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Consolidated bioprocessing of cellulose to isobutanol using *Clostridium thermocellum*

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ABSTRACT

Consolidated bioprocessing (CBP) has the potential to reduce biofuel or biochemical production costs by processing cellulose hydrolysis and fermentation simultaneously without the addition of pre-manufactured cellulases. In particular, *Clostridium thermocellum* is a promising thermophilic CBP host because of its high cellulose decomposition rate. Here we report the engineering of *C. thermocellum* to produce isobutanol. Metabolic engineering for isobutanol production in *C. thermocellum* is hampered by enzyme toxicity during cloning, time-consuming pathway engineering procedures, and slow turnaround in production tests. In this work, we first cloned essential isobutanol pathway genes under different promoters to create various plasmid constructs in *Escherichia coli*. Then, these constructs were transformed and tested in *C. thermocellum*. Among these engineered strains, the best isobutanol producer was selected and the production conditions were optimized. We confirmed the expression of the overexpressed genes by their mRNA quantities. We also determined that both the native ketoisovalerate oxidoreductase (KOR) and the heterologous ketoisovalerate decarboxylase (KIVD) expressed were responsible for isobutanol production. We further found that the plasmid was integrated into the chromosome by single crossover. The resulting strain was stable without antibiotic selection pressure. This strain produced 5.4 g/L of isobutanol from cellulose in minimal medium at 50 °C within 75 h, corresponding to 41% of theoretical yield.

1. Introduction

Lignocellulose instead of sugar as the raw material for biofuel and biochemicals production can potentially provide the quantity needed to make a significant impact, improve net carbon and energy balances, lower production cost, and avoid the food vs. fuel dilemma (Lynd et al., 2005, 2008). However, biomass recalcitrance—resistance to degradation—currently limits the use of lignocellulose. Consolidated bioprocessing (CBP) is a potential solution in which cellulose hydrolysis and fermentation occur simultaneously without added cellulase. *Clostridium thermocellum* is a promising thermophilic CBP host because of its high cellulose deconstruction rate. Recent studies of metabolic features of *C. thermocellum* (Zhou et al., 2013) and advances in genetic modification tools (Tyurin et al., 2004; Tripathi et al., 2010; Argyros et al., 2011) for *C. thermocellum* make the CBP organism an attractive platform for biofuel or biochemical production.

Longer-chain alcohols offer advantages as a gasoline substitute or drop-in fuel (Atsumi et al., 2008). In particular, isobutanol received significant attention because it can be used as fuel or a feedstock chemical. Isobutanol can be dehydrated to form isobutene, which can then be oligomerized to C8 then C12 alkenes to be use as jet fuel. The C8 alkene can also be dehydrocyclized to form p-xylene Peters et al., (2011), which can then be oxidized to form terephthalic acid as a monomer for the common plastic polyethylene terephthalate (PET). Microbial production of isobutanol from renewable sources has been demonstrated in multiple engineered organisms (Atsumi et al., 2008, 2009; Smith et al., 2010; Higashide et al., 2011; Li et al., 2012; Lin et al., 2014), indicating the flexibility of the pathway. Isobutanol production

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from cellulose has also been demonstrated using a cellulolytic organism, *Clostridium cellulolyticum* (Higashide et al., 2011). However, this organism has a low cellulolytic rate and a long doubling time, and is not suitable for CBP . *C. thermocellum* offers much higher cellulose decomposition rate and has the ability to grow at elevated temperatures (50–60 °C), which facilitate cellulose degradation and reduce the chance of contamination compared to the case at mesophilic temperatures. Here, we seek to produce isobutanol directly from cellulose to achieve high titer using *C. thermocellum*.

C. thermocellum genetic tools (Tripathi et al., 2010; Argyros et al., 2011; Guss et al., 2012; Deng et al., 2013) and isobutanol pathway at elevated temperatures (Lin et al., 2014) have been previously reported. In addition, selected *C. thermocellum* promoters have been characterized (Olson et al., 2015). However, the apparent toxicity of the isobutanol pathway genes severely limits the applicability of these genetic systems. Thus, we developed a strategy to overcome this problem and screened for appropriate promoter combinations to express the necessary genes for the pathway, and constructed various strains for isobutanol production using the available plasmid-based system (Argyros et al., 2011; Guss et al., 2012; Deng et al., 2013). We then characterized the strains constructed, determined the gene copy number, identified native enzymes potentially involved in isobutanol biosynthesis, and optimized the production conditions.

2. Methods

2.1. Bacterial strains and plasmids

C. thermocellum DSM 1313 Δ*hpt* was a gift from Katherine Chou from the National Renewable Energy Laboratory. We referred *C. thermocellum* DSM 1313 Δ*hpt* as the wild type strain in this study because the Δ*hpt* is used for the sole purpose of counter-selection when needed, and has no effect on growth and fermentation. *Escherichia coli* BL21 (New England Biolabs, Ipswich, MA) and MDS™42 LowMut Δ*recA* (Pósfai et al., 2006) (SCARAB genomics, Madison, WI) were used as host for plasmid construction. Strains and plasmids used in this study are listed in Table 1.

All plasmids were constructed by DNA assembly techniques. Both vector and inserts (target genes) were amplified by PCR using Phire Hot Start II DNA polymerase (Thermo Scientific, Hudson, NH). PCR products were purified by a PCR purification Kit (Zymo Research, Irvine, CA). The vector and insert were mixed with Gibson Assembly Master Mix (New England Biolabs, Ipswich, MA) and incubated at 50 °C for 1 h. Then the assembly product was transformed to BL21 or MDS™42 LowMut Δ*recA* strain. The presence of correctly cloned inserts was determined by colony PCR and DNA sequencing (Retrogen, San Diego, CA).

2.2. Chemicals and reagents

All chemicals unless otherwise specified were acquired from Sigma-Aldrich (St. Louis, MO) or Thermo Scientific. Phire Hot Start II DNA polymerase was purchased from New England Biolabs.

2.3. Media and cultivation

All *E. coli* strains were grown in LB or TB medium containing appropriate antibiotics at 37 °C on a rotary shaker (250 rpm). Antibiotics were used at the following concentrations: ampicillin, 200 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml.

