9-16-2008

Metabolic Engineering of a Thermophilic Bacterium to Produce Ethanol at High Yield

A. Joe Shaw
Dartmouth College

Kara K. Podkaminer
Dartmouth College

Sunil G. Desai
Dartmouth College

John S. Bardsley
Mascoma Corporation

Stephen R. Rogers
Dartmouth College

See next page for additional authors

Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield

A. Joe Shaw*†, Kara K. Podkaminer*, Sunil G. Desai*, John S. Bardsley†, Stephen R. Rogers*, Philip G. Thorne†, David A. Hogsett‡, and Lee R. Lynd*†‡§

*Thayer School of Engineering, and ‡Department of Biological Sciences, Dartmouth College, Hanover, NH 03755; and †Mascoma Corporation, 16 Cavendish Court, Lebanon, NH 03766

Edited by Roy H. Doi, University of California, Davis, Davis, CA, and approved July 22, 2008 (received for review February 9, 2008)

We report engineering *Thermoanaerobacterium saccharolyticum*, a thermophilic anaerobic bacterium that ferments xylan and biomass-derived sugars, to produce ethanol at high yield. Knockout of genes involved in organic acid formation (acetate kinase, phosphate acetyltransferase, and L-lactate dehydrogenase) resulted in a strain able to produce ethanol as the only detectable organic product and substantial changes in electron flow relative to the wild type. Ethanol formation in the engineered strain (ALK2) utilizes pyruvate:ferredoxin oxidoreductase with electrons transferred from ferredoxin to NAD(P), a pathway different from that in previously described microbes with a homoethanol fermentation. The homoethanologenic phenotype was stable for >150 generations in continuous culture. The growth rate of strain ALK2 was similar to the wild-type strain, with a reduction in cell yield proportional to the decreased ATP availability resulting from acetate kinase inactivation. Glucose and xylose are co-utilized and utilization of mannose and arabinose commences before glucose and xylose are exhausted. Using strain ALK2 in simultaneous hydrolysis and fermentation experiments at 50°C allows a 2.5-fold reduction in cellulase loading compared with using *Saccharomyces cerevisiae* at 37°C. The maximum ethanol titer produced by strain ALK2, 37 g/liter, is the highest reported thus far for a thermophilic anaerobe, although further improvements are desired and likely possible. Our results extend the frontier of metabolic engineering in thermophilic hosts, have the potential to significantly lower the cost of cellulosic ethanol production, and support the feasibility of further cost reductions through engineering a diversity of host organisms.

bioenergy | cellulosic ethanol | thermophile

Motivated by potential sustainability, security, and rural economic benefits, ethanol produced from cellulosic biomass is a leading candidate among alternatives to petroleum-derived transportation fuels (1, 2). Metabolic engineering of microorganisms responsive to the needs of cellulosic ethanol production has received considerable attention and effort over the last two decades with utilization of xylose and other non-glucose sugars as a major focus. In particular, mesophilic recombinant microorganisms producing ethanol at high yield from nonglucose sugars present in biomass have been developed by increasing ethanol yields in enteric bacteria (3, 4) and by conferring the ability to use non-glucose sugars to *Zymomonas mobilis* (5) and yeast (6, 7).

The high cost of converting biomass to sugars is the primary factor impeding establishment of a cellulosic biofuels industry (8, 9). One approach to lowering this key cost is to use thermophilic bacteria for biofuel production. Cellulolytic thermophiles such as *Clostridium thermocellum*, and hemicellulolytic thermophilies of the genera *Thermoanaerobacter* and *Thermoanaerobacterium* could be used in conjunction to hydrolyze and ferment all of the sugars in biomass (10). Use of the hemicellulolytic organisms alone at high temperatures could potentially lower the quantity of added cellulase required. For example, Patel *et al.* (11) report lactic acid production from cellulose at low cellulase loadings, by using a thermophilic *Bacillus* species. *T. saccharolyticum* JW/SL-YS485 is one such hemicellulolytic organism with the ability to hydrolyze xylan and ferment the majority of biomass-derived sugars at thermophilic temperatures.

All described thermophilic saccharolytic anaerobes produce organic acids in addition to ethanol. In *T. saccharolyticum* (12) and most other thermophiles, acetic acid is formed from pyruvate via pyruvate:ferredoxin oxidoreductase (POR), phosphate acetyltransferase, and acetate kinase, while lactic acid is formed from pyruvate by L-lactate dehydrogenase. Extensive efforts using classical mutagenesis techniques to obtain stable strains exhibiting high-ethanol yields over a range of conditions have not been successful (13). Genetic systems suitable for engineering thermophiles have long limited strain development, but have started to emerge (14–16) along with the first reports of metabolic engineering in thermophilic, saccharolytic hosts (17, 18). Here, we report engineering *T. saccharolyticum* JW/SL-YS485 to produce ethanol as the only significant organic product.

