol yield was not increased significantly. Motivated by the above mentioned work, we undertook to investigate an approach based on manipulating the activity of enzymes impacting the supply and demand of nicotinamide cofactors. In particular, we hypothesized that reducing flux through the malate shunt could increase NADH availability and thereby increase the yield of ethanol produced by the NADH-linked bifunctional ADHE enzyme operative in *C. thermocellum*. Results with mutants designed to test this hypothesis are reported herein.

2. Materials and methods

2.1. Media, culture conditions and reagents

C. thermocellum strains were grown on low carbon media (Holwerda et al., 2012) by adding 1 g/L of yeast extract. *C.*

thermocellum was grown anaerobically at 55 °C unless otherwise noted. *Escherichia coli* was grown aerobically at 37 °C in LB broth. *Saccharomyces cerevisiae* was grown in YPD medium or synthetic uracil dropout media (Shanks et al., 2006). The strains and plasmids used in this study are summarized in Table 1. All *C. thermocellum* strains in this study were derived from *C. thermocellum* DSM1313 (WT).

2.2. Insertion of pyruvate kinase into C. thermocellum

The pyruvate kinase gene was amplified by PCR from *T. saccharolyticum* and cloned downstream from the native *C. thermocellum* enolase promoter to generate plasmid pDGO-05(Supplementary information Fig. S1) by using yeast cloning (Argyros et al., 2011; Gibson, 2009). This plasmid was transformed into strain M1354 (*Δhpt* strain) (Olson, 2012) and selected for the insertion of the pyruvate kinase gene at the *C. thermocellum ldh* locus (Argyros et al., 2011; Olson et al., 2010; Tripathi et al., 2010). Those cells were then grown in the presence of 8-azahypoxanthine (8-AZH) to select for colonies having lost the hpt-cat cassette. The resulting strain was designated DS8.

2.3. Down-regulation of phosphoenolpyruvate carboxykinase.

The general process of changing the start codon was as described previously (Argyros et al., 2011; Tripathi et al., 2010). In order to change the start codon from ATG to GTG, the replicating plasmid pYD01 was used (Supplementary information Fig. S2). Mutant selection was carried out using the knockout cassette assembled in the yeast derived from that reported in Olson (2012), Olson et al. (2010) as presented in Supplementary information Fig. S3. Two DNA fragments homologous to upstream regions

Table 2
Enzymatic activities (μmole/min/mg) of the malate shunt in WT and mutants of *C. thermocellum*. The data represent the average of duplicate assays performed with cell extracts from three different cultures.

Enzyme	Strain			
	WT	DS8	YD01	YD02
Pyruvate kinase (ADP)	< 0.001*	0.52 ± 0.05	0.14 ± 0.01	0.84 ± 0.08
Phosphoenolpyruvatecarboxykinase (GDP)	4.14 ± 0.43	4.09 ± 0.05	0.54 ± 0.03	< 0.001*
Phosphoenolpyruvatecarboxykinase (ADP)	< 0.001*	< 0.001*	< 0.001*	< 0.001**
Malate dehydrogenase (NADH)	1.67 ± 0.11	10.89 ± 2.59	0.78 ± 0.16	0.60 ± 0.01
Malate dehydrogenase (NADPH)	< 0.001*	< 0.001*	< 0.001*	< 0.001**
Malic enzyme ($NADP^+$)	1.97 ± 0.25	4.46 ± 1.19	3.01 ± 0.11	< 0.001*
Malic enzyme (NAD^+)	< 0.001*	< 0.001*	< 0.001*	< 0.001*
Pyruvate, phosphate dikinase	< 0.001*	< 0.001*	< 0.001*	< 0.001*

* Below the detection limit; ± means SD.