Except for small scale isobutanol production, all *C. thermocellum* strains were cultured inside a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) in a modified CTFuD medium (Tripathi et al., 2010) at 50 °C incubation . CTFuD medium contains the following components: 3 g/L of sodium citrate tribasic dehydrate, 1.3 g/L ammonium sulfate, 1.43 g/L potassium phosphate monobasic, 1.37 g/L potassium phosphate dibasic, 0.5 g/L cysteine-HCl, 21 g/L MOPS, 6 g/L glycerol-2-phospate disodium, 5 g/L cellobiose, 4.5 g/L yeast extract, 0.01 g/L calcium chloride, 0.011 g/L magnesium chloride, 0.0006 g/L ferrous sulfate heptahydrate, 0.01 g/L thiamin, and 0.001 g/L resazurin. Antibiotics were used at the following concentrations: thiamphenicol 20 ug/ml. In addition, 2.5 g/L sodium bicarbonate was used to enhance *C. thermocellum* growth.

Table 1
List of strains and plasmids used in this study.

Name	Description[a]	Reference
Strain		
E. coli BL21	*fhuA2 [lon] ompT gal [dcm] ΔhsdS*	New England Biolabs
MDS™42 LowMut ΔrecA	MG1655 multiple-deletion strain (1) Δ*dinB* Δ*polB* Δ*umuDC* (2) Δ*IS609* Δ*patD* Δ*ydcV* Δ*ydcU* Δ*ydcT* Δ*ydcS* Δ*ydcR* Δ*hicA* Δ*hicB* Δ*yncJ* Δ*ydcP* Δ*ydcN* Δ*ydcO* Δ*ydcM* Δ*recA* (1819) The *recA* 1819 mutation is a deletion of *recA*	SCARAB genomics
C. thermocellum Δ*hpt*	DSM 1313[T] Δ*hpt*[b]	National Renewable Energy Laboratory[c]
CT24	Δhpt *ilvBN*: P_{rpi}: $kivd_{LL}$ – P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study
CT24*	Strain CT24 with two point mutation on *kivd*[d]	This study
CT242	Δhpt *ilvBN*: P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study
Plasmid		
pDL	Source of thermostable $lacZ_{GS}$	Bacillus Genetic Stock Center
pNW33N	ColE1 and pBC1 *ori*; Cm^R; *E. coli-Bacillus* shuttle vector	Bacillus Genetic Stock Center
pCT24	ColE1 and pBC1 *ori*; Cm^R; P_{rpi}: $kivd_{LL}$ – P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study
pCT228	ColE1 and pBC1 *ori*; Cm^R; P_{rpi}: $kivd_{LL}$: RBS: Geoth_3237 – P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study
pCT229	ColE1 and pBC1 *ori*; Cm^R; P_{rpi}: $kivd_{LL}$: RBS: Geoth_3823 – P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study
pCT24*	ColE1 and pBC1 *ori*; Cm^R; P_{rpi}: $kivd_{LL}$(m2)[d] – P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study
pCT242	ColE1 and pBC1 *ori*; Cm^R; P_{pck}: $ilvBNC_{CT}$ – P_{ilvD}: $ilvD_{CT}$	This study

[a] In plasmid descriptions, subscripts indicate the source of the gene as follows: GS, *Geobacillus stearothermophilus*; LL, *Lactococcus lactis*; CT, *Clostridium thermocellum.*
[b] *C. thermocellum* DSM 1313 Δ*hpt* as wild type strain in this study because the Δ*hpt* is used for the sole purpose of counter-selection, and has no effect on growth and fermentation.
[c] *C. thermocellum* DSM 1313 Δ*hpt* was given by Katherine Chou from National Renewable Energy Laboratory.
[d] CT24* contains a *kivd* gene with two point mutations. However, there is no significant difference in isobutanol production between strains with CT24 and CT24*.

Stock cultures of *E. coli* were maintained at −80 °C in 13% (v/v) glycerol. Stock cultures of *C. thermocellum* were maintained at −80 °C directly.

2.4. C. thermocellum transformation

C. thermocellum electro-competent cells were freshly prepared as described (Guss et al., 2012). Briefly, *C. thermocellum* DSM 1313 Δ*hpt* was grown in CTFuD medium at 50 °C inside a Coy anaerobic chamber till OD_{600} =0.4–1. The culture was chilled on ice for 10 min, and cells were collected by centrifugation in a 500 ml corning bottle at 4 °C and 6000 g for 40 min. Then supernatants were removed aerobically. To minimize disturbance, cell pellets were washed with 400 ml ice MilliQ water (MQ) twice, and centrifuged at 4 °C and 6000*g* for 25 min. Lastly, pellets were resuspended with 200–500 μl electroporation buffer (250 mM sucrose and 10% glycerol) in the anaerobic chamber.

For each transformation, 25 μl of the competent cells were mixed with about 200–1000 ng of DNA in 1-mm-gap pre-chilled electroporation cuvettes (Molecular BioProducts, San Diego, CA). The mixtures were electroporated (1.2 kV, 1.5 m s square pulse) with a BioRad GenePulser XCell (BioRad Laboratories, Hercules, CA). Cells were immediately resuspended in 1 ml pre-warmed CTFuD medium, then plated by mixing with 25 ml molten CTFuD medium (0.8% agar) containing 20 μg/ml thiamphenicol without recovery period. The plates were incubated at 50 °C anaerobically for up to one week.

2.5. Screening isobutanol production strain from C. thermocellum recombinants

To screen for our isobutanol producing strain, 2 ml of engineered *C. thermocellum* DSM 1313 Δ*hpt* cultures were grown until stationary phase (OD_{600}=0.9–1.2) and centrifuged in 2 ml tubes at 6000*g* at 25 °C for 10 min. The pellets were resuspended in 1 ml CTFuD medium containing 100 g/L cellulose and 20 ug/ml thiamphenicol. The production was performed at 50 °C. Isobutanol was measured by gas chromatography after 24 h.

2.6. Optimization of production condition using small scale fermentation

Low-carbon minimal growth medium (LC medium) (Holwerda et al., 2012) was the starting point for optimizing isobutanol production. Optimization was studied using recombinant CT24* (CT24 strain with two point mutations on *kivd*, but no significant difference in isobutanol production compared to CT24). A small scale fermentation process was used in order to have faster experiment turnover rate. First, CT24* was pre-cultured in 50–250 ml of CTFuD medium till log phase (OD_{600}=0.2–0.6) or stationary phase (OD_{600}=0.9–1.2). Then, the culture was concentrated to 3 ml LC medium with 100 g/L cellulose at OD_{600}=3.3 or 16 with varying medium composition to start isobutanol production. During the production, pH was checked by pH strip (EMD Millipore, Billerica, MA) and titrated every 2 h by 45% KOH. Cells and cellulose were vigorous mixed every 2 h.

