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METABOLIC ENGINEERING OF *CLOSTRIDIUM CELLULOLYTICUM* FOR ISOBUTANOL PRODUCTION FROM CELLULOSE

Wendy Higashide[1, §], Yongchao Li[2,§], Yunfeng Yang[2‡], and James C. Liao[1, 3*]

[1]*Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, Los Angeles, CA 90095*

[2]*BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN 37831*

[3]*Institute for Genomics and Proteomics, University of California, Los Angeles, Los Angeles, CA 90095*

*Corresponding author. Mailing address: Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, 5531 Boelter Hall, 420 Westwood Plaza, Los Angeles, California 90095, USA. Phone: (310) 825-1656, Fax: (310) 206-4107. E-mail: liaoj@seas.ucla.edu.

§ Both authors contributed equally to this work and are co-first authors.

‡Current address: Department of Environmental Science & Engineering, Tsinghua University, Beijing 100084, China.

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ABSTRACT

Producing biofuels directly from cellulose, known as consolidated bioprocessing, is believed to reduce costs substantially compared to a process in which cellulose degradation and fermentation to fuel are accomplished in separate steps. Here we present a metabolic engineering example to develop a *Clostridium cellulolyticum* strain for isobutanol synthesis directly from cellulose. This strategy exploits the host's natural cellulolytic activity and the amino acid biosynthetic pathway and diverts its 2-keto acid intermediates for alcohol synthesis. Specifically, we have demonstrated the first isobutanol production to approximately 660 mg/L from crystalline cellulose using this microorganism.

INTRODUCTION

Compared to biofuels produced from corn (starch-rich) and sugar cane (sucrose-rich), biofuels obtained from cellulosic materials could result in lower fuel costs (16), greater petroleum displacement (13), and lower greenhouse gas emissions (16). To meet its potential, technological advances are needed to improve the conversion efficiency of the recalcitrant lignocellulose to fermentable sugars (29). So far improvements in cellulase production (17, 20) and pretreatment techniques (1) have aided in increasing cellulose degradation efficiency in a cost-effective manner.

Another approach which has generated much interest is consolidated bioprocessing (CBP). This process utilizes microorganisms to perform biomass hydrolysis and the fermentation of the sugars into biofuel within a single process (16). Research in this area has taken one of two approaches. In one approach, referred to as the 'recombinant cellulolytic strategy' (14), microorganisms that have previously demonstrated high biofuel yields are engineered to utilize cellulose and/or the sugars resulting from cellulose degradation. These organisms have been genetically engineered to expand their substrate range to include cellulose or the sugars freed from cellulose or hemicellulose degradation, as in the case of ethanologenic organisms such as *Escherichia coli* (23, 33), *Zymomonas mobilis* (4, 18), and *Saccharomyces cerevisiae* (14, 27). Research efforts continue on improving the strains' cellulolytic abilities to industrially relevant levels. For the 'native cellulolytic strategy' (14), research has mainly focused on microorganisms that possess cellulosomes, which are extracellular multi-enzyme complexes that aid in the digestion of cellulose. While these microorganisms are capable of efficiently hydrolyzing cellulose, their biofuel productivities are significantly lower

than existing industrial strains. In addition to improving biofuel productivity (22), research efforts are also focused on increasing ethanol yield (31), eliminating competing pathways (26), and improving ethanol tolerance (30).

Most studies employing the native cellulolytic strategy have been conducted with the thermophlic, cellulolytic *Clostridium thermocellum*. This strain is particularly attractive because it will be able to thrive in high-temperature fermentations, which are conducive to high substrate conversion, low contamination risk, and high product recovery (15). Although *C. thermocellum* has potential to be a CBP organism, issues such as low transformation efficiency (28) and the lack of publications demonstrating successful overexpression of foreign proteins in *C. thermocellum*, significantly impede the engineering progress of this organism to produce synthetic biofuels, such as isobutanol. One way to hasten this progress is to first establish and optimize the desired metabolic pathways in a closely related, more amenable organism. Once the specifics have been determined, such as identifying which genes to be overexpress, mutate, and/or delete, the same strategy can then be adapted to *C. thermocellum*. *Clostridium cellulolyticum*, which was originally isolated from decayed grass (21), is a useful candidate for this initial metabolic engineering work because, like *C. thermocellum*, it belongs to *Clostridium* group III based on 16S rRNA phylogenetic analysis (7), and as a mesophile, many problems that are associated with the heterologous expression of proteins in thermophiles are circumvented. In addition, *C. cellulolyticum* has a sequenced genome (GenBank Accession: NC_011898.1), well-established DNA transfer techniques (24), and methods in gene overexpression (10). As a potential CBP organism in its own right, *C. cellulolyticum* can not only utilize xylose similar to *C. thermocellum*,

but it can also utilize additional sugars freed from hemicellulose degradation, including arabinose, fructose, galactose, mannose, and ribose (9).

Previously, *C. cellulolyticum* has been genetically engineered for improved ethanol production (10). Similarly, most of the research concerning the construction of a CBP organism has focused on ethanol production. Despite this, it has been asserted that higher alcohols, such as isobutanol, are better candidates for gasoline replacement because they have an energy density, octane value, and Reid vapor pressure that are more similar to gasoline (5). Unlike ethanol, isobutanol can also be blended at any ratio with gasoline or used directly in current engines without modification (8). In this study, we have metabolically engineered *C. cellulolyticum* to produce isobutanol. By expressing enzymes that direct the conversion of pyruvate to isobutanol using an engineered valine biosynthesis pathway, we were able to produce up to 660 mg/L of isobutanol using *C. cellulolyticum* growing on crystalline cellulose.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 1. Restriction enzymes, phosphatase (New England Biolabs, Ipswich, MA), ligase (Rapid DNA ligation kit; Roche, Mannheim, Germany), and DNA polymerase (KOD DNA polymerase; EMD Chemicals, San Diego, CA) were used for cloning. Oligonucleotides were synthesized by Eurofins MWG Operon (Huntsville, AL).