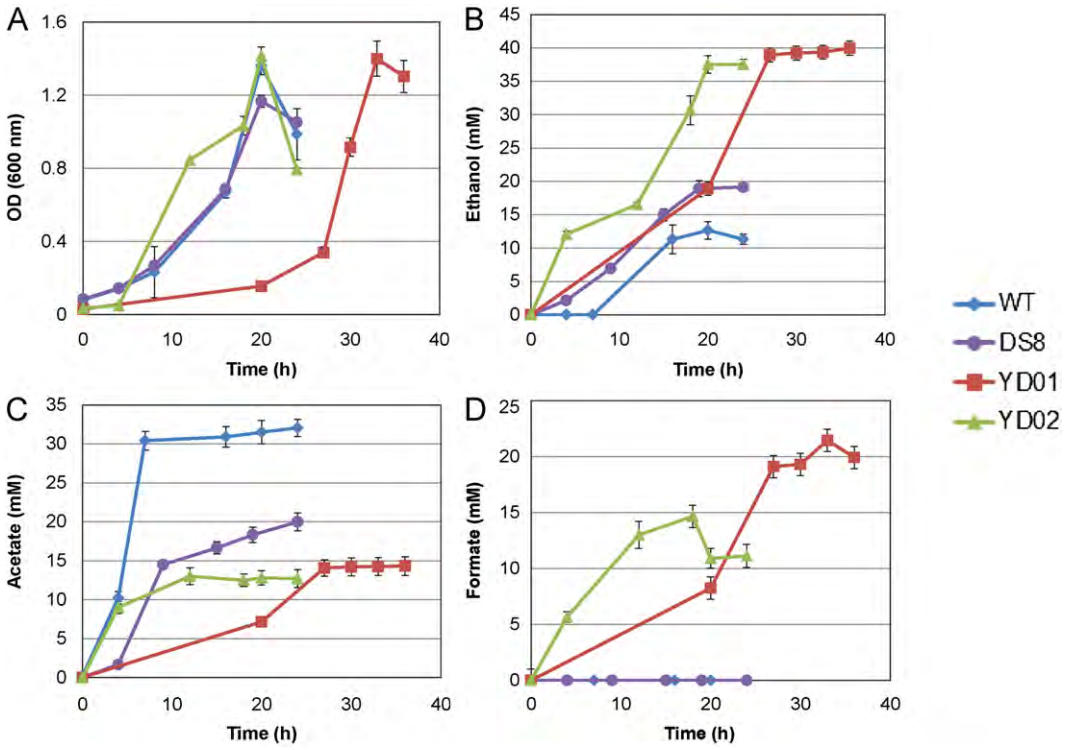

Fig. 2. Cell growth and fermentation products in *C. thermocellum* WT, DS8, YD01. and YD02 on 5 g/L(14.6 mM) cellobiose. (A) Cell growth; (B) Ethanol productions; (C) acetate productions; (D) formate productions.

of PEPCK were synthesized and denoted 5′upstreamA and 5′upstreamB. At the 5′ end of upstreamB, there was a 60 bp coding part of PEPCK containing the start codon GTG. Plasmid pYD01 was transformed into *C. thermocellum* DS8. GTG was introduced into chromosome of DS8 by homologous recombination. Those cells were then grown in the presence of 8-azahypoxanthine (8-AZH) to select for colonies having lost the hpt-cat cassette. The resulting strain was designated YD01.

2.4. Knockout malic enzyme gene in C. thermocellum

The disruption of malic enzyme gene (Clo1313_1879) was based on the protocol described by (Argyros et al. (2011) using replicating plasmid pYD03 (Supplementary information Fig. S4) shown in Supplementary information Fig. S5. A 0.7 kb sequence upstream of Clo1313_1879 and a 0.48 kb sequence downstream of Clo1313_1879 and 0.6 kb internal to Clo1313_1879 were amplified and assembled to form plasmid pYD03 by yeast assembly (Gibson et al., 2008). The 0.48 kb sequence on the downstream of Clo1313_1879 was assembled by 3 pieces of synthetic ultra-oligos (Supplementary information Table S1). After recombination, there were two identical pieces of 0.7 kb sequence, which overlapped each other to eliminate the hpt-cat cassette (Supplementary information Fig. S5).

2.5. Enzymatic assays

To determine enzymatic activities in cell extract, 50 mL of *C. thermocellum* cells were harvested in late exponential growth phase in serum bottles, washed twice by 10 mL 100 mM Tris/HCl buffer, pH 7.5, and resuspended in 1 mL of the same buffer. After disruption of the cells by sonication (Fong and Deng, 2010a) and subsequent centrifugation for 5 min at 15871 × *g* and 4 °C, the supernatant was used for assays (Riedel et al., 2001). Specific activity was defined as: 1 U/mg = 1 μmole-NAD(P)H/min/mg-protein. If the activity was less than 0.001 U/mg, the enzyme was determined to be absent. Unless otherwise indicated, all assays were performed at 55 °C for 1–5 min. The cell extracts from the same strain were obtained from three different cultures with equal inoculum. The quantity of cell extract was varied to ensure that activity was measured in the linear range. The enzymatic results were measured by biological triplicates. All above procedures were conducted under anaerobic conditions.