2.7. Isobutanol production

To examine isobutanol production, engineered *C. thermocellum* DSM 1313 Δ*hpt* cultures were grown till stationary phase (OD_{600}=0.9–1.2) and centrifuged at 4300 rpm at 40 °C for 30 min. Supernatant was removed and pellets were resuspended in LC medium. LC medium contains the following constituents: 100 g/L cellulose, 0.4 g/L urea, 5 g/L cellobiose, 21 g/L MOPS, 2 g/L potassium phosphate monobasic, 3 g/L potassium phosphate dibasic, 0.1 g/L cysteine-HCl, 0.05 g/L calcium chloride, 0.2 g/L magnesium chloride, 0.0035 g/L ferrous sulfate heptahydrate, 2.5 g/L sodium bicarbonate, 0.02 g/L pyridoxamine dihydrochloride, 0.004 g/L PABA, 0.002 g/L biotin, 0.002 g/L B12, and 0.01 g/L thiamin. For *C. thermocellum* cultures, antibiotics were used at the following concentration: thiamphenicol 20 ug/ml. Production of isobutanol was carried out in 5 mL centrifuge tubes with 3 mL of LC medium at pH 7.5, and 50 °C anaerobic incubation. Samples were maintained at pH=7.5 in 2 h intervals.

2.8. Cellulose measurement

Quantitative saccharification assay (Sluiter et al., 2008) was used for cellulose concentration measurement. First, 100 μL homogeneous medium solution were aliquoted into 1.5 mL microcentrifuge tubes and spun down for 2 min at 15,000 rpm. The supernatant was removed and the cellulose pellet was resuspended and vortexed in 1 mL of MQ twice. Following the last wash, samples were incubated overnight at 55 °C to dry pellets. Then, the dried samples were added to 143 μL of 72% H_2SO_4 and incubated samples for 1 h at 30 °C on an Eppendorf Thermomixer (Eppendorf, Hauppauge, NY) until the cellulose was completely dissolved. The solubilized samples were transferred to 5 ml centrifuge tubes with 4 mL of MQ and autoclaved for 1 h. Then, 500 μL autoclaved sample was then filtered to a 2 ml vial. Lastly, cellulose concentration was measured by first degrading to glucose, which was quantitatively measured via high-performance liquid chromatography (Agilent, Hanover, NH) with Aminex HPX-87 column (Biorad Laboratories, Hercules, CA).

2.9. Measuring gene transcription using quantitative real-time PCR (qRTPCR)

C. thermocellum cell samples were prepared using the same procedure as in isobutanol fermentation process. At the predetermined time point, 300 μl cell culture ($1.6*10^{10}$ cfu/ml) was collected and immediately mixed with 2 volumes of Qiagen RNAprotect bacteria agent to stabilize intracellular RNA. The cells were lysed with proteinase K and 15 mg/ml lysozyme in Tris buffer at pH 8.0 for 2 h. Total mRNA was subsequently extracted using Qiagen RNeasy Mini kit following the manufacturer's protocol. RNase-free DNase (Qiagen) was further used to treat the RNA column to minimize the genomic DNA contamination.

The qRTPCR reaction was carried out using iScript Reverse Transcription Supermix (BioRad) following the manufacturer's protocol. A typical reaction of 20 μl contains 10 μl iScript RT Supermix solution, 300 nM of each primer, 500 ng RNA sample, and 0.25 μl reverse transcriptase. The reactions were carried out in a 96-well

Table 2
Primer sequences used in qRTPCR.

Gene name	Primer sequence
recA	f: CTTATTGTTTCCCAGCCGGATACC
	r: CTGAAGACCTACATGGGAATCTCC
kivD	f: CAATTGGATATACATTCCCAGC
	r: CTAATTCTTGCACCGTAAGTTG
ilvB	f: TTATGGTTTCAAGGGCAGGAG
	r: TCCGACAGCATCTCTTTCAAC
ilvN	f: AAGCATACTTTATCGGTCCTGG
	r: GCTGTCAATGTTAAATCCCCTC
ilvC	f: GTTCTTGCTTTTGCCCACG
	r: ACACCTTTTCCCTCCACATAC
ilvD	f: CAGGTATCAGAATGGCAGGAG
	r: CATTCCCGTATGACCCATCG
repB	f: ACAGTTCGTTGGTTGTTTCTCAC
	r: CCGTTGCACGCATAAAACCA

plate using BioRad CFX96 Real Time System. The qRTPCR results were analyzed with the ΔC_T method using *recA* as the reference gene. The list of primers used in qRTPCR is listed in Table 2.

2.10. Measuring plasmid copy number using quantitative real-time PCR

C. thermocellum cell samples were grown in CTFuD rich medium to reach the stationary phase. The cells were subsequently washed with MQ through two cycles of centrifugation and resuspension. The concentrated cell suspensions were then subject to 98 °C for 4 min in a thermal cycler. The insoluble *C. thermocellum* cell debris was removed from the heat-treated samples after centrifugation, and the supernatant that contains *C. thermocellum* cell total DNA was collected for plasmid copy number analysis.

The qRTPCR reaction and the subsequent data analysis to determine plasmid copy number was carried out following the sample procedure as in the previous measurement of gene transcription, except no reverse transcriptase was added in the reaction mixture. *recA* was used as the reference gene in *C. thermocellum* genomic DNA, and *repB* was used as the plasmid-specific gene to determine the plasmid copy number.

2.11. Isobutanol dehydrogenase enzyme assay

The isobutanol dehydrogenase enzyme assay was carried out at 50 °C using an Agilent 8453 UV–vis spectrophotometer. The reaction mixture contains 40 mM Tris–Cl at pH 7.0, 5 mM dithiothreitol (DTT), 300 μM NADH/NADPH, 20 mM isobutaldehyde and crude extract. The reaction was initiated with the addition of isobutaldehyde. The rate of the enzymatic reaction was monitored with the decrease of absorption at 340 nm, corresponding to the consumption of NADH/NADPH. The total protein concentration was quantified using Bradford assay (BioRad)

2.12. Ketoisovalerate ferrodoxin-dependent reductase (KOR) enzyme assay

The KOR enzyme assay procedure was adapted from a previously reported protocol (Heider et al., 1996). The assay was carried out at 50 °C using the Agilent 8453 UV–vis spectrophotometer under strict anaerobic condition. The reaction mixture contained 200 mM potassium phosphate at pH 7.0, 5 mM thiamine pyrophosphate (TPP), 10 mM methyl viologen, 10 mM DTT, 2 mM $MgCl_2$, 2 mM coenzyme A, 6 mM KIV, if indicated, and crude extract. The reaction was initiated with the addition of *C. thermocelllum* crude extract. The rate of the enzymatic reaction was monitored with the increase of absorption at 604 nm, corresponding to the reduction of methyl viologen. The total protein concentration was quantified using Bradford assay (BioRad).