Chemicals. Unless indicated otherwise, commercial reagents, enzymes, and coenzymes were supplied by Sigma Chemical Company (St. Louis, MO).

Media and cultivation. *C. cellulolyticum* was grown at 34˚C in VM medium (11) that has been modified to reduced precipitation. For 1 liter: KH_2PO_4 (1.0 g), K_2HPO_4 (3.4 g), urea (2.14 g), $MgCl_2$ 6·H_2O (1.0 g), $CaCl_2 \cdot 2H_2O$ (0.15 g), $FeSO_4 \cdot 6H_2O$ (1.25 mg), 3-(N-morpholino)propanesulfonic acid (MOPS, 10.0 g), resazurin (2.0 mg), vitamin solution (10 ml), yeast extract (2.0 g), oligoelement solution (1 ml) cysteine-HCl (1.0 g), and cellobiose (5.1345 g). The vitamin solution (100X) contained: biotin (0.08 µM), pyridoxamine (0.02 µM), cyanocobalamin (0.001 µM), ρ-aminobenzoic acid (0.15 µM), thiamine (0.9 µM), and L-alanine (0.22 µM). The 1000X oligoelement solution contained (in grams $liter^{-1}$ unless otherwise indicated): $FeSO_4 \cdot 7H_2O$ (5.0 g), $ZnSO_4 \cdot 7H_2O$ (1.44g), $MnSO_4 \cdot 7H_2O$ (1.12g), $CuSO_4 \cdot 5H_2O$ (0.25 g), $Na_2B_4O_7$ (0.20 g), $(Mo)_7(NH_4)_6O_{24} \cdot 4H_2O$ (1.00 g), $NiCl_2$ (0.04 g), $CoCl_2$ (0.02 g), HBO_3 (0.03 g), Na_2SeO_3 (0.02 g), and HCl (50 ml of 10 M).

For agar plates, 0.8% (wt/vol) of agar (Difco Laboratories, Detroit, MI) was added to the media. To make competent cells, to prepare cell lysates for enzyme assays, daily maintenance, and to determine isobutanol production on cellobiose, the strains were grown in VM modified media. To examine isobutanol, lactate, acetate, and ethanol production on cellulose, the strains were grown on VM modified media, where cellobiose and yeast extract were replaced with 10 g/L of crystalline cellulose (Sigma type 50, 50 um).

Stock cultures of *C. cellulolyticum* were maintained at -80˚C in 15% (vol/vol) glycerol and were grown for one transfer in cellobiose medium before initiation of growth experiments.

Transformation. Cell transformation was conduced as described previously (11) with some modifications. Cells were grown for 17-24 h in 10 ml cultures of modified VM media to late exponential phase (OD600 0.5 – 1.0, 5 x 10^6 c.f.u./ml). The following steps were all performed with anoxic solutions under anaerobic conditions at 4˚C. The cells were washed twice with cold electroporation buffer (270 mM sucrose, 1 mM $MgCl_2$, 5 mM sodium phosphate buffer, pH 7.4). The cells were resuspended in 600 µl of electroporation buffer. For each transformation, 200 µl of the cells was mixed with 2 µg of MspI methylated plasmid DNA. The DNA was methylated overnight with 5 units of MspI methyltransferase (New England Biolabs, Ipswich, MA), then purified with the DNA Clean and Concentrator Kit (Zymo Research Inc., Orange, CA). In 2 mm gap electroporation cuvettes (Molecular BioProducts, San Diego, CA), the cells and plasmid DNA were electroporated (1.5 kV, 25 µF, and 48 Ω) with a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA). The electroporated cells were transferred to 10 ml of fresh modified VM media. The cells were recovered for 24 h at 34˚C and then the cells were collected by centrifugation, and cell pellet was spread on modified VM cellobiose agar plates supplemented with 10 µg/ml erythromycin. The plates were incubated at 34˚C anaerobically for 5 to 7 days. Single colonies were transferred to 10 ml VM cellobiose medium supplemented with 10 µg/ml erythromycin.

Analytical procedures. Bacterial growth was measured spectrophotometrically at 600 nm. For cultures containing cellulose, the cellulose was allowed to settle for at least two hours before samples were taken for measurement.

The produced alcohol compounds were quantified by a gas chromatograph (GC) with a flame ionization detector. The system consisted of model 5890A GC (Hewlett-

147 Packard, Avondale, PA) and a model 7673A automatic injector, sampler, and controller
148 (Hewlett-Packard). The separation of alcohol compounds was carried out using a DB-
149 WAX capillary column (30 m, 0.32 mm-inside diameter, 0.50-μm film thickness)
150 purchased from Agilent Technologies (Santa Clara, CA). The GC oven temperature was
151 initially held at 40°C for 5 min and raised with a gradient of 15°C/min until reaching
152 120°C. It was then raised with a gradient of 50°C/min until 230°C and held for 4 min.
153 Helium was used as the carrier gas, with 9.3-lb/in^2 inlet pressure. The injector and
154 detector were maintained at 225°C. Supernatant of culture broth (0.5 μl) was injected in
155 split injection mode with a 1:15 split ratio. Pentanol was used as the internal standard.
156 **Enzyme Assays.** The cells were grown for 17-24 h in 50 ml cultures of modified VM
157 media to late exponential phase (OD600 0.5 – 1.0, 5 x 10^6 c.f.u./ml). The cells were
158 harvested, washed in 50 mM potassium phosphate buffer, pH 7.5, and resuspended in 0.5
159 ml of the same buffer. Crude extract was prepared under aerobic conditions with 0.1-mm
160 glass beads and a Mini Bead Beater 8 (BioSpec Products, Inc., Bartlesville, OK). Total
161 protein measurements were made with the Bradford protein assay kit from Bio-Rad
162 (Hercules, CA).
163 The AlsS assay was performed as described previously (32), with the exception
164 that the reaction mixture contained 20 mM sodium pyruvate, 100mM MOPS buffer, pH
165 7.0, 1 mM $MgCl_2$, and 100 μM cocarboxylase. The concentration of acetoin produced
166 was determined by a standard curve created using pure acetoin. One specific unit of AlsS
167 activity corresponds to the formation of 1 nmol of acetoin per min per mg of soluble
168 protein at 34°C.