2.5.1. Phosphoenolpyruvate carboxykinase (PEPCK) assay

Phosphoenolpyruvate was converted to oxaloacetate by phosphoenolpyruvate carboxykinase and oxaloacetate was then converted to malate by adding malate dehydrogenase (Sigma-Aldrich) which oxidized NADH. The activity was measured by the decrease of NADH detected by absorbance at 340 nm (Jabalquinto et al., 1999; Kim et al., 2004).

The reaction volume was 1 mL, consisting of 100 mM Tris/HCl buffer, 5 mM $MgCl_2$, 5 mM DTT, 10 mM $NaHCO_3$, 2 mM GDP, 2 mM NADH, 6 U of malate dehydrogenase and 5 mM of phosphoenolpyruvate by adding 20 μL cell extract. The reaction was initiated by adding phosphoenolpyruvate.

2.5.2. Pyruvate kinase assay

The reaction rate was determined in a lactate dehydrogenase-coupled assay system by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH (Pon and Bondar, 1967). The reaction volume was 1 mL, consisting of 100 mM Tris/HCl buffer, 5 mM $MgCl_2$, 5 mM DTT, 10 mM KCl, 2 mM ADP (GDP), 0.2 mM NADH, 10 U of lactate dehydrogenase (Sigma-Aldrich) and 5 mM of phosphoenolpyruvate by adding 50 μL cell extract. The reaction was initiated by adding phosphoenolpyruvate. *T. saccharolyticum* with known pyruvate kinase were used as positive controls.

2.5.3. Malate dehydrogenase

The reaction rate was determined by measuring the decrease in absorbance at 340 nm resulting from oxidization of NADH to NAD^+ (Rokosh et al., 1973). The reaction volume was 1 mL, consisting of 100 mM Tris/HCl buffer, 5 mM $MgCl_2$, 5 mM DTT, 0.2 mM NADH, and 10 mM of oxaloacetate by adding 20 μL of cell extract. The reaction was initiated by adding oxaloacetate.

2.5.4. Malic enzyme assay

The reaction rate was determined by measuring the increase in absorbance at 340 nm resulting from reduction of $NADP^+$ or NAD^+ (Iwakura et al., 1979; Lamed and Zeikus, 1981). The reaction volume was 1 mL, consisting of 100 mM Tris/HCl buffer, 5 mM $MgCl_2$, 5 mM DTT, 5 mM of NH_4Cl, 0.2 mM $NADP^+$ and 2 mM malate by adding 20 μL of cell extract. The reaction was initiated by adding malate.

2.5.5. Pyruvate, phosphate dikinase assay

The reaction rate was determined by measuring the increase in absorbance at 340 nm resulting from reduction of $NADP^+$ or NAD^+ (Iwakura et al., 1979; Lamed and Zeikus, 1981). The reaction volume was 1 mL, consisting of 100 mM Tris/HCl buffer, 5 mM $MgCl_2$, 5 mM DTT, 5 mM of NH_4Cl, 0.2 mM NADH, 2 mM pyruvate, 8 U malate dehydrogenase (Sigma-Aldrich), 8 U phosphoenolpyruvate carboxylase (Sigma-Aldrich), and 2 mM ATP by adding 10 μL of cell extract. The reaction was initiated by adding ATP.

2.6. Gene expression

Gene expression was measured by real-time PCR using the general procedure described by Fong and Deng (2010a, 2010b), Olson and Lynd (2012) with primers as presented in Supplementary information Table S1. Three reference genes, Clo1313_1884 (ATP-binding protein), Clo1313_0023 (thiamine pyrophosphate TPP-binding domain-containing protein), Clo1313_1163 (recA protein) used in this study to normalize the expression.

2.7. Ethanol tolerance

The ethanol was added into the sterile low carbon media with 1 g/L yeast extract in the purged serum bottles to make 10 g/L, 15 g/L, 25 g/L, 35 g/L and 40 g/L ethanol respectively and the total volume was maintained 50 mL. WT, DS8, YD01 and YD02 strains were inoculated into these serum bottles with 1% inoculation size. The serum bottles were incubated at 55 °C. The OD at 600 nm was used to test the cell densities. All the results were measured by biological triplicates.

2.8. Amino acids analysis

The amino acids samples were taken when the strains reached the maximal ethanol yields and samples were frozen immediately by dry ice and then shipped to amino acid.com for measurements. Each strain had at least three independent cultures.

2.9. Analytical techniques

The carbon recovery was calculated according to Ellis et al. (2012). In general, carbon recovery was defined: carbon atoms output system per carbon atoms input the system. The major fermentation products were determined by using a Waters HPLC system with an Aminex HPX-87H column operated at 60 °C.