Results

Knockout mutants of *T. saccharolyticum* were obtained with the following genotypes: L-*ldh*$^-$ (18), *ack*$^-$ *pta*$^-$, and *ack*$^-$ *pta*$^-$ L-*ldh*$^-$ strain ALK1. An analysis of fermentation products in xylose-grown cultures of these strains (Fig. 1) showed that the L-*ldh*$^-$ mutant did not produce detectable lactic acid and produced increased yields of ethanol from pyruvate. The *ack*$^-$ *pta*$^-$ mutant did not produce acetic acid, produced ethanol at increased yield, and produced >25-fold less hydrogen. Strain ALK1 exhibited similarly reduced hydrogen yields and produced ethanol as the only detectable organic product. The fraction of reduced ferredoxin converted to reduced nicotinamide species by ferredoxin:NAD oxidoreductase (FNOR), inferred from stoichiometric analysis [supporting information (SI) Table S1], was 0.48, 0.53, 0.99, and 1.0 in the wild-type, L-*ldh*$^-$, *ack*$^-$ *pta*$^-$, and ALK1 strains, respectively. Hydrogenase activity was present in cell extracts of the ALK1 and wild-type strains at 5.8 ± 1.5 and 2.3 ± 0.6 μmol hydrogen consumed per minute per mg protein, respectively.

Strain ALK2, obtained by cultivating strain ALK1 in continuous culture for approximately 3,000 h with progressively higher

Author contributions: A.J.S., K.K.P., S.G.D., D.A.H., and L.R.L. designed research; A.J.S., K.K.P., S.G.D., J.S.B., S.R.R., and P.G.T performed research; A.J.S., K.K.P., D.A.H., and L.R.L. analyzed data; and A.J.S. and L.R.L wrote the paper.

Conflict of interest statement: Several of the authors are employees or hold a consulting position with the Mascoma Corporation, which has a financial interest in the organism described here.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY278026 (L-*ldh* gene of *T. saccharolyticum*) and EU313773 (*pta* and *ack* genes of *T. saccharolyticum*)].

§To whom correspondence should be addressed. E-mail: lee.lynd@dartmouth.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0801266105/DCSupplemental.

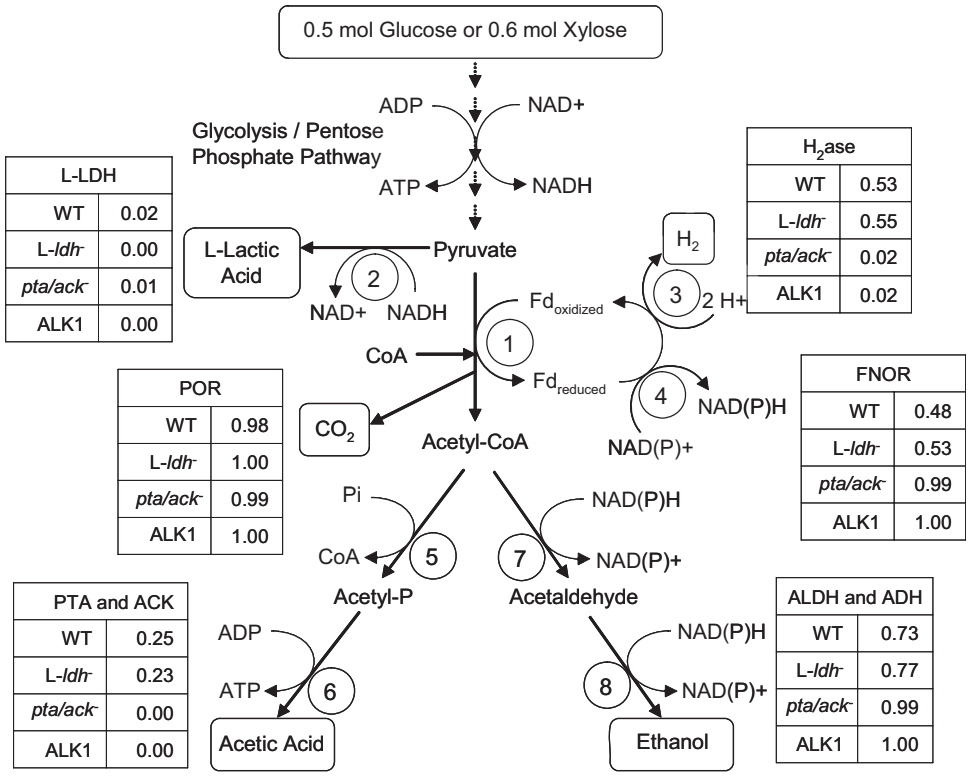

Fig. 1. Fermentative pathway in *T. saccharolyticum* and fluxes in knockout strains. Pyruvate/ferredoxin oxidoreductase, POR (*1*); L-lactate dehydrogenase, L-LDH (*2*); hydrogenase, H_2ase (*3*); ferredoxin/NAD(P)H oxidoreductase, FNOR (*4*); phosphate acetyltransferase, PTA (*5*); acetate kinase, ACK (*6*); acetaldehyde dehydrogenase, ALDH (*7*); and alcohol dehydrogenase, ADH (*8*). Fluxes normalized to pyruvate are shown for the wild-type, L-*ldh*$^-$, *pta/ack*$^-$, and ALK1 strains for batch fermentation of 28 mM xylose at 55°C. Values were determined as indicated in the *Materials and Methods* section and Table S1.

feed xylose concentrations, exhibited a greater capacity for xylose consumption in both batch and continuous culture. More than 99% of the feed xylose was used at concentrations up to 70 g/liter with a mean ethanol yield of 0.46 g of ethanol/g xylose in continuous culture at a pH of 5.2–5.4 without base addition (Fig. S1). The maximum ethanol concentration and volumetric productivity was 33 g/liter and 2.2 g L^{-1} h^{-1}, respectively. No decrease in ethanol yield was observed over hundreds of generations in continuous culture without antibiotic selection.