To measure the reduction of 2-acetolactate to 2,3-dihydroxy-isovalerate, the oxidation of NADPH was monitored by a decrease in absorbance at 340 nm. The substrate, 2-acetolactate, was first produced in a separate reaction as described for the Als assay using purified, heterogeneously expressed *B. subtilis* AlsS in *E. coli* strain BL21. From this reaction 180 µl was added to 200 mM potassium phosphate buffer, pH 7.5, 4 mM $MgCl_2$, and 0.1 mM NADPH. The samples were incubated at 34°C for 5 min, then the reaction was initiated with the addition of cell extracts. The consumption of NADPH was monitored at 340 nm (extinction coefficient, 6.22 $mM^{-1}cm^{-1}$). IlvC activity is expressed as nmol of NADPH oxidized per min per mg of soluble protein at 34°C.

The IlvD assay was performed as described previously (12). The 500 µl reaction mixture contained 5 mM $MgSO_4$, 50 mM Tris-Cl, pH 8.0, cell extract, and 10 mM 2,3-dihydroxy-isovalerate. The substrate, 2,3-dihydroxy-isovalerate, was synthesized as described previously (6). After the reaction mixture was preincubated for 5 min at 34°C, the substrate was added to initiate the reaction. The samples were incubated for 15 min at 34°C. The reaction was terminated by the addition of 125 µl of 10% (wt/vol) trichloroacetic acid, then 250 µl of saturated 2,4-dinitrophenylhydrazine in 2 N HCl was added to the samples. After 20 min at room temperature, 875 µl of 2.5 N NaOH was added and then the samples were incubated for another 30 min at room temperature. The samples were then spun down for 1 min to remove coagulated protein. Sample absorbances were measured at 550 nm. Standard curves were created from known amounts of 2-ketoisovalerate. The specific activity was calculated as 1 nmol of 2-ketoisovalerate synthesized per min per mg of soluble protein at 34°C.

The decarboxylation activity of Kivd was assayed as described previously (34) with some modifications. Kivd activity was measured at 34 °C using a coupled enzymatic assay method. ADH6 was isolated as previously described (34). Excess ADH6 was used to reduce aldehyde into alcohol, and concomitantly, cofactor NADPH was oxidized to NADP+. The assay mixture contained 0.2 mM NADPH, 0.1 μM ADH6 and 20 mM 2-ketoisovalerate in assay buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM MgSO4, 0.5 mM ThDP) with a total volume of 0.2 mL. The reactions were started by the addition of the 2-ketoisovalerate. The consumption of NADPH was monitored at 340 nm (extinction coefficient, 6.22 $mM^{-1}cm^{-1}$). One specific unit of Kivd activity corresponds to the oxidation of 1 nmol of NADPH per min per mg of soluble protein at 34°C.

To measure the alcohol dehydrogenase activities of YqhD and AdhA, the oxidation of NADPH was monitored by a decrease in absorbance at 340 nm. The assay mixture contained 50 mM MOPS, pH 7.0, 25 mM isobutyraldehyde, and 0.2 mM NAD(P)H. The samples were incubated at 34°C for 5 min, then the reaction was initiated with the addition of cell extracts. The consumption of NAD(P)H was monitored at 340 nm (extinction coefficient, 6.22 $mM^{-1}cm^{-1}$). One specific unit of ADH activity corresponds to the oxidation of 1 nmol of NAD(P)H per min per mg of soluble protein at 34°C.

RESULTS

A single base pair insertion in *alsS* enables expression in *C. cellulolyticum*. In order to achieve direct isobutanol production from pyruvate, the genes encoding *B. subtilis* α-acetolactate synthase, *E. coli* acetohydroxyacid isomeroreductase, *E. coli* dihydroxy acid dehydratase, *L. lactis* ketoacid decarboxylase, and *E. coli* alcohol dehydrogenase (Fig. 1A) were cloned into a pAT187 derivative plasmid (25). These specific genes were chosen because they were the same genes utilized for isobutanol production in *E. coli* (2) and *S. elongatus* (3). The different combinations of the genes (Fig. 1B) were cloned as single synthetic operons driven by the constitutive ferredoxin (Fd) promoter from *Clostridium pasteurianum*.

The activities of the first three enzymes in the isobutanol pathway were examined by transforming plasmids expressing *alsS* or *alsS ilvCD* into *C. cellulolyticum*. While *C. cellulolyticum* was successfully transformed with the empty vector, no *C. cellulolyticum* *alsS* or *alsS ilvCD* transformants were obtained. The same results were observed after repeated transformation efforts. Due to the fact that *alsS* and *alsS ilvCD* transformants could not be obtained, the complete isobutanol pathway was then examined. *C. cellulolyticum* was transformed with a plasmid expressing *alsS ilvCD kivd adhA*. While transformants were obtained, sequencing confirmation of the plasmid revealed that a single adenine insertion, which is not found in the wild-type *alsS* sequence, was present 54 bp downstream of the start ATG. This single insertion, by shifting the reading frame, results in a downstream premature stop codon (TGA) and, subsequently, a truncated 37 amino acid protein (Fig. 2). This spontaneous mutation in *alsS* ('**alsS*') was found to have originated in the *E. coli* strain used for cloning.