Sample collection and processing were as reported previously (Shao et al., 2011). The carbon in biomass was measure by TOC/N (Shimadzu); hydrogen was measured by gas chromatography system by Mascoma Corporation (Ellis et al., 2012). Oxidation/ reduction (O/R) balance was calculated as described by Johnson et al. (1931).

3. Results

3.1. Improvements of carbon flux to ethanol

3.1.1. Expression of pyruvate kinase in C. thermocellum

The genome sequence of *C. thermocellum* does not contain a gene identified as pyruvate kinase (EC 2.7.1.40). Consistent with this, pyruvate kinase activity was not detected in cell extracts of *C. thermocellum*. In cell extracts of *T. saccharolyticum*, which were used as a positive control, the enzyme was readily detectable. The pyruvate kinase activity in this organism was 0.44 U/mg. The activity of pyruvate, phosphate dikinase (PPDK) has the reverse reaction catalyzing PEP to pyruvate in some organisms (Tjaden et al., 2006). The PPDK activity was tested in *C. thermocellum* and there was no activity detected (Table 2).

In view of the absence of pyruvate kinase in *C. thermocellum*, it was decided to express the *T. saccharolyticum* gene encoding pyruvate kinase in *C. thermocellum*. The pyruvate kinase gene (Tsac_1363) from *T. saccharolyticum* was inserted into the chromosome of *C. thermocellum* under control of the native enolase promoter by replacing the lactate dehydrogenase gene (Fig. 1B). Insertion of the pyruvate kinase gene was verified by PCR and sequencing, and the resulting strain was denoted DS8.

Strain DS8 grew as fast as the wild-type (Fig. 2A) and it produced 19.1 mM ethanol from 14.6 mM cellobiose whereas the wild-type strain produced 12.0 mM ethanol (Fig. 2B). At the same time, DS8 produced 18.5 mM ethanol from 5 g/L of Avicel compared to 12.4 mM ethanol in WT (Fig. 3A). The ethanol yield of strain DS8 on cellobiose (0.65 mole-ethanol/mole-glucose equivalent) was 1.6-fold higher than that in WT (0.41 mole-ethanol/mole-glucose equivalent) (Fig. 3C). The ethanol yield of strain DS8 on Avicel was (0.59 mole-ethanol/mole-glucose equivalent) 1.48-fold higher than that in WT. WT produced 1.64 moles/mole-glucose equivalent H_2, which is close to 1.6 moles/mole-glucose equivalent found by Levin et al. (2006), but DS8 produced much less H_2 (1.18 moles/mole-glucose equivalent) (Fig. 3B). This result indicates that less-reduced cofactors were consumed to produce H_2 instead of ethanol in DS8. The gene expression level of the heterologous pyruvate kinase in strain DS8 was 5.2 compared to zero in the wild-type strain. The enzymatic activity of pyruvate kinase in DS8 was 0.52 U/mg, comparable to its level in *T. saccharolyticum* (Table 2).

3.1.2. Targeted modifications of malate shunt enzymes

To further direct phosphoenolpyruvate flux through pyruvate kinase in lieu of the malate shunt, we sought to down-regulate phosphoenolpyruvate carboxykinase (PEPCK) by constructing a derivative of strain DS8 in which the ATG start codon of the *pepck* gene was replaced by GTG (Fig. 1C).

Strain YD01 grew much slower than DS8 and wild-type strains (Fig. 2A). It produced 38.8 mM ethanol from 14.6 mM of cellobiose whereas the wild-type and DS8 strains produced 12.0 mM and 19.1 mM ethanol, respectively (Fig. 2B). The ethanol yield of YD01 was 1.33 mole-ethanol/mole-glucose equivalent, twice that of strain DS8, and over 3 times that of the wild type (Fig. 3C). YD01 had good performance on 5 g/L Avicel by producing 36.3 mM ethanol with 1.18 mole-ethanol/mole glucose equivalent yield (Figs. 3A and C).