The growth rate of strain ALK2 is comparable with the wild-type strain, with fermentation of ~80 mM xylose completely used in less than 10 h for both strains (Fig. 2). The cell yield, calculated from the maximum cell concentration observed, was 0.12 g/g xylose for strain ALK2 and 0.15 g/g xylose for the wild-type strain. The 20% lower cell yield observed for strain ALK2 compared with the wild-type strain is similar to the 23% decrease in ATP gain per mole xylose fermented resulting from loss of acetate kinase activity in the mutant (Table S2). A maximum cell specific ethanol production rate from xylose of 1.4 g of ethanol g cells^{-1} h^{-1} is calculated for strain ALK2 from the ratio of the maximum specific growth rate of 0.37 h^{-1}, the cell yield, and the ethanol yield of 0.46 g of ethanol/g xylose.

Specific activities of enzymes in the proposed pathway from pyruvate to ethanol were assayed in the ALK2 strain (Table 1). Activities were found for POR, FNOR, acetaldehyde dehydrongenase (ALDH), and alcohol dehydrogenase (ADH). In contrast to the wild-type strain, FNOR, ALDH, and ADH activities in the ALK2 strain all have higher specific activities with the cofactor NADPH than with NADH.

Fermentation of sugar mixtures and cellulase loading required for cellulose hydrolysis, both matters of considerable applied interest, are examined in Figs. 3 and 4. Strain ALK2 was cultivated in a 1-L fed-batch fermentor with glucose, xylose, galactose, and mannose each present initially at 12.5 g/liter for a total concentration of 50 g/liter. An equiweight mixture of these sugars at a concentration of 330 g/liter was fed at 3 g/h from 8–24 h into the fermentation. Consumption of xylose and glucose was essentially simultaneous (Fig. 3), with both sugars still present after 16 h and below detection limits after 20 h. Consumption of mannose began when the glucose/xylose concentration reached approximately 5 g/liter, and consumption of galatose began when the mannose concentration reached a similar concentration. At the point glucose and xylose concentrations approached zero, the mannose concentration was reduced by 98%, and the galactose concentration was reduced by 92%. A final ethanol concentration of 37 g/liter was produced from mixed sugars with a maximum ethanol productivity of 2.7 g·liter^{-1}·h^{-1} and an average ethanol productivity of 1.5 g·liter^{-1}·h^{-1}.

Simultaneous saccharification and fermentation (SSF) of Avicel, a predominantly crystalline model cellulosic substrate, was undertaken in batch mode at an initial concentration of 50 g/liter by using a commercial cellulase preparation (Spezyme CP) from *Trichoderma reesei.* SSF with *T. saccharolyticum* ALK2 was undertaken at 50°C, the maximum temperature at which the enzyme preparation was stable in our hands, and was carried out without supplemental β-glucosidase because *T. saccharolyticum* is able to ferment cellobiose. SSF with *Saccharomyces cerevisiae* D5A was undertaken at 37°C, close to the maximum temperature tolerated by this organism, both with and without supplemental β-glucosidase because this yeast does not produce this enzyme. Experiments with yeast were performed at a cellulase loading of 10 filter paper units (FPU)/g cellulose, which is representative of conditions anticipated for an industrial process and does not entail substantial saturation of the