The frame shift mutation in the *alsS* sequence was a cause for great concern because of the effect it could have on AlsS activity. Thus, to determine the activities of AlsS and the other enzymes expressed from the synthetic operon, enzymatic assays were performed on lysates of the *C. cellulolyticum* strain expressing **alsS ilvCD kivd adhA* (Fig. 3A). Surprisingly, for the AlsS assay, the **alsS ilvCD kivd adhA* lysates were found to demonstrate an activity of 282 nmol min^{-1} mg^{-1}, which was significantly higher than the 11 nmol min^{-1} mg^{-1} demonstrated by the strain transformed with the vector (Fig. 3A). Thus, despite the insertion mutation, the mutant retained a significant level of activity. However, unlike AlsS, we were not able to detect enzymatic activity for IlvC (Fig. 3B), IlvD (Fig. 3C), Kivd (Fig. 3D), or AdhA (Fig. 3E). There were no statistically significant differences in activity for these enzymes in the lysates of **alsS ilvCD kivd adhA* expressing strain and the vector control strain.

The presence of AlsS activity, despite the stop codon introduced by the frameshift mutation, suggests that the 37 amino acid truncated protein is not the only translation product. It is likely that an alternate Shine-Delgarno (SD) sequence and start site are present downstream of the insertion. After examining the sequence, we have identified a likely candidate for the alternative SD and start site (Fig. 2), which is approximately 8 and 23 bp, respectively, downstream from the adenine insertion. This would result in an AlsS that is 25 amino acids shorter than the wild-type AlsS, and explain the activity in the transformants.

To further analyze the activity of the *alsS* mutation, we compared the AlsS and *AlsS activities in *E. coli* because we were unable to obtain a *C. cellulolyticum* transformant expressing the wild-type *alsS*. Figure 4 compares the AlsS activities of *E.*

coli expressing the **alsS ilvCD* and *alsS ilvCD* constructs. While the **alsS* mutation presented no significant activity in *E. coli*, wild-type *alsS* demonstrated activity that was approximately 1000-fold higher than the empty vector. This result highly suggests that the mutation significantly reduces the activity of AlsS. This difference in activity may explain why *C. cellulolyticum* cannot be transformed with constructs that contain *alsS* as the first gene in the operon, which was the case for *alsS*, *alsS ilvCD*, and *alsS ilvC ilvD kivd adhA* (Fig. 1B).

Production of isobutanol from cellobiose and cellulose. Despite the mutation in *alsS*, **alsS ilvCD kivd adhA* was found to produce isobutanol titers of 140 mg/L from cellobiose over a period of 90 h (Fig. 5C) and 420 mg/L (Fig. 5D) on cellulose over a period of 13 days. These titers are significantly higher than the 17 mg/L and 30 mg/L of isobutanol that is produced by the strain transformed with the empty vector on cellobiose and cellulose, respectively (Fig. 5C and D).

In order to test our hypothesis of the wild-type AlsS's toxic effect on *C. cellulolyticum* growth during transformation, and to obtain transformants with the wild-type AlsS, it was necessary to decrease the activity of the wild-type AlsS. To achieve this, *kivd yqhD alsS ilvCD* was constructed with which *alsS* was the third gene in the operon. Previously, it has been shown that mRNA abundance decreases with increasing distance of the gene from the promoter, irrespective of gene content (19). Specifically, for the operons that they studied, they found that mRNA abundance decreased by approximately 50% from one gene to the next (19). Thus, as the third gene in the operon, it would be expected that the *alsS* mRNA abundance would be less than that if *alsS* was the first gene in the operon. After successful transformation of *kivd yqhD alsS ilvCD*, the

281 resulting transformants were found to produce up to 364 mg/L of isobutanol on
282 cellobiose over a period of 90 h (Fig. 5C) and 660 mg/L of isobutanol on cellulose within
283 7-9 days (Fig. 5D).

284 Although successful transformation suggested that AlsS activity had been
285 successfully attenuated, enzyme assays were performed to quantify the activity of AlsS
286 and the other genes in the operon. As seen in Figure 3A, AlsS activity for the strain
287 expressing *kivd yqhD alsS ilvCD* resulted in approximately 10-fold higher AlsS activity
288 than the vector control, with activities of 133 and 11 nmol min^{-1} mg^{-1}, respectively (Fig.
289 3A). For Kivd activity, the *kivd yqhD alsS ilvCD* expressing strain had 19-fold higher
290 Kivd activity than the strain expressing the vector alone with activities of 147.1 and 7.9
291 nmol min^{-1} mg^{-1}, respectively (Fig. 3D). Unlike AlsS and Kivd, no activity could be
292 detected for IlvC (Fig. 3B), IlvD (Fig. 3C), and YqhD (Fig. 3E) when *kivd yqhD alsS*
293 *ilvCD* was expressed. There were no statistically significant differences in activity for
294 these enzymes when comparing the *kivd yqhD alsS ilvCD* expressing strain and the
295 vector control strain.
296
297
298 **DISCUSSION**
299 Previously, we had successfully shown that *E. coli* can be metabolically
300 engineered to produce the isobutanol by manipulating *E. coli*'s amino acid biosynthetic
301 pathway by diverting the 2-keto acid intermediates towards biofuel production (2). Using
302 the same metabolic engineering strategy, we were able to achieve an isobutanol titer of
303 660 mg/L by the cellulolytic mesophile *C. cellulolyticum* by expressing *kivd yqhD alsS*

304 *ilvCD*. To our knowledge, this is the first demonstration of isobutanol production directly
305 from cellulose.

