Metabolic Engineering 13 (2011) 570–577

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

# Metabolic engineering of *Thermobifida fusca* for direct aerobic bioconversion of untreated lignocellulosic biomass to 1-propanol

Yu Deng [a,b], Stephen S. Fong [a,*]

[a] Department of Chemical and Life Science Engineering, Virginia Commonwealth University, 601W. Main Street, Richmond, VA 23284, USA
[b] Thayer School of Engineering, Dartmouth College, 8000 Cummings Hall, Hanover, NH 03755, USA

ARTICLE INFO

*Article history:*
Received 8 April 2011
Received in revised form
23 June 2011
Accepted 27 June 2011
Available online 3 July 2011

*Keywords:*
*T. fusca*
1-propanol
Aerobic
Biomass
*adhE2*

ABSTRACT

Biofuel production from renewable resources can potentially address lots of social, economic and environmental issues but an efficient production method has yet to be established. Combinations of different starting materials, organisms and target fuels have been explored with the conversion of cellulose to higher alcohols (1-propanol, 1-butanol) being one potential target. In this study we demonstrate the direct conversion of untreated plant biomass to 1-propanol in aerobic growth conditions using an engineered strain of the actinobacterium, *Thermobifida fusca*. Based upon computational predictions, a bifunctional butyraldehyde/alcohol dehydrogenase was added to *T. fusca* leading to 1-propanol production during growth on glucose, cellobiose, cellulose, switchgrass and corn stover. The highest 1-propanol titer (0.48 g/L) was achieved for growth on switchgrass. These results represent the first demonstration of direct conversion of untreated lignocellulosic biomass to 1-propanol in an aerobic organism and illustrate the potential utility of *T. fusca* as an aerobic, cellulolytic bioprocess organism.

© 2011 Elsevier Inc. All rights reserved.

## 1. Introduction

Metabolic engineering approaches to cellulosic biofuel production have largely been directed at improving the rate of cellulose degradation (Aguirre et al., 1986; Bothwell et al., 1993; Caspi et al., 2006; Stephanopoulos, 2008) and expressing appropriate genes for production of desired products including ethanol, higher alcohols or hydrocarbons (Dien et al., 1999; Nonklang et al., 2008; Rao et al., 2007). The culmination of these efforts could be a consolidated bioprocessing (CBP) scheme (Demain et al., 2005; Lopez-Contreras et al., 2000; Rani et al., 1997) where cellulose could be converted to a desired biofuel by a single organism. Thus far, it has been difficult to engineer a microorganism with all of the desired features necessary for an efficient CBP scheme. A key baseline requirement for effective implementation of a CBP scheme is the need for facile genetic manipulation tools for the targeted CBP organism. Most cellulolytic organisms have low product yields and are difficult to genetically manipulate whereas most genetically tractable model organisms lack cellulolytic capabilities.

The application of targeted genetic manipulation can then lead to novel chemical production or improvements in chemical product yields. In the case of biofuel CBP schemes, both the target compound of interest and the improvement of product yields need to be addressed. 1-propanol, along with 1-butanol, has been identified as a potential next-generation gasoline substitute (Nielsen et al., 2009; Shen and Liao, 2008) that is compatible with existing infrastructure and can be potentially used as a straight replacement for petroleum-based gasoline. 1-propanol has been detected in small quantities ($<70$ mg/L) in *Clostridia* species (Janssen, 2004) and yeast (Eden et al., 2001). Recent work on *Escherichia coli* has led to a 1-propanol titer of $\sim$1 g/L (Shen and Liao, 2008) for growth on glucose. For 1-propanol, there are relatively few identified organisms with native production capabilities and overall production yields need to be greatly increased.

Considering the various aspects of a biofuel CBP scheme, we sought to develop the aerobic, thermophilic, cellulolytic actinobacterium *Thermobifida fusca*, as a potential platform organism for biofuel production. *T. fusca* has been studied and characterized for its cellulolytic capabilities (Deng and Fong, 2010a, 2010b; Chen and Wilson, 2007; Lykidis et al., 2007). The genome of *T. fusca* has been sequenced (Lykidis et al., 2007). In previous work, we have also developed a system for chromosomal genetic manipulation specific for *T. fusca* (Deng and Fong, 2010b). In this study, we applied the recently developed systems for chromosomal manipulation of *T. fusca* to generate a designed strain that directly converts untreated plant biomass to 1-propanol. We also show

* Corresponding author. Fax: +1 804 828 3846.
*E-mail addresses:* dengrain@hotmail.com (Y. Deng), ssfong@vcu.edu (S.S. Fong).

1096-7176/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2011.06.007

that *T. fusca* has high native tolerance of 1-propanol and using enzymatic assays, the pathways utilized to produce 1-propanol were identified. Thus, *T. fusca* has been engineered to directly convert untreated plan biomass to 1-propanol and has the potential for further manipulation to increase 1-propanol yield.

## 2. Materials and methods

### 2.1. Strains and culture conditions

Strains used and generated in this study are listed in Table 1. *E. coli* BW25113 was used to propagate pIJ790 (Deng and Fong, 2010b). All the *E. coli* strains were grown in SOC or LB media. *Clostridium acetobutylicum* ATCC 824 was incubated according to Yu et al. (2007).

*T. fusca* YX and B6 were grown in Hagerdahl medium containing different carbon sources at 46 °C. Both strains were cultured in Erlenmeyer flasks with the rubber stoppers to create a semi-aerobic environment. 50 mL pre-cultures of *T. fusca* YX and B6 were grown at 46 °C and 200 rpm for 24 h in a 500 mL Erlenmeyer flask. Growth cultures for testing were inoculated using 5% of the pre-culture and grown at 46 °C and 200 rpm. Cell growth was measure at OD 600 nm.

To test if *T. fusca* could utilize untreated biomass as a carbon source, dry untreated switchgrass and corn stover (obtained from National Renewable Energy Lab, USA) were used. The switchgrass and corn stover were mechanically milled using a coffee grinder before being added to minimal Hagerdahl medium.