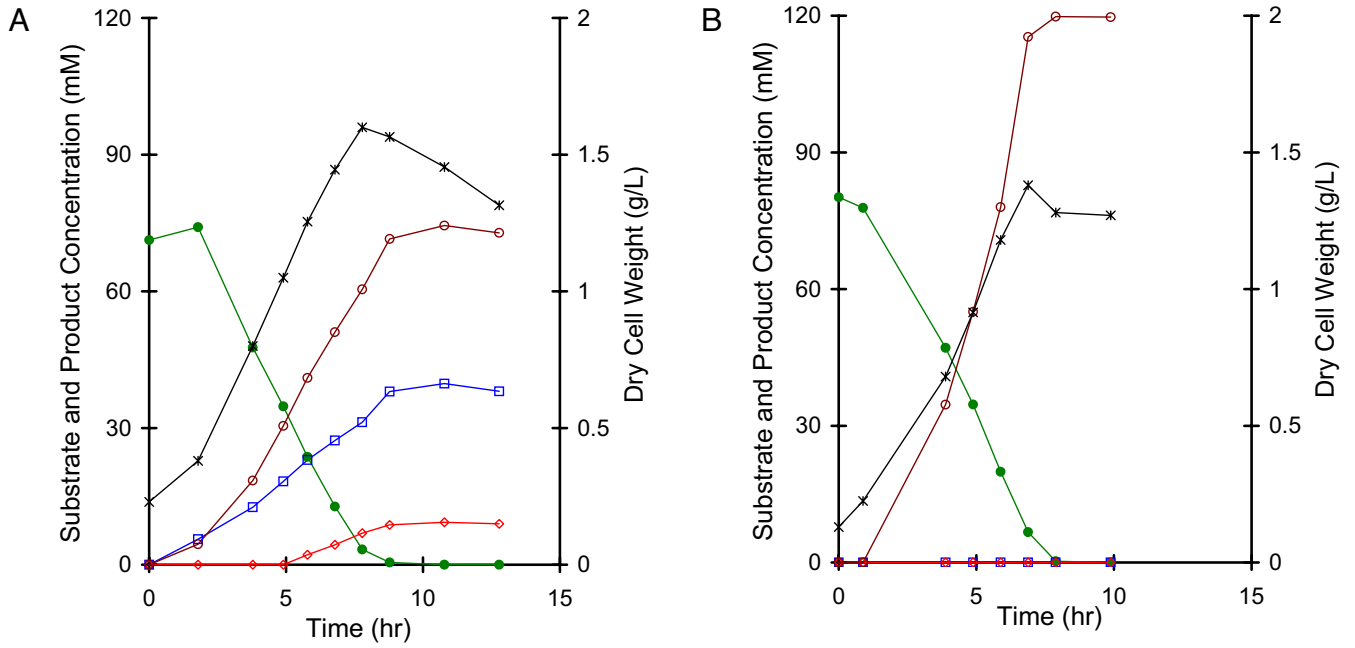

Fig. 2. Batch fermentation of xylose at 55°C in bioreactors controlled at pH 6.0. (*A*) Wild-type strain and (*B*) ALK2 strain. Xylose (filled green circles), ethanol (open brown circles), lactic acid (open red diamonds), acetic acid (open blue squares), and cell dry weight (*) were determined as indicated in the *Materials and Methods* section. Carbon recovery for the wild-type fermentation is 107% and the ALK2 fermentation is 102%.

hydrolysis rate with respect to cellulase loading. Thereafter, experiments with *T. saccharolyticum* were performed iteratively at various cellulase loadings until results matched those obtained with yeast.

As seen in Fig. 4, results at an enzyme loading of 4 FPU/g cellulose obtained with *T. saccharolyticum* ALK2 are very similar to results obtained with *S. cerevisiae* at 10 FPU/g cellulose with supplemental β-glucosidase. Without supplemental β-glucosidase, SSF with *S. cerevisiae* at 37°C and 10 FPU/g cellulose is slower than *T. saccharolyticum* at 50°C with 4 FPU/g cellulose. Concentrations of cellobiose and glucose were low (<0.5 g/liter) after 4 h in all SSF experiments, suggesting that cellulose hydrolysis was rate-limiting and that significant inhibition by bulk phase concentrations of hydrolysis products was not operative.

Discussion

T. saccharolyticum ALK2 produced ethanol as the only significant organic end-product under all conditions examined. This strain differs from engineered mesophilic xylose-using strains that achieve near-theoretical ethanol yields in several significant ways. It converts pyruvate to ethanol via a pathway involving POR with electron transfer from ferredoxin to NAD(P) (Fig. 1), whereas previously developed mesophilic strains use pyruvate decarboxylase. Whereas strain ALK2 utilizes xylose and glucose

Table 1. Specific activities of enzymes in the pyruvate to ethanol pathway

	Specific activities (μmol·min^{-1}·mg^{-1} protein)			
Enzyme	ALK2	SD	WT*	SD*†
POR	1.9	0.1	3.7	0.3
FNOR–NADH	0.73	0.08	1.08	0.01
FNOR–NADPH	2.3	0.5	0.55	0.09
Aldh–NADH	0.005	0.001	0.031	0.015
Aldh–NADPH	0.019	0.005	0.11	0.05
Adh–NADH	0.012	0.004	1.05	0.16
Adh–NADPH	0.23	0.04	0.11	0.06

*Data from ref 12.
†SD, standard deviation.

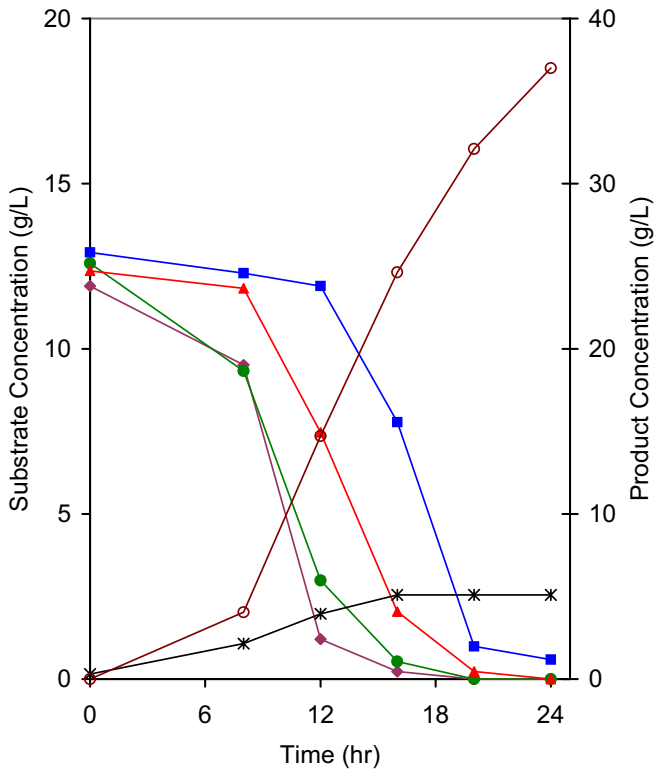

Fig. 3. Mixed sugar fed-batch fermentation. Strain ALK2 at 55°C without pH control (pH 5.2–5.4). Feeding of 3 g·liter^{-1}·h^{-1} commenced at 8 h and ended at 24 h. Xylose (filled green circles), glucose (filled purple diamonds), mannose (filled red triangles), galactose (filled blue squares), ethanol (open brown circles), and cell dry weight (*).