2.13. Strain stability assay

The *C. thermocellum* CT24 was prepared by freshly inoculating antibiotic-free CTFuD rich media from strain freeze stock. Then, the *C. thermocellum* culture at late exponential or early stationary phase (OD_{600}=0.8–1.5) was used for 10% inoculation to a fresh antibiotic-free CTFuD medium. The CT24 culture sample was taken at each cell passage and plated onto CTFuD agar with or without 20 μg/ml thiamphenicol. The difference in the number of colonies subsequently formed is used to determine the percentage of cells that retained the gene integration.

3. Results and discussion

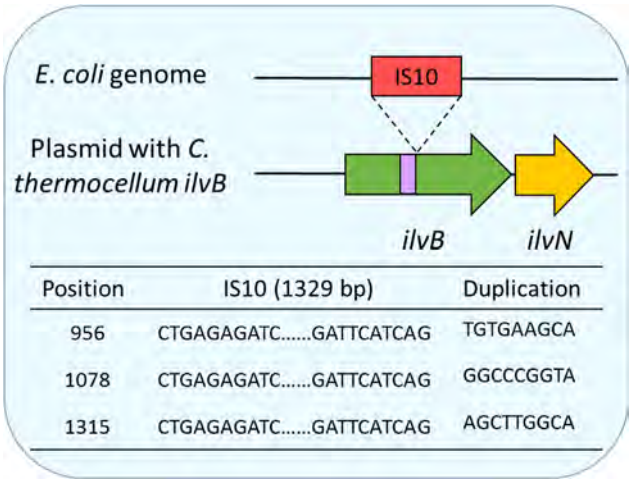
3.1. Toxicity of acetohydroxyacid synthase

Our previous work using *Geobacillus thermoglucosidasius* (Lin et al., 2014) confirms that the isobutanol pathway enzymes, specifically, *Bacillus subtilis* acetolactate synthase (AlsS) and *L. lactis* KIVD, are functional *at an elevated temperature* (50 °C). This result suggests that the pathway should work in *C. thermocellum*. In addition, various genes have been expressed by native glyceraldehyde-3-phosphate dehydrogenase (*gapDH*), cellobiose phosphorylase (*cbp*) and enolase promoters (Tripathi et al., 2010; Deng et al., 2013) in *C. thermocellum*. Based on these results, we began by overexpressing *L. lactis kivd* and *B. subtilis alsS* driven by the *gapDH* promoter. However, *C. thermocellum* transformation of the plasmids containing *gapDH* driven *L. lactis kivd* or *B. subtilis alsS* were unsuccessful after repeated attempts. A similar phenomenon was also observed in *C. cellulolyticum* when transforming a plasmid to express *B. subtilis* AlsS (Higashide et al., 2011). Cloning of genes having the same activity, such as *ilvBN* from various organisms, encountered similar difficulty (Li and Liao, 2015). During the cloning process, the *E. coli* host recognized the foreign promoters used and expressed the gene in an uncontrolled fashion. The metabolic changes then upset the host and resulted in either no colonies or colonies with inactivated genes. Interestingly, the *ilvB* clones almost always contain an insertion sequence IS10 (Fig. 1) at specific positions (956, 1078, and 1315 bp). Kovarík et al. (2001) reported an IS10 transposition event which occurred incidentally during gene cloning. Use of the Clean Genome® strain (Pósfai et al., 2006) alleviated the insertion problem and facilitated plasmid construction.

3.2. Selection of appropriate promoters for expressing isobutanol pathway genes in C. thermocellum

Insufficient expression of the pathway genes cannot produce high titers of isobutanol, while excessive expression may cause toxicity. Therefore, we had to select for a set of appropriate promoters to express the isobutanol pathway in *C. thermocellum* without compromising cell growth.

We started by applying the prevailing strategy (Fig. 2A) for prospecting promoters in *C. thermocellum* using a thermostable *lacZ*



Position	IS10 (1329 bp)	Duplication
956	CTGAGAGATC.....GATTCATCAG	TGTGAAGCA
1078	CTGAGAGATC.....GATTCATCAG	GGCCCGGTA
1315	CTGAGAGATC.....GATTCATCAG	AGCTTGGCA

Fig. 1. Scheme of inactivated *C. thermocellum ilvB* with *E. coli* IS10 during cloning. IS10 commonly inserts into *C. thermocellum ilvB* at specific locations (956, 1078, and 1315 bp). Insertion starts at the end of the duplication sequence (represented here in purple). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