### 2.2. Plasmid construction

The general strategy of adding the *adhE2* gene into the chromosome of *T. fusca* is shown in Fig. 1. The *adhE2* gene was amplified from *C. acetobutylicum* ATCC 824 using primers adhE2F and adhE2R (Table 2) and was integrated into the pGEM-T vector according to the manufacturer's instructions (Promega). The integrity and direction of *adhE2* in pGEM-T was verified by sequencing to ensure that *adhE2* was integrated with the same transcriptional direction as the MSC site. The constructed adhe2-PGEM plasmid was transformed into competent *E. coli* BW25113/pIJ790 (Deng and Fong, 2010b) that had the λ RED system for homologous recombination. To put a selective marker to *adhE2*, the kanamycin resistant gene (*kan*) was used. The *kan* gene was amplified from pET-30a(+) (Novagen) using primers kanadhe2F and kanadhe2R. These two primers contained 39-bp DNA fragments, respectively, which were homologous to the upstream of *adhE2* gene in adhe2-PGEM. The *kan* cassette was then transformed into *E. coli* BW25113/pIJ790 with adhe2-PGEM. With the help of λ RED system, the *kan* gene was integrated upstream of *adhE2* by homologous recombination to form the new plasmid kan-adhE2-PGEM. To replace the *celR* gene, two ends of kan-adhe2 cassette were flanked by two 39-bp DNA fragments homologous to upstream and downstream of *celR*, respectively, by primers celkanadhe2F and celkanadhe2R. The plasmid pGEM2816 was constructed in our lab previously (Deng and Fong, 2010b). The kan-adhe2 cassette was transformed to BW25113/pIJ790 with pGEM2816 and kan-adhe2 cassette replaced *celR* gene and the upstream and downstream of *celR* flanked on the both ends of kan-adhe2 and the new plasmid was named kan-adhe2-celR-PGEM.

**Table 1**
Strains and plasmids used in this study.

| | Relevant genotype/ comments | Source/ref. |
|---|---|---|
| **Strain** | | |
| *E. coli* DH5α | F-, *φ80dlacZΔM15*, *Δ(lacZYA-argF)U169*, *deoR*, *recA1*, *endA1*, *hsdR17(rk1−, mk+)*, *phoA*, *supE44*, *λ−*, *thi-1*, *gyrA96*, *relA1* | Dr. Rita Shiang, Virginia Commonwealth University |
| *E .coli* BW25113 | K12 derivative: *ΔaraBAD*, *ΔrhaBAD* | Bierman et al. (1992) |
| *E. coli* ET12567 | *dam*, *dcm*, *hsdS*, *cat*, *tet* | Gust et al. (2003) |
| *T. fusca* B6 | *ΔcelR*, *kan*, *adhE2* | This study |
| *E. coli K12* | Wild type strain | This study |
| **Plasmid** | | |
| pGEM-T | *bla*, *lacZ* | Promega, USA |
| pIJ790 | *λ*RED (*gam*, *bet*, *exo*), *cat*, *araC*, rep101ts | Deng and Fong (2010b) |
| pGEM2816 | *bla*, *celR*, *lacZ* | Deng and Fong (2010b) |
| adhe2-PEGM | *bla*, *adhE2*, *lacZ* | This study |
| kan-adhe2-PEGM | *bla*, *adhE2*, *kan*, *lacZ* | This study |
| celR-kan-adhe2-PEGM | *ΔcelR*, *bla*, *adhE2*, *kan*, *lacZ* | This study |

### 2.3. Transformation of T. fusca and allelic exchange

The plasmid of kan-adhe2-PGEM was introduced into *E. coli* ET12567/pUZ8002 by electroporation and transformed to *T. fusca* protoplasts and detailed procedure was described by our lab previously (Deng and Fong, 2010b). The kan-adhe2 cassette of transformants was amplified by primers 2816F and 2816R (Table 2) and directly sequenced by Sanger sequencing, thus confirming a positive recombination event.

### 2.4. Cytoplasmic protein measurement

1 mL culture was centrifuged at 10,000 × *g* for 5 min. The pellets were resuspended in fresh media and centrifuged at 10,000 × *g* for 5 min again. Sediments were dissolved in 200 μL 50 mM Tris–HCl buffer (pH6.8) containing 2% SDS, 0.1 M DTT and 50% glycerol. Samples were then pulsar sonicated at 80% strength for 10 min in an ice bath. After centrifuging at 10,000 × *g* for 5 min, the proteins in the supernatant were measured using the Bradford protein assay (Bradford, 1976).

### 2.5. Metabolite detection

The produced alcohols were detected by gas chromatograph–mass spectrometry (GC–MS) equipped with a flame ionization detector. The culture broth supernatant was injected in split injection mode (1:15split ratio) using isopropanol as the internal standard. Detailed procedures are described in Hanai et al. (2007). Other chemicals were detected by HPLC system (Dionex Ultimate3000) equipped with Bio-Rad HPX-87H ion exclusion column. The mobile phase was 0.005 mol/L $H_2SO_4$ at the rate of 0.6 mL/min using IR and UV detection.

### 2.6. Enzymatic assays

#### 2.6.1. Threonine dehydratase

The assay was based on the conversion between α-ketobutyrate and α-hydroxybutyrate catalyzed by lactic dehydrogenase, with the corresponding oxidation of NAD to NADH monitored at 340 nm (Cohn and Phillips, 1974). The reaction mixture (200 μL) contained 0.1 μmol of NADH, 1 μmol of DTT, 1.5 units of lactic dehydrogenase, 25 μmole of L-threonine, 20 μmole of potassium phosphate buffer (pH 7.5) and cell extract. All assays were conducted with a minimum of three biological replicates and *E. coli* BW25113 was used as a positive control.