As an alternative approach to directing flux away from the malate shunt, a NADP-dependent malic enzyme (Clo1313_1879)

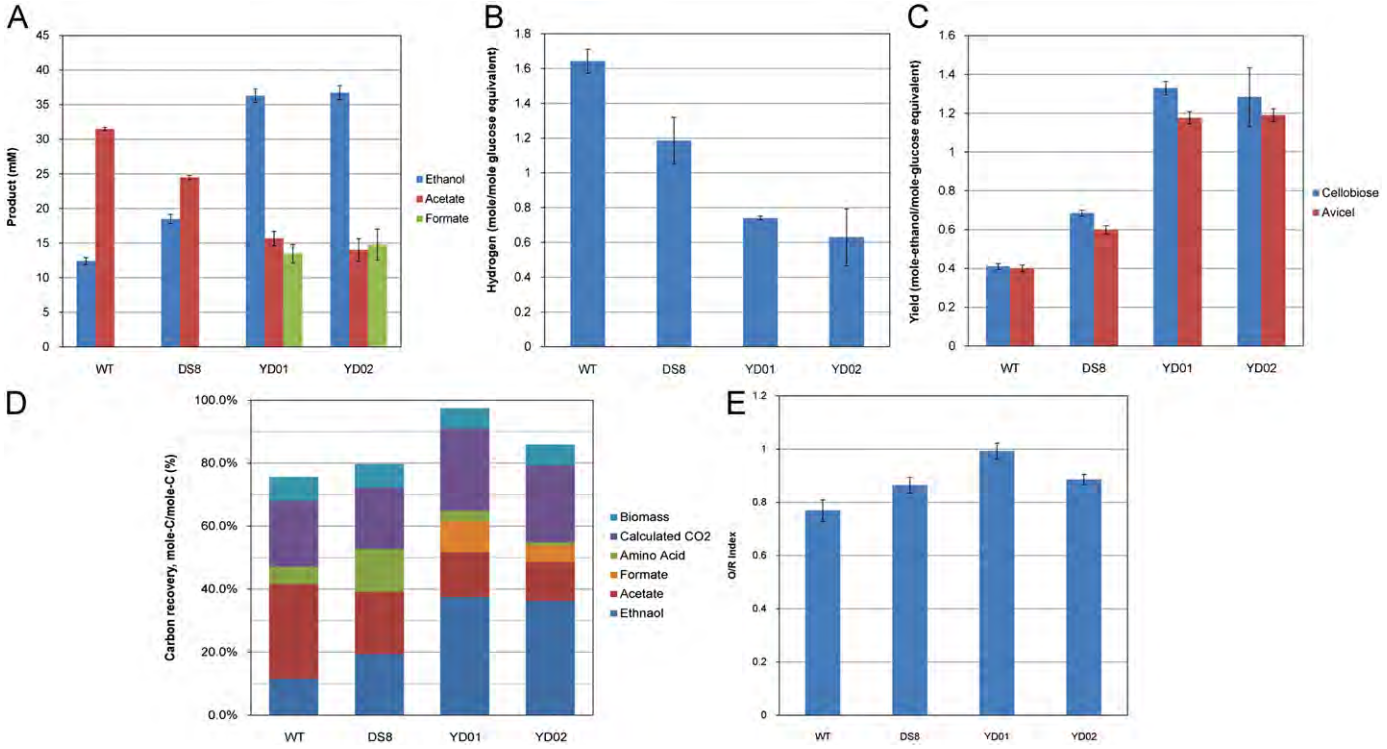

Fig. 3. Characteristics of *C. thermocellum* WT, DS8, YD01 and YD02 strains. (A) Fermentation results of *C. thermocellum* strains on 5 g/L Avicel; (B) H_2 productions in different strains. (C) Ethanol yield of *C. thermocellum* strains on cellobiose and Avicel. (D) The carbon recovery of different strains on 5 g/L (14.6 mM) cellobiose. (E) The oxidation/ reduction (O/R) index of different strains on 5 g/L (14.6 mM) cellobiose. The ethanol yield was calculated based on moles of ethanol produced per mole of glucose equivalent consumed.

was deleted in strain DS8 using plasmid pYD03, resulting in strain YD02 (Fig. 1D). YD02 grew faster than WT and DS8 strains. The ethanol yield of YD02 on cellobiose (1.28 mole-ethanol/mole glucose equivalent) was 3-fold higher than the wild-type. The ethanol yield of YD02 on Avicel was 1.19 mole-ethanol/mole glucose equivalent (Fig. 3C). The ethanol yields of YD01 and YD02 strains on Avicel were a little lower than those on cellobiose.

YD01 produced 0.74 mole/mole-glucose H_2 and YD02 produced 0.63 mole/mole-glucose H_2. H_2 productions in YD01 and YD02 strains were reduced by 55% and 62%, respectively. Both strains' H_2 productions were much lower than WT and DS8, meaning more NADH was supplied to AdhE in YD01 and YD02 to produce ethanol.

Oxidation/reduction (O/R) Index for WT, DS8, YD01 and YD02 on cellobiose was 0.77, 0.86, 0.99 and 0.88, respectively (Fig. 3E).