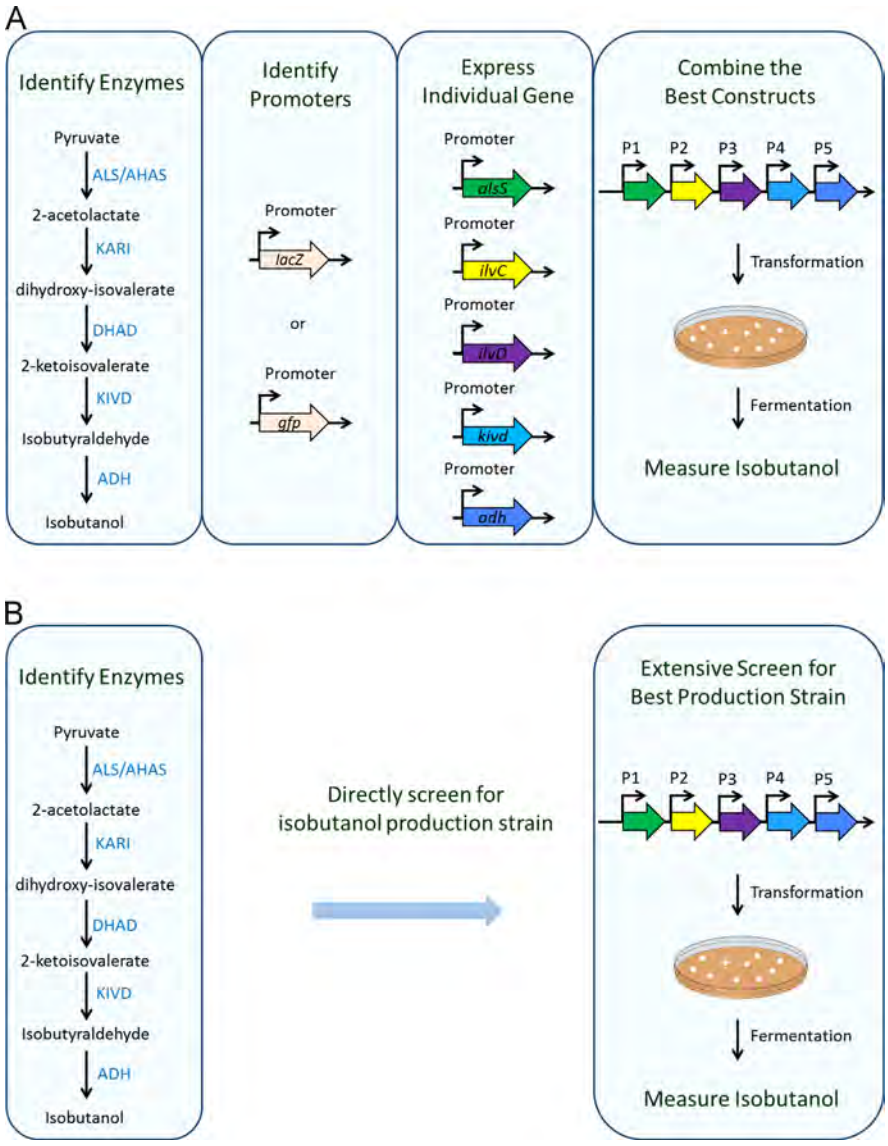


Fig. 2. (A) Current metabolic approach for constructing isobutanol pathway in *C. thermocellum*. (B) A streamline approach to directly screen for best *C. thermocellum* isobutanol production recombinant.

as a reporter. Promoters of various strengths were chosen to overexpress enzymes in the isobutanol pathway. However, the success of individual promoters does not necessarily translate to a functional pathway when combined, particularly because of the metabolic imbalance issue that may lead to toxicity. Therefore, we applied an alternative strategy (Fig. 2B) to directly screen for isobutanol production. We cloned all of the necessary genes (*kivd* from *L. lactis*, *alsS* from *B. subtilis or ilvBN* from *C. thermocellum*, and *ilvCD* from *C. thermocellum*) for isobutanol production under varying promoters to create different constructs. We included *C. thermocellum* AHAS (coded by *ilvBN*) as the enzyme to catalyze the first step in the isobutanol pathway, because this enzyme is relatively insensitive to feedback inhibition. These constructs excluded alcohol dehydrogenase (Fig. 3A) because the enzyme activity (NADPH-dependent) is present in *C. thermocellum* crude extract (Fig. S1).

However, cloning remains challenging due to the toxicity of overexpressing enzymes in the isobutanol pathway. All target plasmids containing the *alsS* gene driven by *C. thermocellum* promoters were unable to be constructed . We constructed 120 different plasmids using various promoters at the P1 and P2 positions and the native *ilvD* promoter for the P3 position (Table 3 and Table. S1 and S2) (Fig. 3A). 21 plasmids were

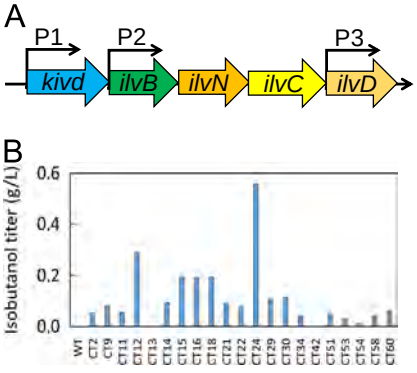


Fig. 3. (A) Plasmid constructs for overexpressing isobutanol pathway in *C. thermocellum*. P1 and P2 represent individual promoters and P3 is the native *C. thermocellum ilvD* promoter. (B) Screening of isobutanol production from engineered *C. thermocellum* in CTFuD medium within 24 h. Recombinant strains were grown in CTFuD medium to $OD_{600}=1$, then concentrated to CTFuD medium with 80 g/L cellulose at $OD_{600}=2$.

sequence-verified and transformed into *C. thermocellum*. Then, these engineered *C. thermocellum* strains were tested in the rich CTFuD medium at 50 °C for isobutanol production. The best strain

Table 3
List of plasmid constructs tested in *C. thermocellum* for isobutanol production.

Plasmid name	Promoter for *kivd*	Promoter for *ilvBNC*
pCT04	clo1313_0295	clo1313_0099
pCT09	clo1313_2131	clo1313_0099
pCT11	clo1313_1798	clo1313_0099
pCT12	clo1313_1616	clo1313_0099
pCT13	clo1313_0184	clo1313_0099
pCT14	clo1313_1983	clo1313_0099
pCT15	clo1313_2092	clo1313_0099
pCT16	clo1313_2093	clo1313_0099
pCT18	clo1313_1717	clo1313_0099
pCT21	clo1313_1616	clo1313_0099
pCT22	clo1313_0184	clo1313_1616
pCT24	clo1313_0184	clo1313_0415
pCT29	clo1313_1616	clo1313_1983
pCT30	clo1313_1616	clo1313_2131
pCT34	clo1313_1616	clo1313_1364
pCT42	clo1313_0184	clo1313_1717
pCT51	clo1313_0184	clo1313_1983
pCT53	clo1313_1983	clo1313_2092
pCT54	clo1313_1983	clo1313_2942
pCT58	clo1313_1798	clo1313_1616
pCT60	clo1313_1798	clo1313_1818

(CT24) produced 0.6 g/L of isobutanol within 24 h in the un-optimized condition (Fig. 3B).

We tested the effect of overexpressing isobutanol dehydrogenase. Two thermostable isobutanol dehydrogenase enzymes from *G. thermoglucosidasius*, one NADH-dependent (Geoth_3237) and one NADPH-dependent (Geoth_3823) (Lin et al., 2014), were cloned onto the pCT24 backbone to make pCT228 and pCT229. These plasmids were transformed to *C. thermocellum* to make strains CT228 and CT229. However, both CT228 and CT229 had no significant effect on isobutanol production compared to CT24 strain (Fig. S2). This suggests isobutanol dehydrogenase is not the limiting step in isobutanol production of our recombinant strain CT24.