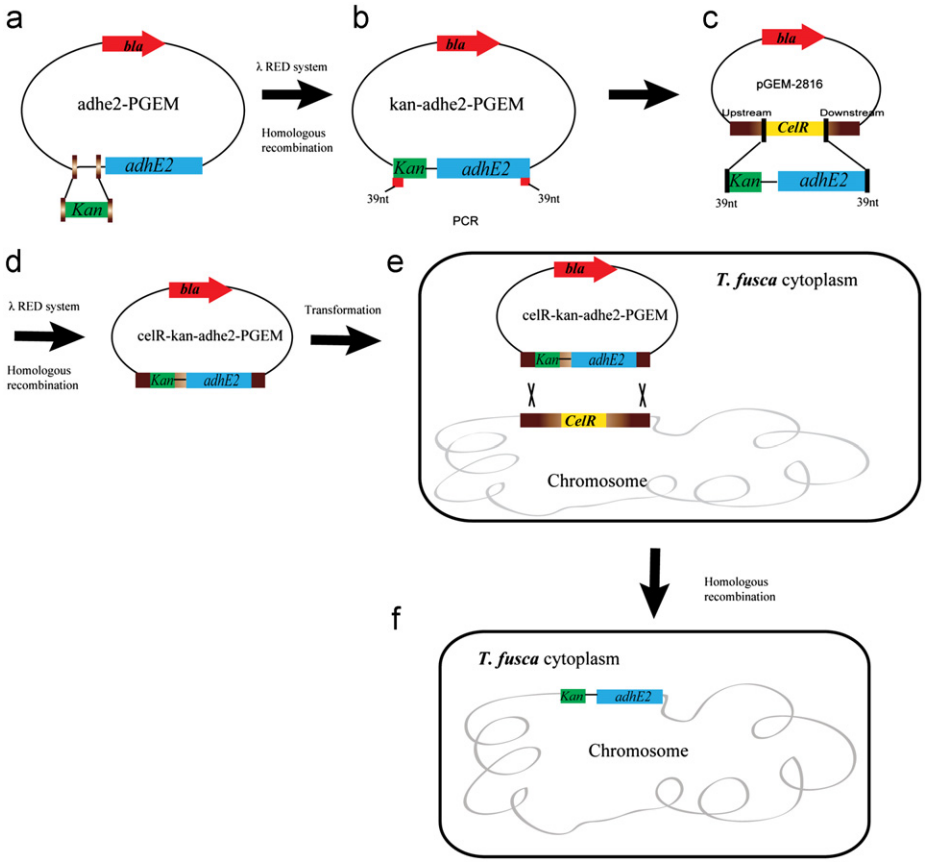


**Fig. 1.** Strategy for replacing *celR* by *adhE2* gene in the chromosome of *T. fusca*. (a) The *adhE2* gene was added into the pGEM-T vector. The *kan* was obtained by PCR using primers containing 39-nt homology extensions corresponding to the upstream of *adhE2*. The PCR product was transformed to *E. coli* BW25113/pIJ790 (expressing the λ-Red recombination functions), containing *adhE2*-PGEM. (b) The *kan-adhE2* cassette was amplified by the primers with 39-nt homologous extension corresponding to the upstream and downstream of *celR* gene in *T. fusca*. (c) *celR* was replaced by kan-adhE2 cassette by homologous recombination. (d), (e) The new plasmid celR-kan-adhe2 was introduced in the *T. fusca* protoplasts. (f) The homologous recombination helped to replace *celR* gene in *T. fusca* by kan-adhe2 cassette.

**Table 2**
PCR primers used in this study.

| Primer name | Sequence 5′-3′ | Generation of |
|---|---|---|
| adhE2F | C**GAGCTC**TATAAGGCATCAAAGTGTGT | *adhE2* |
| adhE2R | G**GCATGC**GTCTATGTGCTTCATGAAGC | *adhE2* |
| kanadhe2F | TATAAGGCATCAAAGTGTGTTATATAATACAATA AGTTT ATGAGCCATATTCAACGGGA | *Kan* flanked by 39 nucleotides homologous to upstream of *adhE2* |
| kanadhe2R | TTATATACACTCCTTTATAAAATTTATTA TTAGTTTGAT TTAGAAAAACTCATCGAGCA | *Kan* flanked by 39 nucleotides homologous to upstream of *adhE2* |
| celkanadhe2F | GATGCCGGGACGGCGCGGTCGTGGGTTGG GGGAACAAGG ATGAGCCATATTCAACGGGA | kan-adhe2 cassette flanked by 39 nucleotides homologous to upstream of *celR* |
| celkanadhe2R | CGTCCACCGCGGGCGCGGATCCAGGGCGACG TGCGGACC GTCTATGTGCTTCATGAAGC | kan-adhe2 cassette flanked by 39 nucleotides homologous to downstream of *celR* |
| 2816F | CCC**AAGCTT**TGCCGTGATGCGGGACTT | *celR* and upstream and downstream sequences |
| 2816R | CACCGTGAACGGGAAAACC | |

The 39 nucleotides homologous to *celR* nucleotides are underlined. Bold type indicates restriction enzyme sites.

### 2.6.2. *Pyruvate oxidase*

Oxidative pyruvate decarboxylation was determined by oxidative coupling of the reaction product, $H_2O_2$, with 4-aminoantipyrine in the presence of horsedish peroxidase and sodium 2-hydroxy-3.5-dichlorobenzene-sulfonate (Risse et al., 1992). The assay mixture consisted of 0.05 M potassium phosphate (pH 6.5), 10% (v/v) glycerol, 40 mM pyruvate, 2 mM 4-aminoantipyrine, 7 mM 2-hydroxy-3.5-dichlorobenzene-sulfonate and 0.4 U/L horseradish peroxidase. The resulting colored was measured at 546 nm. All assays were conducted with a minimum of three biological replicates and *E. coli* K12 MG1655 was used as a positive control.

### 2.6.3. *Propionyl-CoA: succinyl-CoA transferase*

The reaction was conducted in the 96-well microplate. The reaction mixture had 250 μL consisting of Mixture 1 (250 mM Tris/HCl buffer (pH 8.0), 1 mM sodium, malate, 2.5 mM NAD), 10 mL of 1.5 M sodium, acetate, 10 mL of Mixture 2 (220 units of malate dehydrogenase, 35 units of citrate synthase), 0.1 M

potassium, phosphate buffer (pH 6.8) to make up a 1 mL volume), 10 mL of 15 mM succinyl CoA and 60 mL of the cell extract (Suwannakham and Yang, 2005). The reaction rate at 25 °C was followed by measuring the absorbance at 340 nm, on the microplate reader, which increased linearly with time, for 3–5 min. All assays were conducted with a minimum of three biological replicates.

### 2.7. Measurement of tolerance to 1-propanol

*T. fusca* YX and *E. coli* K12 were used to measure the tolerance to 1-propanol.