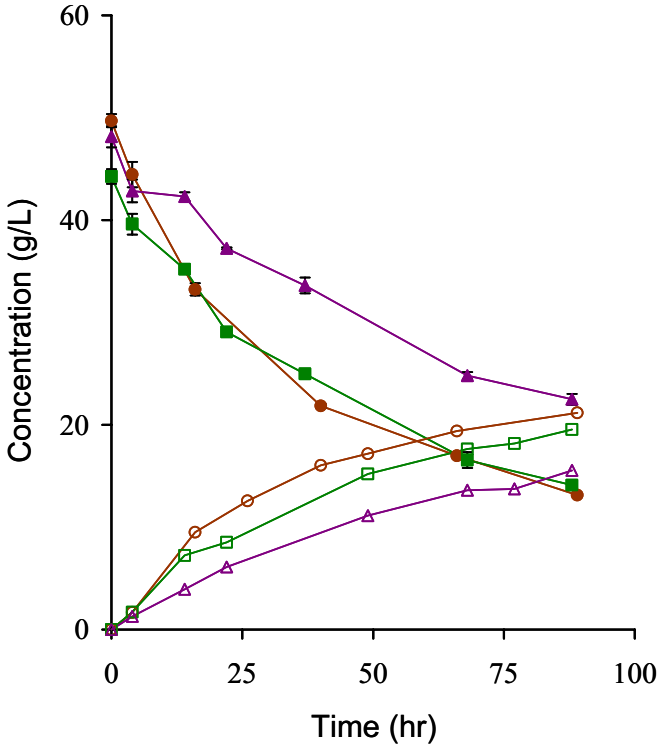

Fig. 4. Comparison of enzyme loading requirements for thermophilic SSF and mesophilic SSF. Batch fermentations were conducted with Avicel and *T. reesei* derived cellulase. Thermophilic SSF was at 50°C with *T. saccharolyticum* ALK2 and 4 FPU cellulase/g cellulose; Avicel (filled brown circles), ethanol (open brown circles). Mesophilic SSF was at 37°C with *S. cerevisiae* D5A, 10 FPU cellulase, and 40 units β-glucosidase; Avicel (filled green squares), ethanol (open green circles). Mesophilic SSF without additional β-glucosidase and otherwise similar conditions; Avicel (filled purple triangles), ethanol (open purple triangles).

simultaneously and co-ferments mannose and galactose with glucose to a significant extent (Fig. 3), the described mesophilic strains use glucose preferentially to xylose. Previously developed mesophilic strains ferment well at ≤37°C and thus require substantially higher cellulase loadings compared with strain ALK2 which ferments well between 50 and 60°C (Fig. 4). The volumetric productivity of xylose fermentation to ethanol using *T. saccharolyticum* ALK2 compares favorably with productivities reported for the mesophilic strains listed in Table 2, while ethanol yields are similar.

In *T. saccharolyticum* ALK2, one of the two moles of NAD(P)H required to make a mole of ethanol from acetyl-CoA originates from glycolysis, and the second originates from the action of FNOR. A similar redirection of electron flow was recently reported for a pyruvate decarboxylase-minus strain of *E. coli*-metabolizing pyruvate via NADH-forming pyruvate dehydrogenase (19). The results reported here support the feasibility of engineering the large group of bacteria that metabolize pyruvate by POR to achieve high ethanol yields, including *C. thermocellum* which exhibits one of the highest rates of cellulose utilization known (13).

The shift from NADH to NADPH specificity in key ethanol pathway enzymes observed in the ALK2 strain was also observed in a non-engineered *Thermoanaerobacter ethanolicus* strain adapted for tolerance to 4% v/w exogenous ethanol (20). The NADH/NAD+ ratio in the *T. ethanolicus* wild-type strain was elevated during pulses of ethanol in continuous culture, whereas the adapted strain was largely unaffected. The NADH to NADPH shift is likely an adaptive response to overcome NADH inhibition of glycolysis at moderate ethanol concentrations (21).

The observed 2.5-fold reduction in cellulase requirements for SSF using *T. saccharolyticum* ALK2 at 50°C compared with yeast at 37°C is significant in light of the dominant impact of the cost of hydrolysis, and cellulase in particular, on process economics (9, 22). Our choice of 50°C for SSF was dictated by the limited temperature stability of the cellulase preparation used rather than the organism. Thus, further reductions in cellulase loading may be anticipated as more thermostable cellulases are developed.

