Consolidated bioprocessing of cellulose to isobutanol using Clostridium thermocellum

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A B S T R A C T

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.

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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 (Tyrin 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 used as jet fuel. The C8 alkene can also be dehydroxylized 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...
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 Δhpt was a gift from Katherine Chou from the National Renewable Energy Laboratory. We referred C. thermocellum DSM 1313 Δhpt as the wild type strain in this study because the Δhpt 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 ΔrecA (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 ΔrecA 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.

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 phosphate dibasic, 0.5 g/L cytochrome-HCl, 21 g/L MOPS, 6 g/L glycerol-2-phosphate 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

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
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<tr>
<td>E. coli BL21</td>
<td>fhua2 [lon] ompT gal [dcm] ΔhodS</td>
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<tr>
<td>MDS™-42 LowMut</td>
<td>MG1655 multiple-deletion strain (1) ΔdinB ΔpolB ΔamuDC (2) ΔIS509 ΔaperD ΔydvΔψ ΔycdU ΔycdT ΔydcS ΔydcR ΔhicB ΔhicC ΔrecA (1819) The recA 1819 mutation is a deletion of recA</td>
<td>SCARAB genomics</td>
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<td>C. thermocellum Δhpt</td>
<td>DSM 1313 Δhpt</td>
<td>National Renewable Energy Laboratory&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>CT24</td>
<td>Δhpt iibBN: P&lt;sub&gt;recA&lt;/sub&gt; kivd&lt;sub&gt;Δ2&lt;/sub&gt; – P&lt;sub&gt;recA&lt;/sub&gt; iibBN&lt;sub&gt;Δ2&lt;/sub&gt; – P&lt;sub&gt;recA&lt;/sub&gt; iibDN&lt;sub&gt;Δ2&lt;/sub&gt;</td>
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<td>CoE1 and pBC1 ori; Cm&lt;sup&gt;2&lt;/sup&gt;; E. coli-Bacillus shuttle vector</td>
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<sup>a</sup> In plasmid descriptions, substrates indicate the source of the gene as follows: GS, Geobacillus stearothermophilus; LL, Lactococcus lactis; CT, Clostridium thermocellum.

<sup>b</sup> C. thermocellum DSM 1313 Δhpt was given by Katherine Chou from National Renewable Energy Laboratory.

<sup>c</sup> CT24<sup>2</sup> contains a kivd gene with two point mutations. However, there is no significant difference in isobutanol production between strains with CT24 and CT24<sup>2</sup>.
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 Δhpt was grown in CTFuD medium at 50 °C inside a Coy anaerobic chamber till OD₆₀₀ = 0.4–1. The culture was chilled on ice for 10 min, and cells were collected by centrifugation in a 500 ml conning 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 ms 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 Δhpt cultures were grown until stationary phase (OD₆₀₀ = 0.9–1.2) and centrifuged in 2 ml tubes at 6000g at 25 °C for 10 min. The pellets were resuspended in 1 ml CTFuD medium containing 100 g/L cellulose and 20 μg/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 difference in isobutanol production compared to CT24). A small scale fermentation process was used in order to have faster disturbance, cell pellets 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.

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### 2.7. Isobutanol production

To examine isobutanol production, engineered *C. thermocellum* DSM 1313 Δhpt cultures were grown until stationary phase (OD₆₀₀ = 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 cellulobiose, 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 μg/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 twi. 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% H₂SO₄ 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 (qRT-PCR)

*C. thermocellum* cell samples were prepared using the same procedure as in isobutanol fermentation process. At the predetermined time point, 300 μl cell culture (1.6×10⁷ 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 RNasy 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 qRT-PCR 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>
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<th>Gene name</th>
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<td>recA</td>
<td>f: CTTATGGTTTCCCAACCGGATAAC&lt;br&gt;r: CGAGACATCTACAGGGAATCTCC</td>
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<td>kivD</td>
<td>f: CTAAATCTTGGACCGTGTAATTT&lt;br&gt;r: CAAAATTGGATATACATTCCCAGC</td>
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<td>ilvB</td>
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<td>repB</td>
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</table>

Table 2

| Primer sequences used in qRT-PCR. |
3. Results and discussion

3.1. Toxicity of acetohydroxyacid synthase

Our previous work using Geobacillus thermoglucoptidase (Lin et al., 2014) confirms that the isobutanol pathway enzymes, specifically, Bacillus subtilis acetalactate 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), cellibiose phosphorylase (cbb) 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). Kovarik 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 reporter gene (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. 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.)
as a reporter. Promoters of various strengths were chosen to over-express 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 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
(CT24) produced 0.6 g/L of isobutanol within 24 h in the unoptimized condition (Fig. 3B).

We tested the effect of overexpressing isobutanol dehydrogenase. Two thermostable isobutanol dehydrogenase enzymes from *C. thermohydrosulphinus*, 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 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.

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}=0.2, then concentrated to the various LC media with 80 g/L cellulose at OD_{600}=3.3.

### 3.4. Confirming pathway overexpression with qRT-PCR

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 (qRT-PCR) 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 qRT-PCR 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. Similarly transcriptional pattern was observed for CT24 during the growth phase (Fig. S5, Table 3

<table>
<thead>
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<th>Plasmid name</th>
<th>Promoter for kivD</th>
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Fig. 4. LC medium optimization for (A) isobutanol and (B) ethanol production at 50 C. CT24 strain (CT24 strain with two point mutations on kind, 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.
This KOR enzyme activity was quantified 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).

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-type strain without genetic engineering is capable of producing isobutanol (Fig. 5A) indicates that a KIVD-type strain without genetic engineering is capable of producing isobutanol. Examination of C. thermocellum DSM 1313 genome points to ketoisovalerate ferredoxin-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. 5B). 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, qRT-PCR 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 \textit{C. thermocellum} to produce isobutanol directly from cellulose. We first addressed the cloning difficulty in \textit{E. coli} caused by an IS10 insertion which occurred due to the enzyme toxicity of acetohydroxyacid synthase (encoded as \textit{ilvB/N}). 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 \textit{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.jybeem.2015.07.001.

References