All the measurements were conducted on the 96-well microplate with the cover and the plate was well sealed by the tape. Cells were grown to the exponential phase where the cells were sensitive to the alcohol and then 200 μL of cell culture was aliquoted to the microplate with different concentration of 1-propanol. Cell growth was monitored using the microplate reader until the cultures reached stationary phase. A minimum of three biological replicates were conducted for each condition tested.

### 2.8. RNA preparation and real-time PCR

To study molecular-level differences in the cultures of *T. fusca*, gene expression was studied using real-time PCR using cells harvested at the mid-log growth phase and early stationary phase corresponding to experimental points exhibiting different cellulase activity and details were described in Deng and Fong (2010b).

## 3. Results

### 3.1. Identification of pathways to 1-propanol production

Based upon the genome and annotation of the cellulolytic, thermophilic actinobacterium, *T. fusca* (Lykidis et al., 2007), we used metabolic pathway analysis to partially reconstruct a constraint-based model of the metabolic network of *T. fusca*, similar to the model that was constructed for the anaerobic cellulolytic ethanologen, *Clostridium thermocellum* (Roberts et al., 2010). While no previous reports document the native metabolic end-products secreted by *T. fusca*, our computational analysis and

**Table 3**
Gene expression of propionic acid-related genes.

| Gene name | Relative gene expression | Protein | Function |
|---|---|---|---|
| *Tfu_1546* | 1.07 ± 0.15 | Acetyl-CoA synthetase | Catalyze the reactions between propionic acid and propanoyl-CoA |
| *Tfu_2971* | 0.93 ± 0.19 | acetate kinase | Catalyze the reactions between propionic acid and propanoyl phosphate |
| *Tfu_0948* | 1.00 ± 0.19 | Propionyl-CoA carboxylase | Catalyze the reaction between propanoyl-CoA and 2-methyl-malonyl-CoA |

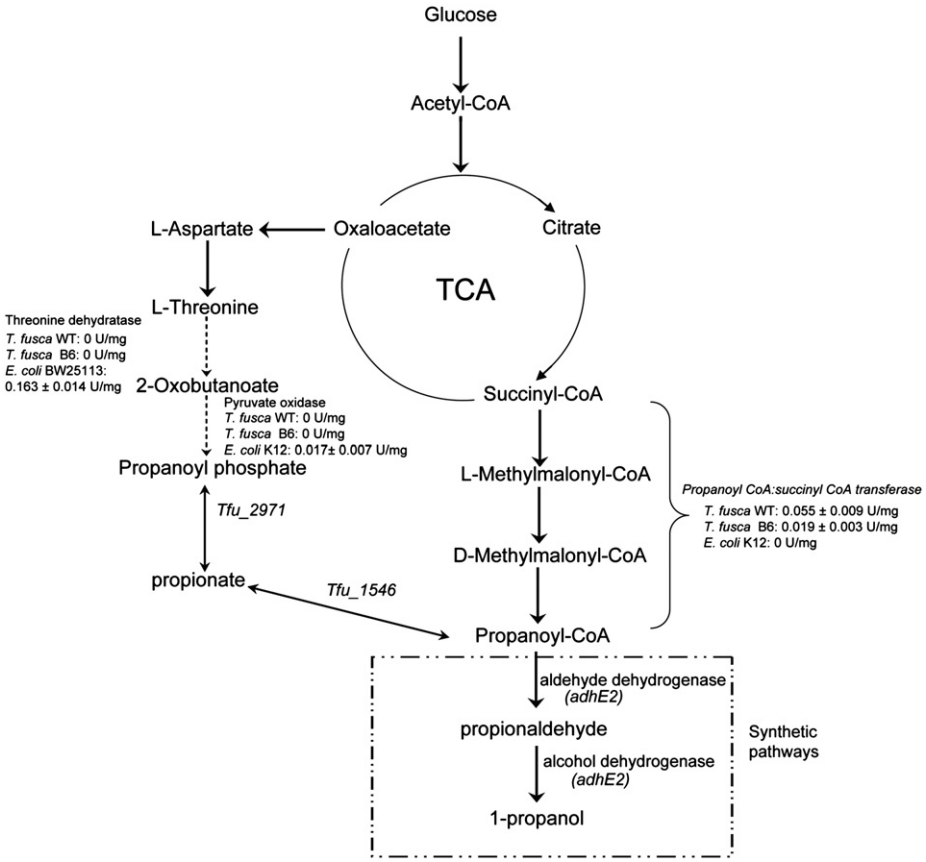


**Fig. 2.** Schematic illustration of 1-propanol production via the threonine and succinyl-CoA: propanoyl-CoA pathways in the genetically engineered *T. fusca*. The enzymatic activities of threonine dehydratase, pyruvate oxidase and propionyl CoA: succinyl CoA transferase are also shown. Pathways from propanoyl-CoA to propanoyl phosphate were proposed based on the mRNA measurements of *Tfu_2971* and *Tfu_1546* as well as the detection of propionate. The succinyl-CoA:propanoyl-CoA pathways were proposed based on propanoyl CoA:succinyl CoA transferase activity.

experimental results demonstrate that *T. fusca* produces propionic acid. Secretion of propionic acid was observed experimentally using HPLC (data not shown) and mRNA transcription of key genes (*Tfu_2971* and *Tfu_1546*) were measured using real-time PCR (Table 3) as an indicator of *in vivo* transcription activity.

Propionic acid is four biochemical reactions removed from 1-propanol (Fig. 2). The conversion of propionate to propanoyl-CoA can be theoretically achieved by an acetyl-CoA synthetase (gene *Tfu_1546*) that is natively found in *T. fusca*. The remaining two reactions to convert propanoyl-CoA to propionaldehyde then to 1-propanol can be achieved using a single bifunctional butyraldehyde/alcohol dehydrogenase such as the *adhE2* gene found in *C. acetobutylicum*. The butyraldehyde/alcohol dehydrogenase in *C. acetobutylicum* catalyzes a broad range of substrates including the reactions from propanoyl-CoA to propionaldehyde and from propionaldehyde to 1-propanol.(Palosaari and Rogers, 1988)

Based upon the genome annotation of *T. fusca*, two coherent pathways natively exist that can produce propionate or propanoyl-CoA. The first pathway originates from oxaloacetate in the TCA cycle going through aspartate and threonine. This is the canonical keto-acid synthesis pathway and has been utilized previously for production of 1-propanol in *E. coli* (Shen and Liao, 2008). The second pathway originates from succinyl-CoA in the TCA cycle goes through methylmalonyl-CoA to propanoyl-CoA.