306 We encountered several difficulties with *C. cellulolyticum* that we did not meet
307 with *E. coli* in regards to the expression of the isobutanol pathway. One of these
308 difficulties arose from the lack of an inducible expression system in *C. cellulolyticum*.
309 Without the ability to control gene expression, the toxicity of some of the genes had a
310 greater effect on the microorganism's growth than they would have otherwise.
311 Specifically, the expression of the gene that encodes for acetolactate synthase, *alsS*,
312 appears to have a toxic effect in *C. cellulolyticum* which is evidenced by the lack of *alsS,*
313 *alsS ilvCD*, and *alsS ilvCD kivd adhA* transformants. Moreover, this problem with
314 transformation is alleviated when the amount of *alsS* mRNA is decreased, as in the case
315 for **alsS, *alsS ilvCD*, **alsS ilvCD kivd adhA*, and *kivd yqhD alsS ilvCD* constructs. It is
316 likely that the control conferred by an inducible system would aid in tempering the
317 expression level of AlsS, and subsequently, its inhibitory growth effects.

318 Another difficulty we encountered was the lack of detectable activity for IlvC,
319 IlvD, and the alcohol dehydrogenases (ADHs) AdhA and YqhD. From the enzyme
320 activity assays (Fig. 3), we were unable to detect activities that were significantly greater
321 than that found for the vector control. However, despite the results of the enzyme assays,
322 it appears that some activity is present. For example, although no activity was detected
323 for Kivd and AdhA in *C. cellulolyticum* transformed with **alsS ilvCD kivd adhA*, it
324 appears that there is *in vivo* activity as the **alsS ilvCD kivd adhA* transformants were
325 found to produce an isobutanol titer of 428 mg/L, while **alsS ilvCD* transformants had a
326 titer of 278 mg/L. It is not surprising that the lack of these enzyme activities did not