3.3. Optimization of production conditions from cellulose

Although many defined minimal growth medium for *C. thermocellum* have been previously reported (Fleming and Quinn, 1971; Johnson et al., 1981; Holwerda et al., 2012), medium composition has not been optimized for isobutanol production. We chose the LC medium (Holwerda et al., 2012) with Avicel cellulose as the starting point for optimizing isobutanol production. To accelerate the turnover rate of the experiment and explore better production conditions, we developed a small scale high density fermentation as mentioned previously. Initially, the target strain was grown in CTFuD medium for the fast growth rate. Then, the culture was concentrated to higher density to achieve higher productivity and to mimic industrial processes.

We varied the medium composition (vitamin, bicarbonate, urea and pH). Lower urea concentration (from 7.5 g/L to 1 g/L) improved isobutanol production titer 2.4 fold (Fig. 4A), but had no significant effect on ethanol production (Fig. 4B). High urea concentration favored valine production, while low urea shifted the product to isobutanol, as expected (Fig. S3). Further, we focused on optimizing the pre-culture condition and density (OD_{600}) after suspension. Fig. S4 shows that cell harvesting at the stationary phase (OD_{600}= 1.1) increased both isobutanol and ethanol production almost twofold as a result of less valine and lactate production. Pre-culture with cellulose and mixing during production had no significant effect on isobutanol production (Fig. S4A), yet increased density (OD_{600}=3.3–16) enhanced isobutanol production fivefold (Fig. S4A), as expected.

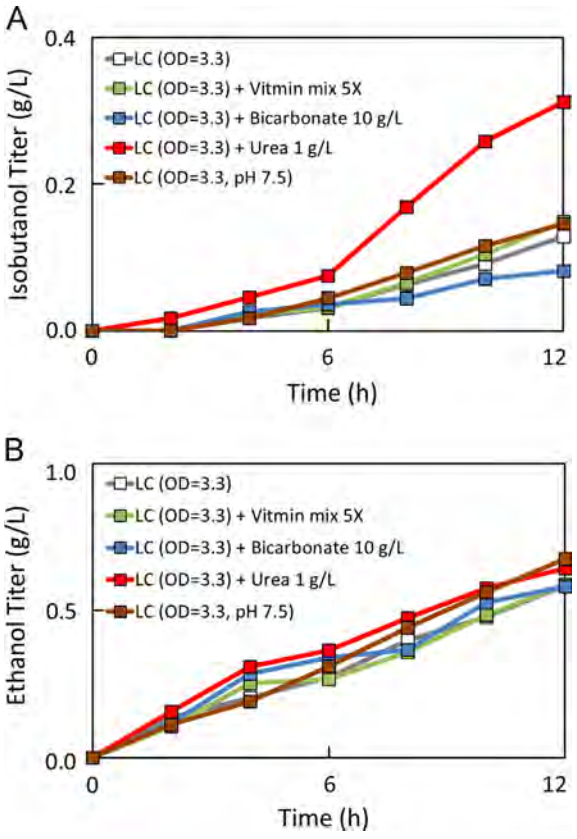


Fig. 4. LC medium optimization for (A) isobutanol and (B) ethanol production at 50 °C. CT24* strain (CT24 strain with two point mutations on *kivd*, but no significant difference in isobutanol production compared to CT24) was grown in CTFuD medium to OD_{600}=0.2, then concentrated to the various LC media with 80 g/L cellulose at OD_{600}=3.3.

With the improved procedure, we tested isobutanol production from cellulose in LC medium using CT24. The strain was grown in CTFuD medium to OD_{600}=1–1.5, then concentrated to LC medium (80 g/L cellulose, pH=7.5, urea=0.4 g/L) at OD_{600}=16. Under this production protocol, 5.4 g/L of isobutanol was produced during 75 h (Fig. 5A). Since the initial cellulose concentration was 80 g/L, and the final concentration of cellulose was 46.4 g/L with 1.6 g/L glucose left in the medium, the yield roughly 41% of the theoretical maximum. The major byproducts during the CT24 fermentation were lactate, acetate and ethanol (Fig. 5B). The wild-type control produced about 1.5 g/L of isobutanol, with a significant amount of valine (0.54 g/L for WT, 1.1 g/L for CT24) produced.

3.4. Confirming pathway overexpression with qRTPCR

In order to ascertain that the observed boost in isobutanol titer from our best production strain CT24 was a direct result of isobutanol pathway overexpression, quantitative real-time PCR (qRTPCR) was performed to measure the relative transcription level of five genes (*ilvB*, *ilvN*, *ilvC*, *ilvD* and *kivd*) constituting this pathway. The *recA* gene was selected as the reference in qRTPCR following previous studies (Stevenson and Weimer, 2005; Wei et al., 2014). Under the optimized fermentation condition (Fig. 6A), a significant increase in mRNA levels was detected for all genes of interest (Fig. 6B). The most pronounced change, approximately fivefold increase in comparison to parental *C. thermocellum*, was observed for *ilvB*, *ilvN* and *ilvC*, all of which were under the control of native phosphoenolpyruvate carboxykinase (*pck*) promoter. Furthermore, the successful transcription of the non-native gene *kivd* was also confirmed in this experiment. A similar transcriptional pattern was observed for CT24 during the growth phase (Fig. S5,

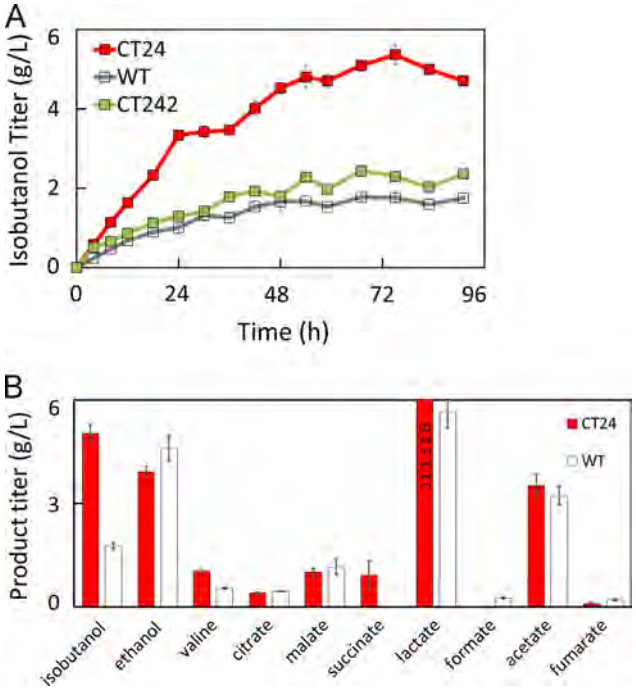


Fig. 5. (A) Isobutanol production and (B) fermentation products formation during 75 h in the optimized LC medium. Wild type, CT24 and CT242 strains were grown in CTFuD medium to OD_{600}=1–1.2, then concentrated to LC medium (0.4 g/L urea) with 80 g/L cellulose at OD_{600}=16. Error bar represents the standard deviation (n=3).