### 3.2. B6 mutant strain construction

The *adhE2* gene from *C. acetobutylicum* was cloned and used to generate a DNA construct with the *adhE2* gene, a kanamycin resistance gene (*kan*), and flanking regions with homology to the *celR* (*Tfu_0938*) gene in *T. fusca* (Fig.1). Using a protoplast fusion protocol customized for thermophiles (Deng and Fong, 2010b), the constructed kan-adhe2-celR-PGEM cassette was used to simultaneously remove the chromosomal *celR* gene in *T. fusca* and add the *kan* and *adhE2* genes resulting in a strain named *T. fusca* B6. The CelR protein in *T. fusca* is a regulator of cellulase activity (Deng and Fong, 2010a, 2010b; Spiridonov and Wilson, 1999), thus with a single recombination event we removed the key regulator of cellulase activity and added the bifunctional *adhE2* gene.

### 3.3. Characterization of T. fusca B6

Initial characterization of the engineered *T. fusca* B6 strain was conducted for growth on glucose supplemented minimal medium at 46 °C and compared to wild-type growth (ATCC BAA-629) and previously described *celR*-deletion strains (Deng and Fong, 2010b) under identical conditions (Fig. 3a). Genetically, the *T. fusca* B6 strain differs from the wild-type strain as it contains the *adhE2* gene from *C. acetobutylicum* and no longer has the native *celR* gene, which is a key regulator for cellulase expression (Deng and Fong, 2010b). The doubling time of the engineered B6 strain (2.82 h) was slightly slower than for the wild-type (2.01 h) and *celR*-deletion strain (2.29 h), Table 4. The overall biomass yield for the B6 strain was comparable to the wild-type strain (Table 4) and significantly higher than the biomass yield for the *celR*-deletion strain. The engineered B6 strain also reached stationary growth on simple substrates (glucose and cellobiose) much faster than either the *celR*-deletion strain or the wild-type strain (Fig. 3a).

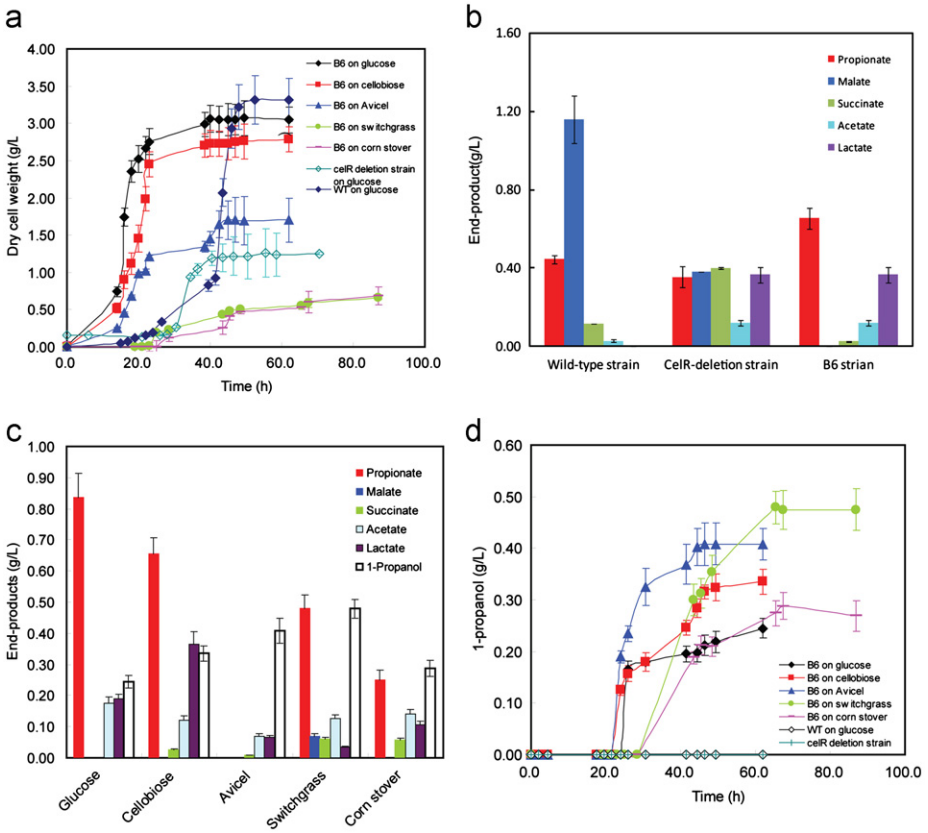


**Fig. 3.** Growth characteristics of *Thermobifida fusca*. (a) The cell growth of *T. fusca* B6 on glucose, cellobiose, Avicel, switchgrass and corn stover and *T. fusca* wild-type and *celR*-deletion strains on glucose. (b) End-product secretions of *T. fusca* wild-type, celR-deletion and B6 strains for growth on cellobiose (c) End-product secretion of *T. fusca* B6 on glucose, cellobiose, Avicel, switchgrass and corn stover. Values shown are the highest measured product titer for each condition. (d) 1-propanol production by *T. fusca* B6 on glucose, cellobiose Avicel, swtichgrass and corn stover and by *T. fusca* wild-type strain and *celR*-deletion strain on glucose.

**Table 4**
Growth characteristics of *T. fusca* strains.

| Strains | Doubling time (h) | Maximum biomass yield (g/L) |
|---|---|---|
| WT on glucose | 2.01 | $3.32 \pm 0.32$ |
| celR-deletion strain on glucose | 2.29 | $1.26 \pm 0.30$ |
| B6 on glucose | 2.82 | $3.07 \pm 0.23$ |
| B6 on cellobiose | 3.11 | $2.79 \pm 0.17$ |
| B6 on Avicel | 4.11 | $1.70 \pm 0.29$ |
| B6 on switchgrass | 16.83 | $0.73 \pm 0.02$ |
| B6 on corn stover | 16.47 | $0.76 \pm 0.03$ |

In terms of metabolic end-product secretion profile, shifts in metabolic pathway utilization were observed between the wild-type, *celR* deletion and engineered B6 strains (Fig. 3b). The wild-type strain of *T. fusca* secretes malate as its major metabolic end-product with lower amounts of propionate, and succinate when grown on cellobiose minimal medium. The *celR* deletion strain secretes comparable amounts of propionate as the wild-type strain, but reduces the amount of malate secreted while increasing the amounts of succinate and lactate produced. The *T. fusca* B6 strain secretes propionate as its major metabolic end-product with 1-propanol, acetate, lactate and small amounts of succinate also being secreted (Fig. 3b). These initial results demonstrated that the genomic integration of *adhE2* into *T. fusca* was successfully expressed and effectively redirected metabolic fluxes to the propionate/1-propanol pathway.