327 preclude isobutanol production because *C. cellulolyticum* possesses native enzymes that
328 can perform the same functions. Homologues of *ilvC* and *ilvD* are part of *C.*
329 *cellulolyticum*'s valine biosynthesis pathway and *C. cellulolyticum* possesses ADHs for
330 ethanol fermentation. Still, additional IlvC, IlvD, and ADH activity would most likely
331 lead to higher isobutanol titers. Differences in GC content and codon usage frequencies
332 between *C. cellulolyticum* and *E. coli* may explain the lack of expression of the *E. coli*
333 genes in the host *C. cellulolyticum*. The utilization of *C. cellulolyticum ilvC*, *ilvD*, and
334 *ADH* genes, or the codon optimization of the *E. coli* genes may resolve this problem.
335 A significant amount of research has been dedicated to engineering organisms
336 that are capable of consolidated bioprocessing. These CBP organisms are anticipated to
337 have the ability to efficiently degrade cellulose and to convert the resulting sugars to
338 biofuels at high productivities. Towards this goal, the production of isobutanol from
339 cellulose has been shown to be feasible in the mesophilic *C. cellulolyticum*. Both the
340 successes and problems encountered in establishing this pathway in *C. cellulolyticum* will
341 aid in the adaptation of this strategy in related cellulolytic thermophiles, such as *C.*
342 *thermocellum* and *Caldicellulosiruptor bescii*.
343
344
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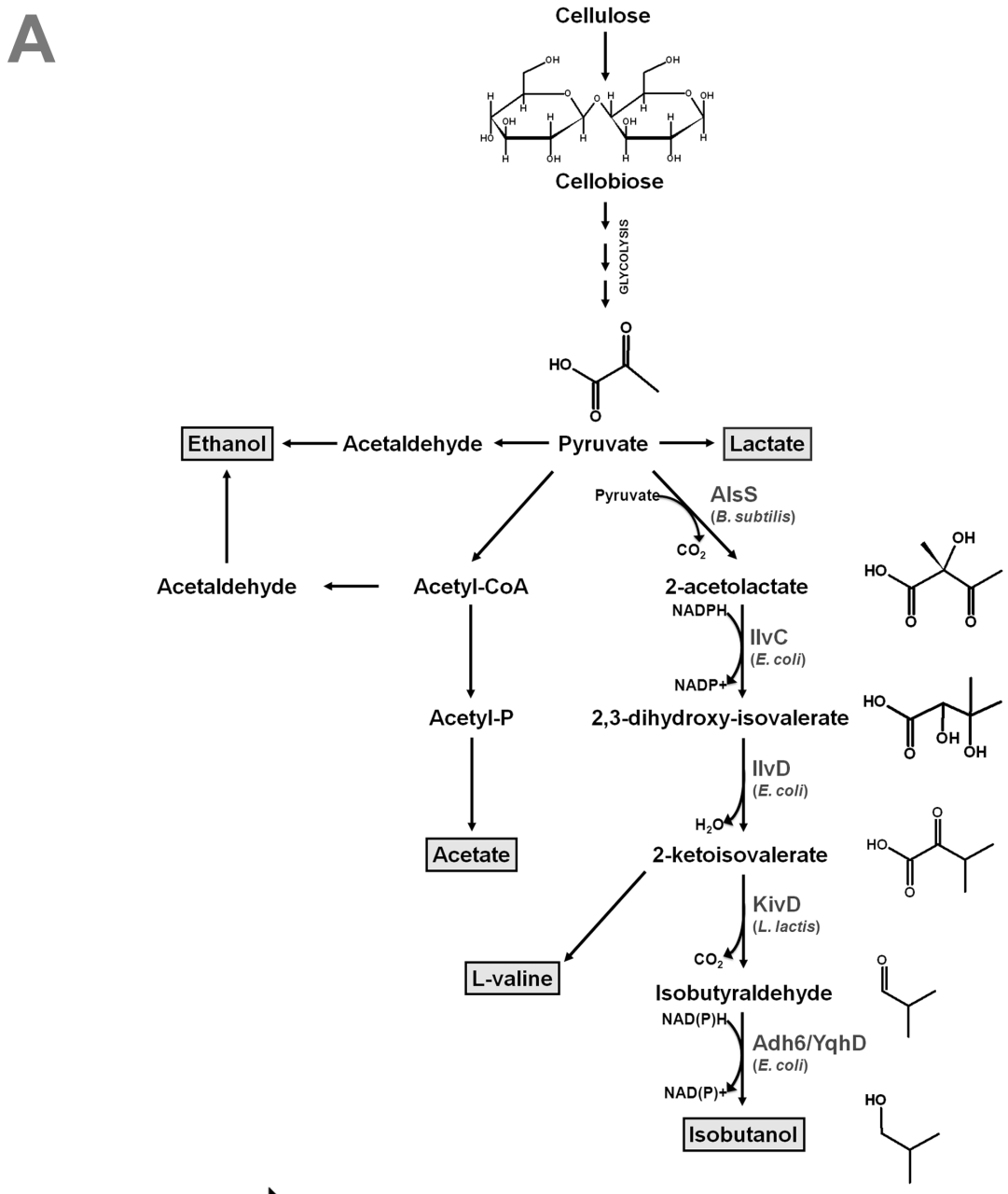
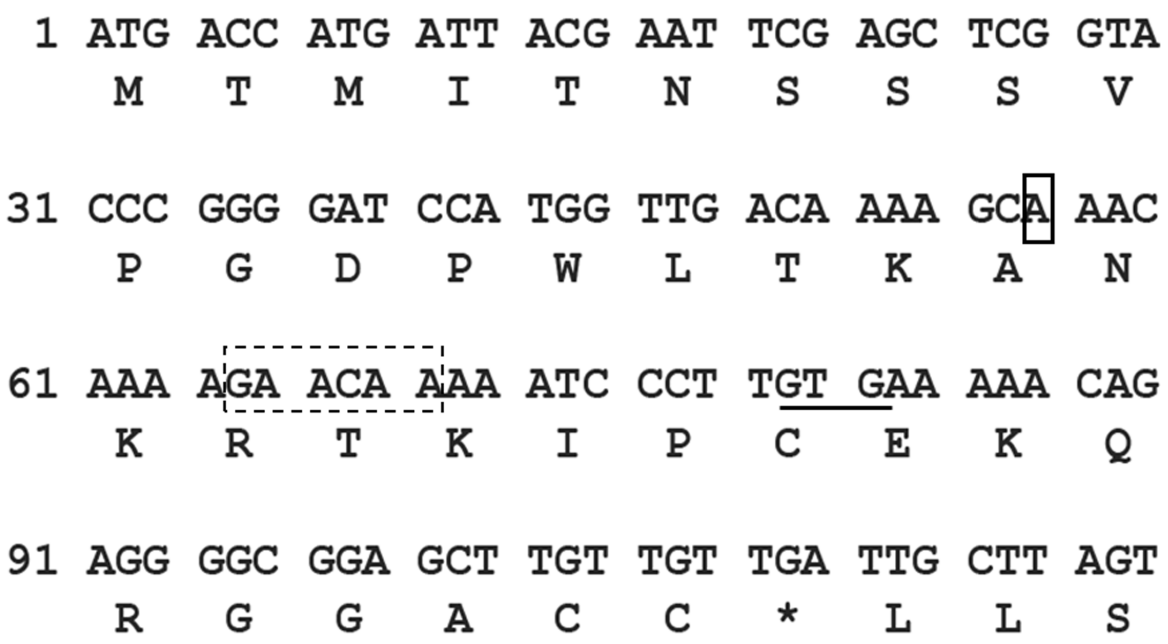
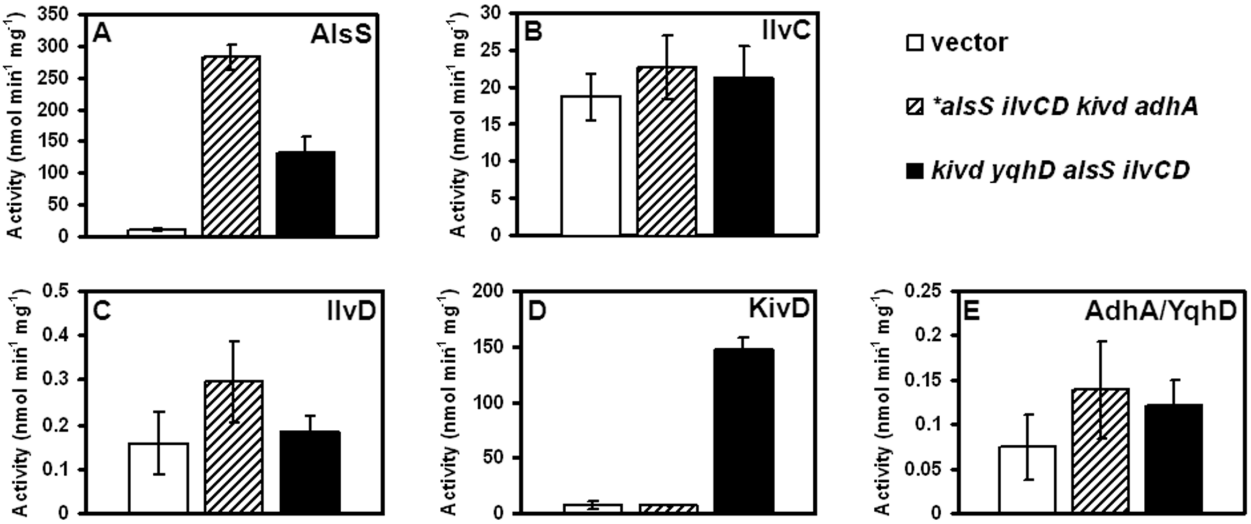
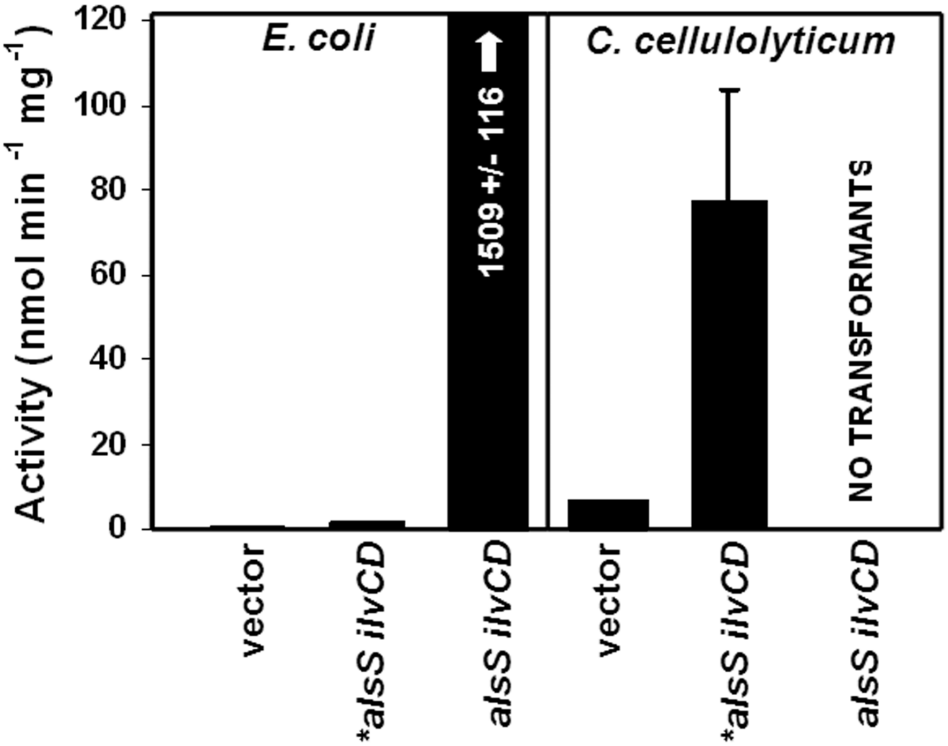
Strain or Plasmid	Phenotype, genotype, or construction description	Source
Strains		
E. coli XL10-Gold	Tet^r, Δ(*mcr*A)183, Δ(*mcr*CB-*hsd*SMR-*mrr*)173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac* Hte, [F', *proAB*, *lacI*^{q}ZΔM15 ,Tn10(tet^r) Amy Cam^r]^a	Stratagene
C. cellulolyticum H10	ATCC 35319	ATCC
Plasmids		
pAT187	Km^R; Broad host range plasmid	(24)