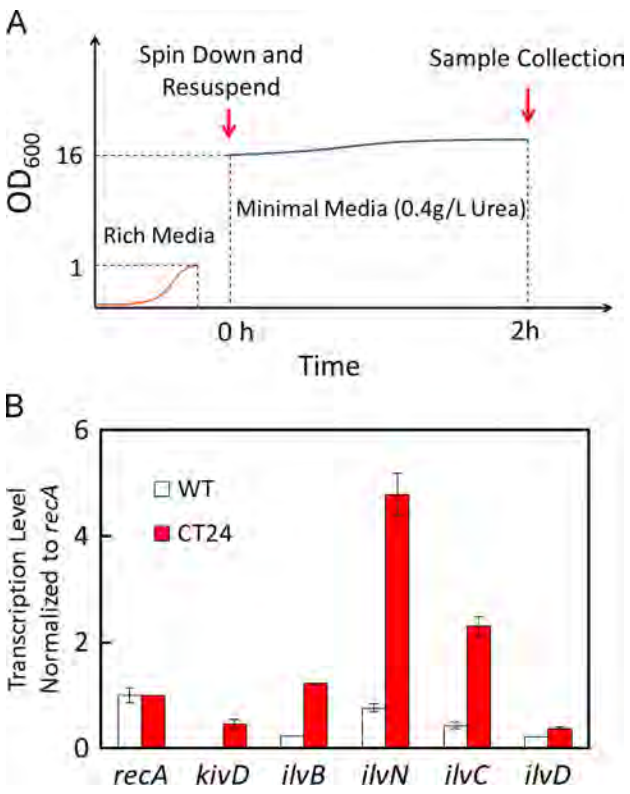


Fig. 6. (A) Scheme for collecting qRTPCR samples under isobutanol fermentation condition. (B) Gene transcription comparison between wild type *C. thermocellum* and isobutanol production strain (CT24) during fermention. *recA* was used as the reference gene in all samples. Error bar represents the standard deviation (n=3).

CTFuD medium and OD_{600}=1 medium cell density) . Taken together, these results indicate that the isobutanol pathway overexpression was responsible for the increase in isobutanol titer.

3.5. Enzymes catalyzing KIV decarboxylation

Ketoisovalerate (KIV) is the divergent point between valine biosynthesis and isobutanol production. As reported previously Holwerda et al. (2014), we also found that *C. thermocellum* wild type strain without genetic engineering is capable of producing detectable amounts of isobutanol (Fig. 5A) indicates that a KIVD-independent native pathway exists in this organism to convert KIV to isobutanol. Examination of *C. thermocellum* DSM 1313 genome points to ketoisovalerate ferrodoxin-dependent reductase (KOR) as the most likely native enzyme to carry out the KIV decarboxylation reaction in the wild type strain (Fig. 7A). KOR had been previously reported in several anaerobic thermophiles (Heider et al., 1996). Three putative KOR genes (Clo1313_0020-0023, Clo1313_0382-0385 and Clo1313_1353-1356) were also annotated in *C. thermocellum* genome.

To verify that *C. thermocellum* has functional KOR to decarboxylate KIV to isobutyryl-CoA using coenzyme-A as a cofactor, we performed an anaerobic KOR enzyme assay following a previously reported protocol using wild type crude extract (Heider et al., 1996). The methyl viologen-based end point assay showed the presence of oxygen-sensitive KOR in *C. thermocellum* crude extract. This KOR enzyme activity was quantified in a subsequent kinetic assay to be 2.4 μmol/min/mg (Fig. 7 B and C).

The confirmation of native KOR activity naturally led us to consider whether KIVD overexpression is indeed needed for isobutanol production in CT24. In order to answer this question, we constructed a *C. thermocellum* strain to overexpress *ilvB, ilvN, ilvC and ilvD* using the same promoters as in CT24 while leaving out *kivd*. The resulting strain, termed CT242, showed an isobutanol titer measurably higher than wild type strain but significantly lower than CT24 (Fig. 5A and Fig. S6). Presumably, the overexpression of *ilvB, ilvN, ilvC and ilvD* was able to increase the flux to ketoisovalerate and the native KOR diverts this intermediate to isobutanol production in CT242. These results demonstrated that the KIV decarboxylation step in CT24 was carried out by a combined contribution from both KOR and KIVD.

3.6. Genome integration and strain stability

To determine the copy number of the plasmid in CT24, qRTPCR was used to quantify the gene copy number of *recA* (genome specific), *repB* (plasmid specific) and *ilvC* (exist both on genome and plasmid) using *C. thermocellum* CT24 total DNA (Skulj et al. 2008). The resulting ratio of *recA*:*repB*:*ilvC*=1:1:2 (Fig. 8A) indicates that, regardless of its DNA form, the plasmid exists as a single copy inside the cell. This result suggests that the plasmid may be integrated into the chromosome by single crossover. PCR was then used to amplify CT24 *ilvBNC* and *ilvHD* operons. As these two operons share sequence similarity with the plasmid, they are the potential sites for homologous recombination. The PCR products were then sequenced and the results conclusively showed that the plasmid expressing isobutanol pathway was integrated into the *ilvBNC* operon in the *C. thermocellum* genome via a single crossover (Fig. 8B).

Interestingly, this single-crossover genome insertion was observed in our study to be very stable even without antibiotic selection (Fig. 8C). Furthermore, genetically engineered CT24 strain was found to outcompete wild type *C. thermocellum* in a prolonged semi-continuous mixed culture using antibiotic-free CTFuD rich medium (data not shown), which suggested that the overexpression of isobutanol pathway may increase *C. thermocellum* fitness under certain growth conditions. This finding was further confirmed by growth measurement (Fig. 8D). This result