Initially, it was assumed that propionic acid and propanoyl-CoA were being generated by the keto-acid biosynthesis pathway (routing through aspartate and threonine) as previously demonstrated in *E. coli* (Shen and Liao, 2008). Further investigation identified that *T. fusca* also potentially can produce propanoyl-CoA from succinyl-CoA as a branch off the TCA cycle. To determine which pathways were being utilized to produce the propanoyl-CoA as a precursor to 1-propanol, we conducted *in vitro* enzymatic assays of several key enzymatic steps. For the keto-acid route, threonine dehydratase (using *E. coli* BW25113 as a positive control) and pyruvate oxidase (using *E. coli* K12 MG1655 as a positive control) were found to have no activity in either the wild-type or the B6 strains of *T. fusca* (Fig. 2).

Propanoyl CoA:succinyl CoA transferase activity was found in both the wild-type and the B6 strains for *T. fusca* with no detected activity in *E. coli* (negative control). We also tested the gene expression level of a key gene *Tfu_0948* encoding propionyl-CoA carboxylase, which catalyzes the reaction between propanoyl-CoA and 2-methyl-malonyl-CoA (Table 3). These data suggest that in the conditions we tested, *T. fusca* utilizes the succinyl-CoA to propanoyl-CoA pathway, but does not utilize the keto-acid pathway (Fig. 2) to produce propanoyl-CoA. This finding is consistent with the shifts in end-product secretion where succinate secretion is decreased as propionic acid (and 1-propanol) secretion increases.

### 3.4. Production of 1-propanol

After comparing basic growth and end-product secretion profiles for the wild-type and B6 strains of *T. fusca* on glucose, a variety of additional carbon sources were tested. Two additional standard laboratory substrates, cellobiose and microcrystalline cellulose (Avicel), and two standard raw biomass substrates, switchgrass and corn stover, were tested. In all conditions, the effective equivalent concentration of carbon substrate was kept roughly the same (5 g/L for glucose, cellobiose and Avicel, and 20 g/L for switchgrass and corn stover using an approximation of ~30% cellulose content). Growth characteristics on the different carbon sources were as expected with progressively slower growth rates and lower biomass occurred with increasing complexity of the carbon source (fastest growth and highest biomass on glucose, slowest growth and lowest biomass on switchgrass and corn stover), Fig. 3a. Interestingly, the highest observed 1-propanol titers were measured for growth on raw switchgrass (0.48 g/L) and Avicel (0.41 g/L), Fig. 3c, but the highest titers on raw switchgrass and corn stover were achieved much later than other substrates (Fig. 3d). In a related fashion, the highest 1-propanol production per dry cell weight (DCW) occurred on the raw biomass, switchgrass (0.87 g propanol/g DCW) and corn stover (0.73 g propanol/g DCW). The B6 strain had the highest yield of 1-propanol (37.3% of the theoretic yield) on switchgrass and corn stover compared to the other substrates (Table 5).

### 3.5. Tolerance to 1-propanol

A major limitation in biofuel production (or solventogenesis) is end-product inhibition where the desired product may be toxic to cells. In many organisms, the upper-limit product titer is dictated by the organism's chemical tolerance (Nicolaou et al., 2010). A series of batch culture experiments were conducted with increasing amounts of 1-propanol (Fig. 4) to evaluate the native tolerance of *T. fusca* to 1-propanol. The cells of *T. fusca* and *E. coli* were obtained at the middle log-phase during the fermentation and then washed by the fresh medium. The cells were inoculated in the 96-well plate with the cover, which was sealed carefully by the tape to prevent 1-propanol from escaping during the test. While the addition of 1-propanol did decrease the overall cell

**Table 5**
Theoretical yield of 1-propanol on different substrates.

| Substrate | Amount (g/L) | % of theoretical yield of 1-propanol |
|---|---|---|
| Glucose | 5 | $16.2 \pm 1.3$ |
| Cellobiose | 5 | $21.6 \pm 1.5$ |
| Avicel | 5 | $35.7 \pm 2.9$ |
| Switchgrass | 20 | $37.3 \pm 1.1$ |
| Corn stover | 20 | $37.3 \pm 1.3$ |

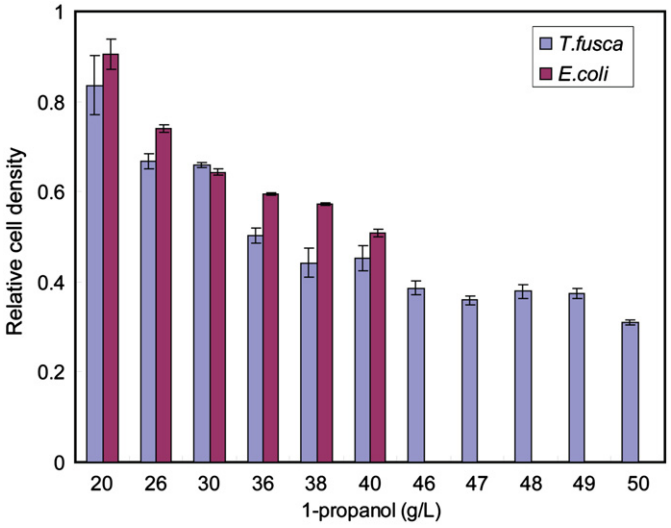


**Fig. 4.** Relative cell density of *T. fusca* and *E. coli* for growth in increasing concentrations of 1-propanol. Relative cell densities were calculated as a ratio of cell densities at the test 1-propanol concentration compared to cell densities for growth with no 1-propanol.