Notwithstanding the noteworthy capabilities of strain ALK2, further work remains before the organism will be suitable for industrial application. Robust fermentation of sugars arising from pretreated lignocellulose will need to be demonstrated in media with minimal supplementation. The concentrations of ethanol produced by strain ALK2, 37 g/liter in fed batch culture and 33 g/liter in continuous culture, are higher than previously reported for thermophilic bacteria (13). These concentrations are, however, only approximately half the maximum concentration of added ethanol that will cease growth by ethanol-adapted thermophilic strains (23–27). Moreover, whereas ethanol can readily be recovered at the maximum concentrations tolerated by thermophiles, this is not the case for the maximum concentrations produced thus far. Closing the gap between the maximum concentrations of ethanol produced and maximum concentrations tolerated is still to be achieved for *T. saccharolyticum*, although examples of success in closing similar gaps have been reported for other organisms (10).

Materials and Methods

Gene Cloning and Plasmid Construction. The *pta*, *ack*, and L-*ldh* (18) genes (GenBank no. EU313773 and AY278026) from *T. saccharolyticum* were identified by degenerate primer PCR of conserved protein sequences and subsequent direct sequencing of genomic DNA. The *pta* and *ack* genes are located adjacent to each other on the genome.

By using standard cloning techniques, 1.2- and 0.6-kb regions 5′ and 3′ of the *pta* and *ack* genes were amplified with primer sets P1, P2 and A1, A2 and

Table 2. Fermentation parameters for xylose-utilizing recombinant organisms

Organism	Xylose, g/liter	Fermentation mode	Ethanol yield, grams of ethanol per gram of substrate	Volumetric productivity (g ethanol liter^{-1}·h^{-1})	Final ethanol, g/liter	Ref.
T. saccharolyticum ALK2	70	Continuous	0.46	2.20	33.1	this study
S. cerevisiae RWB217	20	Batch	0.44	0.48	8.7	6
E. coli LY01 (KO11 der.)	95	Batch	0.47	0.88	42.4	29
E. coli FBR5	50	Continuous	0.44	1.0	22	30
Z. mobilis CP4:pZB5	60	Batch	0.48	0.32	23	31
S. cerevisiae 1400	50	Batch	0.46	0.3	23	32

An attempt was made to find the best parameters reported in the literature for each strain under conditions as comparable as possible. The experiments shown were not done under constant conditions however, and variables such as medium composition and cell concentration may have a significant impact.

inserted into the cloning vector pBLUESCRIPT II SK (+) (Stratagene). Knockout plasmid pSGD9 was created by inserting the thermostable *S. faecalis* kanamycin resistance cassette (14) between the *pta* and *ack* homology regions. Knockout plasmid pSGD8E targeting L-*ldh* was constructed with the same 5′ and 3′ homology regions reported earlier (18) with a fusion of the kanamycin promoter region (primers K1, K2) and adenine methylase gene (primers E1, E2) conferring erythromycin resistance (15) inserted between them.

P1 – 5′ACATGCATGCCCATTTGTCTATAATAGAAGGAAG3′
P2 – 5′CGTCAACAATATCTCTATAGCTGC3′
A1 – 5′GCTCTAGAGCATAGAATTAGCTCCACTGC3′
A2 – 5′ACATGCATGCCGACGCCTCCCATAGCTGCTGCAT3′
K1 – 5′TGGATCCGCCATTTATTATTTCCTTCCTCTTTTC3′
K2 – 5′TTCTAGATGGCTGCAGGTCGATAAACC3′
E1 – 5′GCGGATCCCATGAACAAAAATATAAAATATTCTC3′
E2 – 5′GCGAATTCCCTTTAGTAACGTGTAACTTTCC3′

Transformation of *T. saccharolyticum*. Transformation of *T. saccharolyticum* was performed as previously described (14, 16) with modifications during selection on erythromycin. Cells transformed with pSGD8E were allowed to recover at 48°C for 4 h and subsequently plated on solid medium at pH 6.0 containing erythromycin at 5 μg/ml and incubated at 48°C for 4 days. To create strain ALK1, cells were first transformed with pSGD9 followed by pSDG8E. Double homologous integrations were screened by PCR and confirmed by DNA sequencing.

Media Composition and Strain Storage. *T. saccharolyticum* JW/SL-YS485 was cultured in MTC medium (25). Carbohydrate and complex additives varied between fermentation types as described in the following paragraph. *S. cerevisiae* D5A was grown in YPD medium. All reagents used were from Sigma–Aldrich unless otherwise noted.