3.1.3. Enzyme activity assays

Enzymatic activities associated with the malate shunt measured in WT and engineered strains are presented in Table 2. The activity of pyruvate, phosphate dikinase (PPDK) was not detected. Neither ADP-dependent nor GDP-dependent pyruvate kinase activities were detected in the WT strain. The ADP-dependent pyruvate kinase activities in DS8, YD01 and YD02 were 0.52 U/mg, 0.14 U/mg and 0.84 U/mg, respectively, which were comparable to the activity (0.44 U/mg) detected in the native host, *T. saccharolyticum*

Activity of PEPCK in cell extracts of strain YD01 was 0.54 U/mg, significantly lower than in the WT (4.14 U/mg) and DS8 (4.09 U/mg) strains, indicating that PEPCK in strain YD01 was attenuated significantly. Surprisingly, PEPCK activity was not detectable in strain YD02, suggesting that another C3 to C4 pathway may exist although the assay was repeated several times with independent-prepared extracts and consistently positive controls.

NADH-linked malate dehydrogenase activities in strains YD01 (0.78 U/mg) and YD02 (0.60 U/mg) were lower than in the WT (1.67 U/mg) and DS8 (10.89 U/mg) strains. No NADPH-linked malate dehydrogenase activities were detected in any of the strains (< 0.001 U/mg).The unexpected large difference between malate dehydrogenase activities in the wild-type and DS8 strains was observed repeatedly in independently-prepared extracts.

NADP-dependent malic enzyme activity in YD01 was 3.01 U/mg, which was similar to activity in strains DS8 (4.46 U/mg) and the WT (1.97 U/mg). No malic enzyme activity was detected in strain YD02, and no NADH-dependent activity was detected in any of the strains tested.

3.2. Additional phenotypic effects

In addition to ethanol yield, the WT, DS8, YD01, and YD02 strains were examined with respect to four phenotypic properties, all of which might be expected to be impacted by the supply of NADPH: overall carbon recovery, the accumulation of formate, amino acid production and ethanol tolerance.

Carbon recoveries calculated based on described fermentation products (Fig. 3), amino acids (Fig. 4), and cell mass were 97.4% for strain YD01, 94.2% for strain YD02, 94.1% for strain DS8 and 89.3% for the wild-type.

There was no formate accumulated in WT and DS8 strains. In YD01 and YD02, the formate was 20.4 mM and 10.9 mM, respectively on cellobiose and 13.5 mM and 14.8 mM, respectively on Avicel, meaning pyruvate-formate lyase was activated (Fig. 2A).

Amino acid productions with 0.1 mM or higher are presented in Fig. 4 (all information of amino acids is in supplementary Table S2). It may be observed that L-valine was produced by the WT and DS8 strains at final concentrations of 2.0 mM and 2.9 mM, respectively. However, L -valine production was not detected in strains YD01 and YD02. Total amino acid production in the wild type (2.4 mM) was lower than strain DS8 (6.3 mM) but higher than strains YD01 (1.9 mM) and YD02 (0.4 mM).

The ability of strain YD01 to grow in the presence of added ethanol was compared to that of the wild-type. Both strains had not been previously exposed to elevated ethanol prior to the experiment. As shown in Fig. 5, the wild-type grew at 15 g/L