**Table 6**
Compositions of untreated switchgrass and corn stover.

| Compositions | Glucan % (wt/wt) | Xylan % (wt/wt) | Galactan % (wt/wt) | Arabinan % (wt/wt) | Lignin % (wt/wt) | Mannan % (wt/wt) | Extractives % (wt/wt) | Ashes % (wt/wt) | Total carbon source % |
|---|---|---|---|---|---|---|---|---|---|
| Chemical formula | n(C6H10O5) | n(C5H8O4) | n(C6H10O5) | n(C5H8O4) | n(C9H10O2) | n(C6H10O5) | N/A | N/A | |
| Switch grass | 31 | 20.4 | 0.9 | 2.8 | 17.6 | 0.3 | 17 | 5.8 | 73 |
| Corn stover | 36.4 | 18 | 1 | 3 | 16.6 | 0.6 | 7.3 | 9.7 | 75.60 |

densities of *T. fusca* when compared to cultures with no added 1-propanol, *T. fusca* continued to grow under all concentrations tested up to a concentration of 50 g/L of 1-propanol (maximum concentration tested). Comparison of 1-propanol tolerance between *T. fusca* and *E. coli* showed that *T. fusca* has a higher native tolerance to 1-propanol (Fig. 4). *E. coli* ceased to grow at concentrations of 40 g/L.

## 4. Discussion

When considering the application of bioprocessing for industrial-scale production of biofuel (or other chemical products), a diversity of issues are involved. A large and growing body of work utilizes the main workhorse microorganisms—*Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus subtilis* (Zhang et al., 2011)—for many applications, but it is becoming necessary to develop additional platform organisms that can utilize specialized cellular activities or function that are not found in the established workhorse organisms. In this study, we have focused on the aerobic, cellulolytic actinobacterium, *Thermobifida fusca*, to demonstrate its potential utility as a platform organism for cellulolytic bioprocessing. Overall, we have engineered *T. fusca* to directly convert untreated lignocellulosic biomass to 1-propanol, characterized its native tolerance to 1-propanol and studied the activity of intracellular pathways to identify avenues for further strain improvement.

*T. fusca* is an aerobic organism, and the oxygen concentration is an important variable, which will influence cellulolytic, growth and production capabilities. To test the affect of oxygen on 1-propanol production, we conducted shaken, unstoppered flask experiments with B6 strain, and we found that higher oxygen level hindered the 1-propanol production (data not shown). A series of controlled bioreactor experiments will be conducted to study more about oxygen's affect on 1-propanol production.

The engineered *T. fusca* B6 strain from this work can produce 1-propanol from a variety of carbon sources ranging from standard laboratory substrates (glucose, cellobiose and Avicel) to potential commercial substrates (untreated switchgrass and corn stover). The 1-propanol titer achieved by our designed strain (maximum of 0.48 g/L in this study) is on the same order of magnitude as the highest reported 1-propanol titer from any organism to date (~1 g/L for *E. coli* grown on glucose) (Shen and Liao, 2008). This work represents the first iteration of strain design for *T. fusca*. Given the high native tolerance of *T. fusca* to 1-propanol (up to 50 g/L), there is a high potential for significantly increasing the productivity of *T. fusca*.

In profiling the metabolic end-products secreted by both the wild-type and engineered B6 strains of *T. fusca* (Fig. 3b), it was interesting to observe that the highest amount of 1-propanol produced occurred for growth on switchgrass and cellulose (Avicel) with the lowest amounts occurring for growth on glucose. While initially counterintuitive, we believe these results may allude to a metabolic bottleneck in the last steps of 1-propanol production with the heterologously expressed *adhE2* gene. For growth on cellulose, a host of cellulolytic enzymes need to be expressed and require time to hydrolyze cellulose into glucose monomers for cellular utilization. In this case, the amount of readily available glucose and the glucose flux are low. For growth on glucose, extracellular glucose is readily available and can be transported directly into the cell. Comparing these two scenarios, growth on glucose will subject the metabolic network to higher pathway fluxes (due to the higher input flux of glucose) than for growth on cellulose.

In the engineered B6 strain of *T. fusca*, there is a branch point at propanoyl-CoA where propionic acid can be secreted using genes native to *T. fusca* or 1-propanol can be secreted using the *adhE2* gene from *C. acetobutylicum*. During situations of high carbon flux (i.e. growth on glucose), if the activity of the heterologously expressed *adhE2* cannot handle the high carbon load, excess propanoyl-CoA would accumulate and must be secreted as propionic acid. Under low carbon flux (i.e. growth on cellulose), the activity of the *adhE2* is sufficient to process the carbon load and thus 1-propanol is secreted with no detectable amounts of propionic acid being secreted.

Thus, we believe the enzymatic activity of the heterologously expressed *adhE2* gene from *Clostridium acetobutylicum* appears to be a bottleneck step for 1-propanol production and improvements in controlling expression of this gene should be able to increase 1-propanol production titers. This hypothesis worked very well on the defined substrates – glucose, cellobiose and cellulose – but did not work on switchgrass and corn stover, which were slow carbon sources because the ratio of propionate and 1-propanol remained roughly equally on these two substrates. The roughly equal ratio of propionate and 1-propanol in switchgrass and corn stover is potentially caused by the diversity of sugars contained in these two naturally heterogeneous materials. The two materials are not pre-treated, and they had a variety of the sugars and other carbon sources that can be use in *T. fusca* B6 strain (Table 6) that can potentially affect the carbon flow to produce propionate.

## Acknowledgments

The authors would like to thank Dr. David B. Wilson of Cornell University for kindly providing plasmid pUZ8002, Dr. Rita Shiang of Virginia Commonwealth University for providing a stock of *E. coli* DH5α and the John Innes Center in the UK for providing the plasmid pIJ790.

## References

Aguirre, M.V., Phillips, J.A., Bostwick, L.J., Montenecourt, B.S., 1986. Comparison of the cellulase complexes of *Trichoderma reesei* Rl-P37 and *Thermomonospora fusca* YX. Chem. Eng. Commun. 45, 93–109.

Bierman, M., Logan, R., O'Brien, K., Seno, E.T., Rao, R.N., Schoner, B.E., 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to Streptomyces spp. Gene 116, 43–49.