Fermentation Conditions. Batch fermentations with the wild-type strain, L-*ldh*$^-$, *pta/ack*$^-$, and ALK1 shown in Fig. 1 included 4 g/liter xylose, 2.5 g/liter yeast extract, and 10 g/liter MES buffer at pH 6.2. Fermentations were at 55°C without shaking in anaerobic tubes with a nitrogen gas headspace and a 5% vol/vol inoculation. Continuous cultures of ALK1 and ALK2 contained 20–70 g/liter xylose, 10 g/liter yeast extract, and 5 g/liter tryptone. Temperature was kept at 55°C and pH control was not necessary beyond the buffering provided by MTC medium. During steady states the pH was between 5.2 and 5.5. Fermentations were performed in either a 2-L bioreactor (Applikon Instruments) with a 0.5-L working volume or in custom-made vessels (NDS Technologies) with a 0.25-L working volume. Fermentors were made anaerobic through sparging with nitrogen for 30 min to 1 h, at which point the oxygen-indicating dye resazurin became clear. Sparging was then ended, and carbon dioxide was allowed to accumulate with venting through a condensing tube into a water-containing vessel. Batch fermentations of the wild-type and ALK2 strains contained 12 g/liter xylose and 5 g/liter yeast extract. Fermentation conditions were maintained at 55°C, a pH of 5.5, and 200 rpm in a 3-L bioreactor with a 1-L working volume (Sartorious). Inoculation was with 0.1 g/liter dry-weight cells in the exponential growth phase that were centrifuged and resuspended in fresh media before inoculation. Mixed sugar fed-batch fermentations were initiated with 12.5 g/liter each of glucose, xylose, mannose, and galactose and maintained at 55°C, 150 rpm, and a pH 5.2–5.4 without active pH control. After 8 h of batch fermentation, a concentrated feed at 330 g/liter of the same ratio of sugars was fed at a rate of 0.15 ml/min until 24 h. The mixed sugar fed-batch inoculum was from a batch overnight culture grown in 10 g/liter xylose. The fermentation volume was initially 1-L in a 3-L bioreactor. Thermophilic SSF reactions contained 50 g/liter Avicel PH-105 (FMC), 10 g/liter yeast extract, and 5 g/liter tryptone. The pH was maintained at 5.0 and run at 50°C in 3-L reactors with a 1.5-L working volume. Mesophilic SSF reactions contained 50 g/liter Avicel PH-105 in YP medium, maintained at a pH of 5.0 and run at 37°C. Both SSF reactions were inoculated at 25% vol/vol from a continuous culture fed with 20 g/liter glucose in either MTC medium at 50°C with *T. saccharolyticum* ALK2 or YPD at 37°C with *S. cerevisiae* D5A with a 24-h residence time. *Trichoderma reesei* derived cellulases (Spezyme CP, Genencor) and β-glucosidase (Novozyme 188, Novozymes) were added as indicated in the text.

Analytical Methods. Fermentation metabolites were analyzed by HPLC (28) with an Aminex HPX-87H column (Bio-Rad Laboratories). Hydrogen was analyzed by GC on a carboxen-1000 column with nitrogen as the carrier gas with a TCD detector (Perkin–Elmer). Residual cellulose was determined by quantitative saccharification (28). Dry weights were determined from 10 mL samples after filtration, washing, and drying for 16 h at 90°C. Carbon balances presented in Fig. 2 were determined by measurements of initial carbohydrate concentrations and final carbon-containing end products, including cell dry weight by using the general empirical formula for cell composition of $CH_2N_{0.25}O_{0.5}$. Carbon dioxide was accounted for by stoichiometric correlations to ethanol and acetic acid formation. Carbon contained in yeast extract and extracellular protein was not included in the carbon recovery. Total carbon was calculated based on the following equation, and carbon recovery was calculated as the quotient of the average of the first-two and last-two time points taken during the fermentation.

$$C_t = \frac{60}{150}X + \frac{36}{90}L + \frac{36}{60}A + \frac{36}{46}E + \frac{12}{25.5}CDW$$

C_t = total carbon, X = xylose, L = lactic acid, A = acetic acid, E = ethanol, and CDW = cell dry weight. All units are in grams per liter.

Enzymatic Assays. Crude cell extracts were prepared by sonication and assayed anaerobically at 60°C at the reaction concentrations and conditions reported previously (12). The assays were performed as follows: Hydrogenase–methyl viologen reduction by hydrogen, POR–methyl viologen reduction by pyruvate, FNOR–benzyl viologen reduction by NAD(P)H, ALDH–NAD(P)H reduction by acetyl-CoA, ADH–NAD(P)H reduction by acetaldehyde. Reported data are the average of three assays. Hydrogenase activity was determined with a cell extract from strain ALK1, all other assays were determined with cell extracts from ALK2.

ACKNOWLEDGMENTS. We thank Mike Adams for useful discussions. This work was supported by National Institute of Standards and Technology Grant 60NANB1D0064, Mascoma Corporation, and Link Foundation Fellowships (to A.J.S. and S.G.D.).