pWH159	Km^R; 5.5 kb *Eco*RI fragment of pAT187 was ligated with the *Eco*RI fragment of the PCR product of pAT187, For oligo WH177, Rev oligo WH178.	This study
pWH168	Em^R Km^R; *ermC* was cloned into pWH159 by ligating the *Aat*II-*Pst*I fragment of PCR product, For oligo WH248, Rev oligo WH249, with pECN2 (11) as the template	This study
pWH199	Em^R Km^R; The ferredoxin promoter and multiple cloning site (Sup Fig. 1) were cloned into pWH168 with the *Bss*HII-*Age*I fragment of PCR product, For oligo WH194, Rev oligo WH195; The template was synthesized by PCR assembly, using 18 primers (FD1 to FD18).	This study
pWH203 (**alsS-ilvC-ilvD*)	Em^R Km^R; 0.8 kb *Bam*HI-*Xba*I fragment of pWH315 was cloned into the same sites of pWH277	This study
pWH277 (*alsS-ilvC-ilvD*)	Em^R Km^R; *alsS* from *B. subtilis* and *ilvCD* from *E. coli* was amplified from pSA69 (2), For oligo WH301, Rev oligo WH302; *Bam*HI-*Not*I fragment of PCR product ligated into the *Bam*HI and *Not*I sites of pWH199	This study
pWH278 (*alsS*)	Em^R Km^R; *alsS* from *B. subtilis* was amplified from pSA69 (2), For oligo WH261, Rev oligo WH262; *Bam*HI-*Not*I fragment of PCR product ligated into the same restriction sites as pWH199	This study
pWH314 (*alsS-ilvCD-kivd-adhA*)	Em^R Km^R; *kivd* from *L. lactis* and *adhA* from *E. coli* were amplified by PCR amplification using pSA65 (2) as a template, For oligo WH886, Rev oligo WH885; *Not*I-*Bam*HI fragment of PCR product ligated into *Not*I and *Bgl*II sites of pWH203	This study
pWH315 (**alsS-ilvC-ilvD-kivd-adhA*)	Em^R Km^R; Spontaneous mutation in *alsS*	This study
pWH318 (*kivd-yqhD*)	Em^R Km^R; *kivd* from *L. lactis* and *yqhD* from *E. coli* were amplified by PCR from pCS97 (C. Shen, unpublished), For oligo WH888, Rev oligo WH887; *Acc*65I-*Bam*HI fragment of PCR product ligated into the *Acc*65I and *Bam*HI sites of pWH199	This study
pWH320 (*kivd-yqhD-alsS-ilvC-ilvD*)	Em^R Km^R; *alsS* from *B. subtilis* and *ilvCD* from *E. coli* was amplified from pSA69 (2), For oligo WH900, Rev oligo WH901; *Spe*I-*Not*I fragment of PCR product ligated into the *Xba*I and *Not*I sites of pWH318	This study