Bothwell, M.K., Walker, L.P., Wilson, D.B., Irwin, D.C., Price, M., 1993. Synergism between pure *Thermomonospora fusca* and *Trichoderma reesei* cellulases. Biomass. Bioenerg. 4, 293–299.

Bradford, M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principal of protein-dye binding. Anal. Biochem. 72, 248–254.

Caspi, J., Irwin, D., Lamed, R., Shoham, Y., Fierobe, H.P., Wilson, D.B., Bayer, E.A., 2006. *Thermobifida fusca* family-6 cellulases as potential designer cellulosome components. Biocatal. Biotransfor. 24, 3–12.

Chen, S.L., Wilson, D.B., 2007. Proteomic and transcriptomic analysis of extracellular proteins and mRNA levels in *Thermobifida fusca* grown on cellobiose and glucose. J. Bacteriol. 189, 6260–6265.

Cohn, M., Phillips, A., 1974. Purification and characterization of a B6-independent threonine dehydratase from *Pseudomonas putida*. Biochemistry 13, 1208–1214.

Demain, A.L., Newcomb, M., Wu, J.H.D., 2005. Cellulase, clostridia, and ethanol. Microbiol. Mol. Biol. Rev. 69, 124–154.

Deng, Y., Fong, S., 2010a. Influence of culture aeration on the cellulase activity of *Thermobifida fusca*. Appl. Microbiol. Biotechnol. 85, 965–974.

Deng, Y., Fong, S., 2010b. Development and application of a PCR-targeted gene disruption method for studying CelR function in *Thermobifida fusca*. Appl. Environ. Microbiol. 76, 2098–2106.

Dien, B., Iten, L., Bothast, R., 1999. Conversion of corn fiber to ethanol by recombinant *E. coli* strain FBR3. J. Ind. Microbiol. Biotechnol. 22, 575–581.

Eden, A., Van Nedervelde, L., Drukker, M., Benvenisty, N., Debourg, A., 2001. Involvement of branched-chain amino acid aminotransferases in the production of fusel alcohols during fermentation in yeast. Appl. Microbiol. Biotechnol. 55, 296–300.

Gust, B., Challis, G., Fowler, K., Kieser, T., Chater, K., 2003. PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA 100, 1541–1546.

Hanai, T., Atsumi, S., Liao, J., 2007. Engineered synthetic pathway for isopropanol production in *Escherichia coli*. Appl. Environ. Microbiol. 73, 7814–7818.

Janssen, P., 2004. Propanol as an end product of threonine fermentation. Arch. Microbiol. 182, 482–486.

Lopez-Contreras, A., Claassen, P., Mooibroek, H., De Vos, W., 2000. Utilisation of saccharides in extruded domestic organic waste by *Clostridium acetobutylicum* ATCC 824 for production of acetone, butanol and ethanol. Appl. Microbiol. Biotechnol. 54, 162–167.

Lykidis, A., Mavromatis, K., Ivanova, N., Anderson, I., Land, M., DiBartolo, G., Martinez, M., Lapidus, A., Lucas, S., Copeland, A., Richardson, P., Wilson, D.B., Kyrpides, N., 2007. Genome sequence and analysis of the soil cellulolytic actinomycete *Thermobifida fusca* YX. J. Bacteriol. 189, 2477–2486.

Nicolaou, S., Gaida, S., Papoutsakis, E., 2010. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. Metab. Eng. 12, 307–331.

Nielsen, D., Leonard, E., Yoon, S.-H., Tseng, H.-C., Yuan, C., Prather, K., 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metab. Eng. 11, 262–273.

Nonklang, S., Abdel-Banat, B., Cha-Aim, K., Moonjai, N., Hoshida, H., Limtong, S., 2008. High-temperature ethanol fermentation and transformation with linear DNA in the thermotolerant yeast *Kluyveromyces marxianus* DMKU3-1042. Appl. Environ. Microbiol. 74, 7514–7521.

Palosaari, N., Rogers, P., 1988. Purification and properties of the inducible coenzyme a-linked butyraldehyde dehydrogenase from *Clostridium acetobutylicum*. J. Bacteriol. 170, 2971–2976.

Rani, K., Swamy, M., Seenayya, G., 1997. Increased ethanol production by metabolic modulation of cellulose fermentation in *Clostridium thermocellum*. Biotechnol. Lett. 19, 819–823.

Rao, K., Chaudhari, V., Varanasi, S., Kim, D., 2007. Enhanced ethanol fermentation of brewery wastewater using the genetically modified strain *E. coli* KO11. Appl. Microbiol. Biotechnol. 74, 50–60.

Risse, B., Stempfer, G., Rudolph, R., Mollering, H., Jaenicke, R., 1992. Stability and reconstitution of pyruvate oxidase from Lactobacillus plantarum: dissection of the stabilizing effects of coenzyme binding and subunit interaction. Protein Sci. 1, 1699–1709.

Roberts, S., Gowen, C., Brooks, J., Fong, S., 2010. Genome-scale metabolic analysis of *Clostridium thermocellum* for bioethanol production. BMC systems biology BMC. Syst. Biol. 4, 31–47.

Shen, C., Liao, J., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. Metab. Eng. 10, 312–320.

Spiridonov, N.A., Wilson, D.B., 1999. Characterization and cloning of CelR, a transcriptional regulator of cellulase genes from *Thermomonospora fusca*. J. Biol. Chem. 274, 13127–13132.

Stephanopoulos, G., 2008. Metabolic engineering: enabling technology for biofuels production. Metab. Eng. 10, 293–294.

Suwannakham, S., Yang, S., 2005. Enhanced propionic acid fermentation by Propionibacterium acidipropionici mutant obtained by adaptation in a fibrous-bed bioreactor. Biotechnol. Bioeng. 91, 325–337.

Yu, Y., Tangney, M., Aass, H., Mitchell, W., 2007. Analysis of the mechanism and regulation of lactose transport and metabolism in *Clostridium acetobutylicum* ATCC 824. Appl. Environ. Microbiol. 73, 1842–1850.

Zhang, X.-Z., Sathitsuksanoh, N., Zhu, Z., Zhang, Y.-H., 2011. One-step production of lactate from cellulose as sole carbon source without any other organic nutrient by recombinant cellulolytic *Bacillus subtilis*. Metab. Eng.. doi:10.1016/j.ymben.2011.04.003.