1. Greene N, *et al.* (2004) *Growing Energy: How Biofuels Can Help End America's Oil Dependence* (Natural Resources Defense Council, New York).
2. Farrell AE, *et al.* (2006) Ethanol can contribute to energy and environmental goals. *Science* 311:506–508.
3. Yomano LP, York SW, Ingram LO (1998) Isolation and characterization of ethanol-tolerant mutants of *Escherichia coli* KO11 for fuel ethanol production. *J Ind Microbiol Biotechnol* 20:132–138.
4. Dien BS, Cotta MA, Jeffries TW (2003) Bacteria engineered for fuel ethanol production: Current status. *Appl Microbiol Biotechnol* 63:258–266.
5. Zhang M, Eddy C, Deanda K, Finkelstein M, Picataggio S (1995) Metabolic engineering of a pentose metabolism pathway in ethanologenic *Zymomonas mobilis. Science* 267:240–243.
6. Kuyper M, *et al.* (2005) Metabolic engineering of a xylose-isomerase-expressing Saccharomyces cerevisiae strain for rapid anaerobic xylose fermentation. *FEMS Yeast Res* 5:399–409.
7. Ho NWY, Chen Z, Brainard A, Sedlak M (1999) Successful design and development of genetically engineered Saccharomyces yeasts for effective cofermentation of glucose and xylose from cellulosic biomass to fuel ethanol. *Adv Biochem Eng Biotechnol* 65:164–192.
8. Himmel ME, *et al.* (2007) Biomass recalcitrance: Engineering plants and enzymes for biofuels production. *Science* 315:804–807.
9. Lynd LR, *et al.* (2008) How biotech can transform biofuels. *Nat Biotechnol* 26:169–172.
10. Lynd LR, van Zyl WH, McBride JE, Laser M (2005) Consolidated bioprocessing of cellulosic biomass: An update. *Curr Opin Biotechnol* 16:577–583.
11. Patel MA, Ou MS, Ingram LO, Shanmugam KT (2005) Simultaneous saccharification and cofermentation of crystalline cellulose and sugar cane bagasse hemicellulose hydrolysate to lactate by a thermotolerant acidophilic *Bacillus sp. Biotechnol Prog* 21:1453–1460.
12. Shaw AJ, Jenney FE, Adams MWW, Lynd LR (2008) End-product pathways in the xylose fermenting bacterium, Thermoanaerobacterium saccharolyticum. *Enzyme Microb Technol* 42:453–458.
13. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS (2002) Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol Mol Biol Rev* 66:506–577.
14. Mai V, Lorenz WW, Wiegel J (1997) Transformation of Thermoanaerobacterium sp strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. *FEMS Microbiol Lett* 148:163–167.
15. Klapatch TR, Guerinot ML, Lynd LR (1996) Electrotransformation of Clostridium thermosaccharolyticum. *J Ind Microbiol* 16:342–347.
16. Tyurin MV, Sullivan CR, Lynd LR (2005) Role of spontaneous current oscillations during high-efficiency electrotransformation of thermophilic anaerobes. *Appl Environ Microbiol* 71:8069–8076.
17. Mai V, Wiegel J (2000) Advances in development of a genetic system for Thermoanaerobacterium spp: Expression of genes encoding hydrolytic enzymes, development of a second shuttle vector, and integration of genes into the chromosome. *Appl Environ Microbiol* 66:4817–4821.
18. Desai SG, Guerinot ML, Lynd LR (2004) Cloning of L-lactate dehydrogenase and elimination of lactic acid production via gene knockout in Thermoanaerobacterium saccharolyticum JW/SL-YS485. *Appl Microbiol Biotechnol* 65:600–605.
19. Kim Y, Ingram LO, Shanmugam KT (2007) Construction of an *Escherichia coli* K-12 mutant for homoethanologenic fermentation of glucose or xylose without foreign genes. *Appl Environ Microbiol* 73:1766–1771.
20. Lovitt RW, Shen GJ, Zeikus JG (1988) Ethanol production by thermophilic bacteria: Biochemical basis for ethanol and hydrogen tolerance in Clostridium thermohydrosulfuricum. *J Bacteriol* 170:2809–2815.

21. Burdette DS, Jung SH, Shen GJ, Hollingsworth RI, Zeikus JG (2002) Physiological function of alcohol dehydrogenases and long-chain (C_{30}) fatty acids in alcohol tolerance of Thermoanaerobacter ethanolicus. *Appl Environ Microbiol* 68:1914–1918.
22. Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. *Trends Biotechnol* 25:153–157.
23. Georgieva T, Mikkelsen M, Ahring B (2007) High ethanol tolerance of the thermophilic anaerobic ethanol producer Thermoanaerobacter BG1L1. *Cent Eur J Biol* 2:364–377.
24. Williams TI, Combs JC, Lynn BC, Strobel HJ (2006) Proteomic profile changes in membranes of ethanol-tolerant Clostridium thermocellum. *Appl Microbiol Biotechnol* 72:422–432.
25. Baskaran S, Ahn HJ, Lynd LR (1995) Investigation of the ethanol tolerance of Clostridium thermosaccharolyticum in continuous culture. *Biotechnol Prog* 11:276–281.
26. Rani KS, Seenayya G (1999) High ethanol tolerance of new isolates of Clostridium thermocellum strains SS21 and SS22. *World J Microbiol Biotechnol* 15:173–178.
27. Tailliez P, Girard H, Longin R, Beguin P, Millet J (1989) Cellulose fermentation by an asporogenous mutant and an ethanol-tolerant mutant of Clostridium thermocellum. *Appl Environ Microbiol* 55:203–206.
28. Lu YP, Zhang YHP, Lynd LR (2006) Enzyme-microbe synergy during cellulose hydrolysis by Clostridium thermocellum. *Proc Natl Acad Sci* 103:16165–16169.
29. Tao H, *et al.* (2001) Engineering a homo-ethanol pathway in *Escherichia coli*: Increased glycolytic flux and levels of expression of glycolytic genes during xylose fermentation. *J Bacteriol* 183:2979–2988.
30. Martin GJ, Knepper A, Zhou B, Pamment NB (2006) Performance and stability of ethanologenic Escherichia coli strain FBR5 during continuous culture on xylose and glucose. *J Ind Microbiol Biotechnol* 33:834–844.
31. Lawford HG, Rousseau JD (1999) The effect of glucose on high-level xylose fermentations by recombinant Zymomonas in batch and fed-batch fermentations. *Appl Microbiol Biotechnol* 77- 79:235–249.
32. Sedlak M, Ho NW (2004) Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose cofermentation by a recombinant. Saccharomyces yeast. *Yeast* 21:671–684.