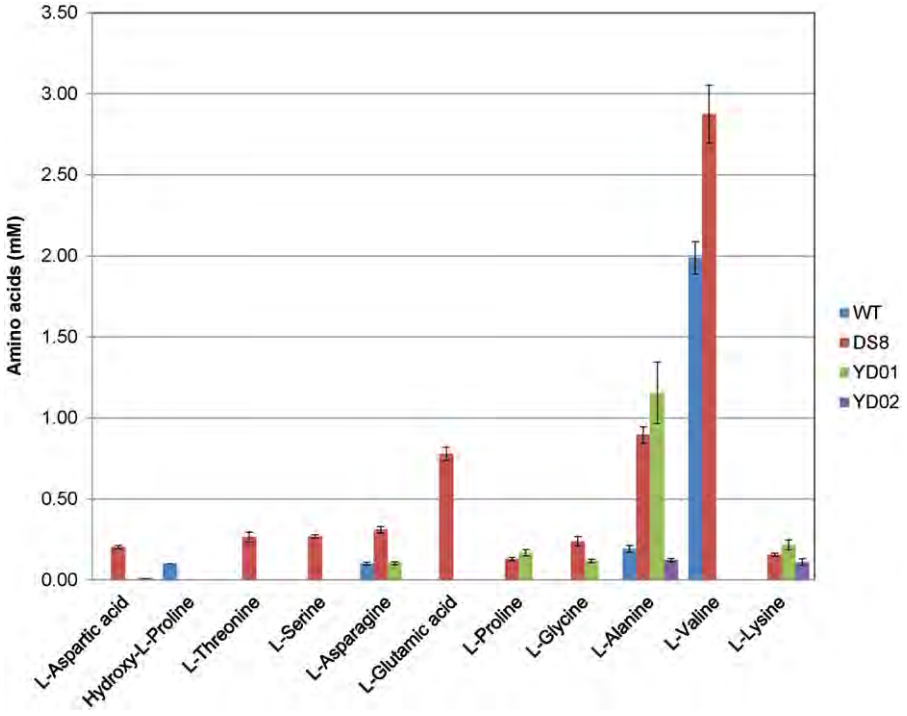

Fig. 4. The amino acids secretions in *C. thermocellum* strains on 5 g/L (14.6 mM) cellobiose. Amino acids with more than 0.1 mM are shown in this figure.

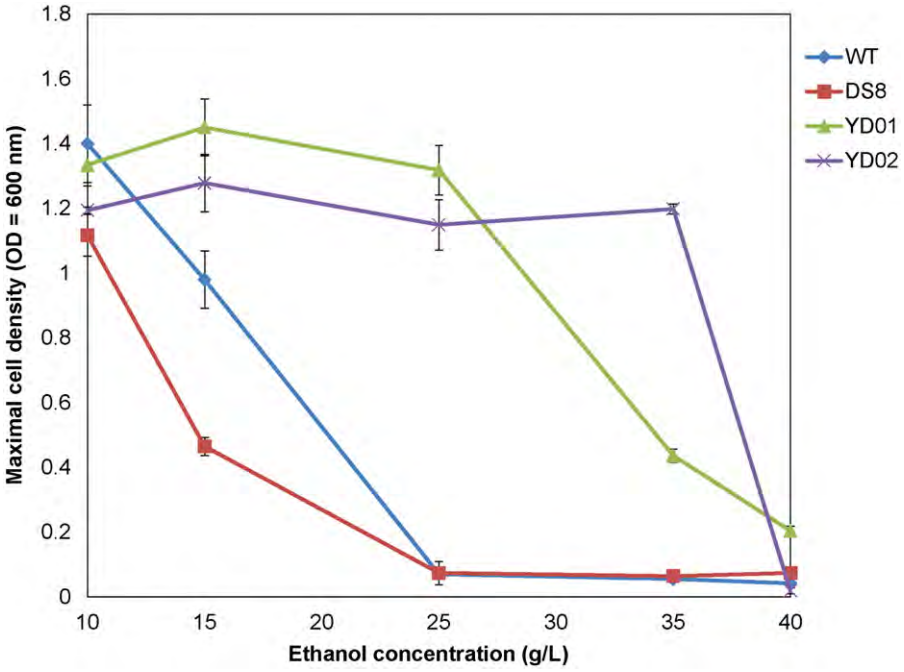

Fig. 5. The ethanol tolerance of *C. thermocellum* WT, DS8, YD01 and YD02.

ethanol but not over 15 g/L ethanol, strain DS8 grew at 15 g/L ethanol very well but not at over 25 g/L ethanol. The strains YD01 and YD02 could grow at 35 g/L ethanol but did not grow over 40 g/L ethanol. Shao et al. (2011) described two evolved *C. thermocellum* strains E50A and E50C with ability to grow well on 40 g/L ethanol but not over 50 g/L ethanol.

4. Discussion

Several lines of evidence support the hypothesis that conversion of PEP to pyruvate in *C. thermocellum* occurs with concomitant conversion of NADH to NADPH via a malate shunt featuring reaction of PEP to OAA, OAA to malate, and malate to pyruvate mediated by PEP carboxykinase, malate dehydrogenase, and malic enzyme, respectively. These include the cofactor specificities of malate dehydrogenase and malic enzyme as noted by Lamed and Zeikus (1980) and all three required enzymes have been detected. The absence of both an identifiable pyruvate kinase gene in the genome and of pyruvate kinase activity has been verified.