* Abbreviations: For, forward primer; Rev, reverse primer.









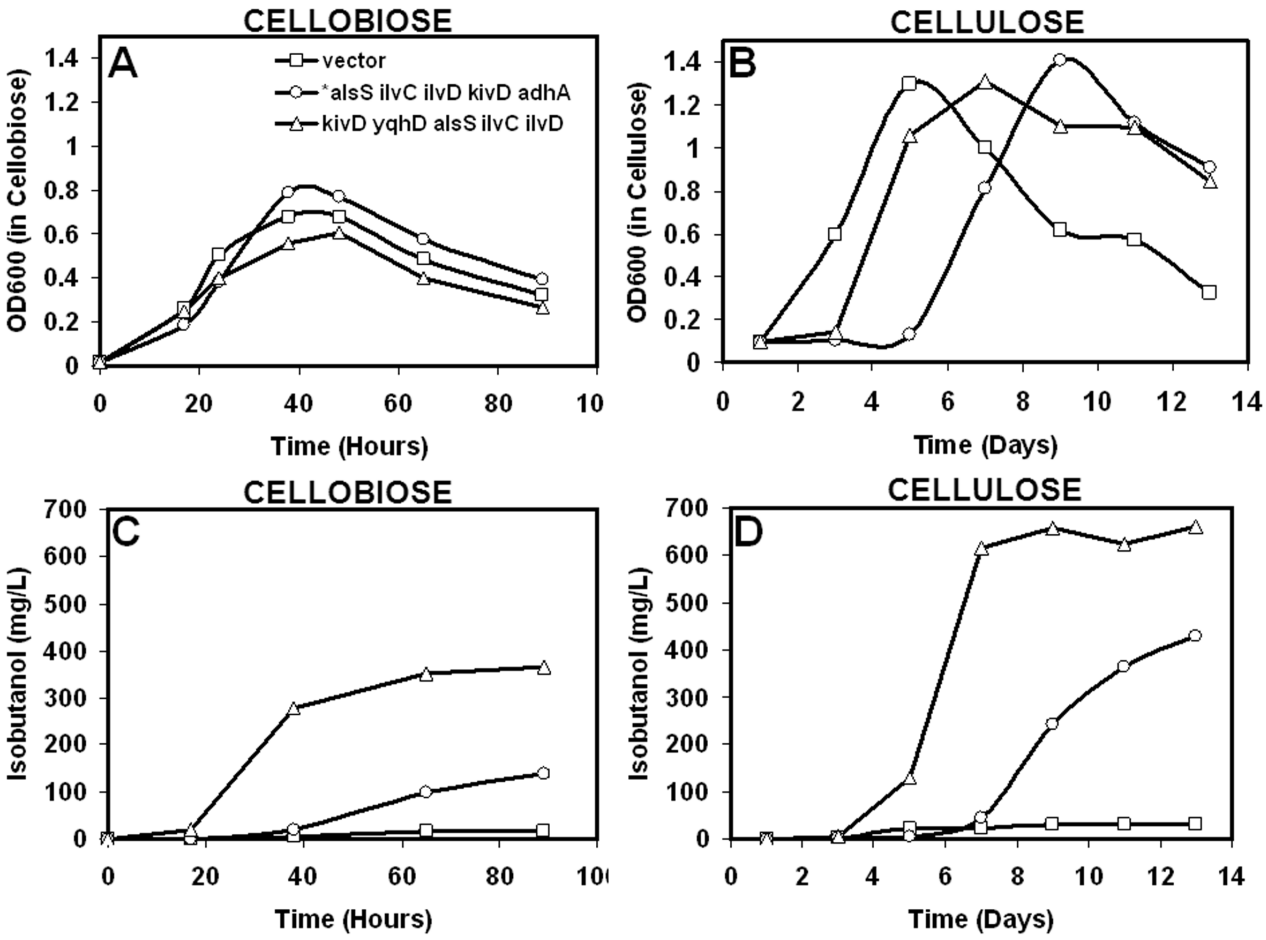


Table 1. List of plasmids and strains used in this study.

FIG. 1. (**A**) The pathway for isobutanol production in *C. cellulolyticum* and (**B**) the ferredoxin promoter (black arrow) driven operons used in this study. The "*" indicates the presence of the adenine insertion in the *alsS* gene sequence.

FIG. 2. The first 120 bp of the *alsS* sequence with the adenine insertion mutation. The adenine insertion (solid box), the putative start GTG, which restores the *alsS* reading frame (underline), the premature stop codon (*), and the putative Shine Delgarno sequence (dashed box) are indicated.

FIG. 3. Activity assays of isobutanol pathway enzymes for *C. cellulolyticum* strains expressing the empty vector (white box), **alsS ilvCD kivd adhA* (black striped box), and *kivd yqhD alsS ilvCD* (black box), determining the activity for (**A**) AlsS (one specific unit of Als activity corresponds to the formation of 1 nmol of acetoin per min per mg of soluble protein at 37°C, (**B**) IlvC (one specific unit of IlvC activity corresponds to the oxidation of 1 nmol of NADPH per min per mg of soluble protein at 37°C, (**C**) IlvD (one specific unit of IlvD activity corresponds to the formation of 1 nmol of 2-ketoisovalerate per min per mg of soluble protein at 37°C), (**D**) Kivd (one specific unit of Kivd activity corresponds to the oxidation of 1 nmol of NADPH per min per mg of soluble protein at 30°C), and (**E**) AdhA and YqhD activity (one specific unit of ADH activity corresponds to the oxidation of 1 nmol of NAD(P)H per min per mg of soluble protein at 37°C).

FIG. 4. AlsS activity of *E. coli* and *C. cellulolyticum* expressing the vector, **alsS ilvCD* contruct, or the *alsS ilvCD* construct. One specific unit of AlsS activity corresponds to the formation of 1 nmol of acetoin per min per mg of soluble protein at 37°C.

FIG. 5. Growth of *C. cellulolyticum* strains on (**A**) cellobiose and (**B**) cellulose and the isobutanol production (mg/L) on (**C**) cellobiose and (**D**) cellulose. The figure shows one representative data set of three independent experiments, all three showing comparable results.