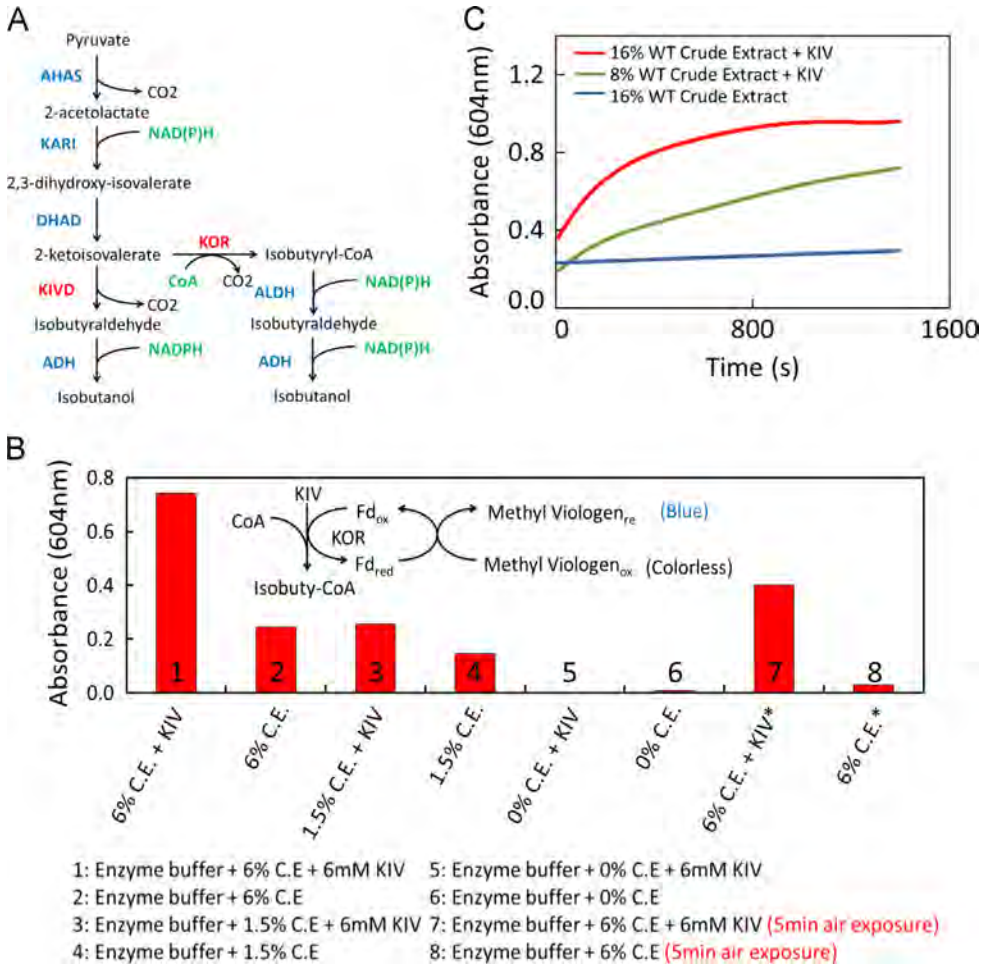


Fig. 7. (A) Two conversion routes of 2-ketoisovalerate to isobutanol in engineered *C. thermocellum* isobutanol production strain. (B) Anaerobic end point enzyme assay of native KOR enzyme activity using wild type *C. thermocellum* crude extract. (C) Anaerobic kinetic enzyme assay of native KOR enzyme activity using wild type *C. thermocellum* crude extract.

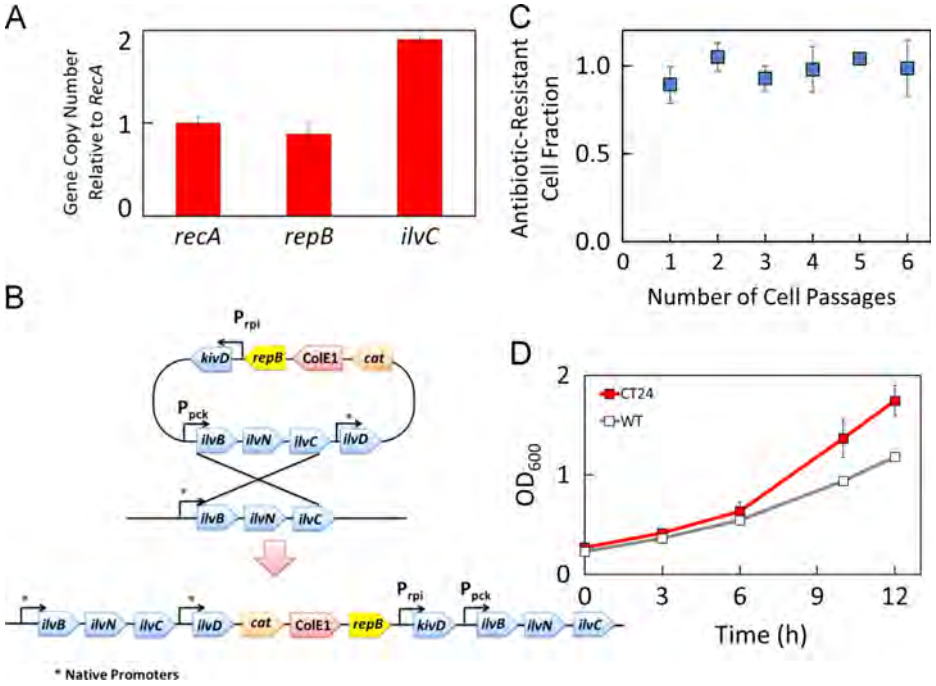


Fig. 8. (A) Determination of gene DNA copy number in CT24 using qRTPCR. (B) Sequencing verified plasmid genome integration after a single crossover in CT24. (C) Strain stability measured by the retention of antibiotics marker after growth and passage in antibiotic-free rich media. (D) Growth curve of CT24 and wild type strain in CTFuD medium without antibiotic pressure. Error bar represents the standard deviation (n=3).

also attests to the practical applicability of this strain and single crossover as a strain construction strategy.

4. Conclusion

In this work, we engineered *C. thermocellum* to produce isobutanol directly from cellulose. We first addressed the cloning difficulty in *E. coli* caused by an IS10 insertion which occurred due to the enzyme toxicity of acetohydroxyacid synthase (encoded as *ilvBN*). Then we applied a strategy to select the best isobutanol producing engineered strain without compromising cell growth. The successful pathway overexpression was subsequently verified with qRTPCR. The activity of the native ketoisovalerate oxidoreductase (KOR), a key enzyme in the native isobutanol pathway, was also demonstrated. We further discovered that the plasmid in the best production strain was chromosomally integrated by a single crossover event. However, this strain was stable without the antibiotic selection pressure. The best engineered strain produced 5.4 g/L of isobutanol from cellulose in optimized minimal medium at 50 °C within 75 h, corresponding to 41% of theoretical yield. The success of this strain demonstrates that *C. thermocellum* is a promising CBP organism for isobutanol production from cellulose.

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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.2015.07.001.

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