Although there were no pyruvate kinase or PPDK activities detected in *C. thermocellum*, phosphotransferase system (PTS) could be the candidate to convert phosphoenolpyruvate (PEP) to pyruvate. However, there was only one gene *Clo1313_0325* found to be related to PTS system. Importantly, for the PTS system to work, it would need several genes, including the E1 enzyme and a few others for the phosphorylation cascade from PEP to transported carbohydrate (Deutscher et al., 2006). There are no such genes existing in *C. thermocellum*. Moreover, PTS proteins do not necessarily point to the presence of a complete PTS system. Some components of the PTS system have regulatory functions only (Deutscher et al., 2006). There is no evidence proves that PTS system exists in *C. thermocellum* to convert PEP to pyruvate.

This hypothesis of the transhydrogenation of malate shunt is consistent with genetic manipulations impacting enzymes involved in the malate shunt. In an *Δldh* strain of *C. thermocellum* with no pyruvate kinase, there is no obvious organic electron acceptor for the NADPH produced by the malate shunt, because NADH is the electron donor for the bifunctional alcohol dehydrogenase(AdhE) present in *C. thermocellum* (Argyros et al., 2011). If the malate shunt were in fact a major route of carbon flux in the wild type, manipulations that decrease flux through this shunt would be expected to decrease NADPH formation, increase NADH, and consequently increase ethanol yield and reduce H_2 production in a *Δldh* host. This is in fact observed: the ethanol yields increased 1.6-fold when pyruvate kinase was heterologously expressed (strain DS8), and over 3-fold when pyruvate kinase was expressed in conjunction with either down-regulation (strain YD01) or knockout (strain YD02) of enzymes associated with the malate shunt. In addition, H_2 productions in YD01 and YD02 strains were reduced by 55% and 62%, respectively.

We speculate that NADPH oversupply due to transhydrogenation associated with the malate shunt in *C. thermocellum* may be relieved in nature by nfnAB mediating the reaction:

$$2\text{NADPH} + \text{NAD}^+ + \text{Fd(ox)} \leftrightarrow 2\text{NADP}^+ + \text{NADH} + \text{Fd(red)}$$

followed by subsequent transfer of electrons to hydrogen, (Wang et al., 2010), and more work needs to be performed on this enzyme.

In addition to increasing ethanol yield, routing flux through pyruvate kinase in lieu of the malate shunt was accompanied by four other phenotypic effects: higher carbon recovery, decreased amino acid production, increased formate production and increased ethanol tolerance. The observation of higher carbon recovery in conjunction with metabolic manipulations that decrease NADPH production suggests that high intracellular concentrations of NADPH drive secretion of normally intracellular metabolites due to mass action. In support of this interpretation, it may be noted that NADPH is the electron donor for described synthesis pathways for the amino acids produced in the greatest amounts by the wild-type (L-valine), and also that amino acid production is reduced when flux is diverted away from the malate shunt in strains YD01 and YD02. Notably, amino acid production in strain YD02, for which the malate shunt is rendered inoperative by knockout of malic enzyme, is decreased by over 5-fold compared to the wild-type.

Our observation that ethanol tolerance roughly doubles in strains YD01 and YD02 compared to the wild-type provides further support for involvement of redox metabolism, and perhaps particularly over-

abundance of reduced pyridine nucleotides, in mediating ethanol inhibition in *C. thermocellum* (Brown et al., 2011). High ethanol concentration could inhibit the activity of AdhE and reduced ferredoxin-NAD^+ reductase in Clostridia species, causing the over-accumulated NADH and high NADH/NAD^+ is toxic to the cells (Lovitt et al., 1988). In YD01 and YD02, pyruvate-formate lyase was activated to produce acetyl-CoA by diverting some carbon flux from ferredoxin-NAD^+ reductase, because there was a lot formate produced in these two strains. The pyruvate-formate lyase was not sensitive to the ethanol and reduced cofactors and it eliminated the accumulation of NADH. Elucidation of the mechanistic basis for this effect is an interesting direction for future study.

The carbon recovery of WT was much lower than DS8, YD01 and YD02. In the WT strain, the O/R index was only 0.77, and the rest reduced potential was used for other chemical productions (Ellis et al., 2012). The O/R index for the engineered strains was much higher than WT, indicating most of the reduced potential was used for generating major products, such as acetate, ethanol, H_2 etc.

Production of ethanol in thermophilic organisms such as *C. thermocellum* is more complicated than in yeast and engineered strains of *E. coli*. In particular, whereas yeast and engineered *E. coli* strains use only one electron carrier (NADH) to produce ethanol from sugars, all three major electron carriers (NAD(H), NADP(H), and ferredoxin) appear to have important roles to play in ethanol production in both native and engineered strains of *C thermocellum* and other thermophiles. We also see the increasing evidence that manipulation of redox reactions is promising for increasing ethanol yields and tolerance in thermophiles.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.11.006.

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