Metabolic Engineering 21 (2014) 17–25

Contents lists available at ScienceDirect

Metabolic Engineering

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

# Metabolic engineering of *Thermoanaerobacterium saccharolyticum* for *n*-butanol production

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ARTICLE INFO

*Article history:*
Received 27 January 2013
Received in revised form
1 October 2013
Accepted 30 October 2013
Available online 9 November 2013

*Keywords:*
Metabolic engineering
Biofuels
*n*-Butanol
Thermophiles

ABSTRACT

The thermophilic anaerobe *Thermoanaerobacterium saccharolyticum* JW/SL-YS485 was investigated as a host for *n*-butanol production. A systematic approach was taken to demonstrate functionality of heterologous components of the clostridial *n*-butanol pathway via gene expression and enzymatic activity assays in this organism. Subsequently, integration of the entire pathway in the wild-type strain resulted in *n*-butanol production of 0.85 g/L from 10 g/L xylose, corresponding to 21% of the theoretical maximum yield. We were unable to integrate the *n*-butanol pathway in strains lacking the ability to produce acetate, despite the theoretical overall redox neutrality of *n*-butanol formation. However, integration of the *n*-butanol pathway in lactate deficient strains resulted in *n*-butanol production of 1.05 g/L from 10 g/L xylose, corresponding to 26% of the theoretical maximum.

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## 1. Introduction

There is an intensive effort underway to develop technology for sustainable production of transportation fuel from plant biomass (Grayson, 2011; Lynd et al., 2008; Olson et al., 2012) and *n*-butanol has attracted attention in this context (Dong et al., 2012; Dürre, 2008). Whereas over 80 billion liters of ethanol per year are currently used as a transportation fuel (Smith et al., 2012), butanol is not used today as a transportation fuel to a significant extent, despite having similar potential thermodynamic conversion efficiency from carbohydrates, a higher energy density, and physical properties more fungible with gasoline, such as easy shipment via pipeline (Dürre, 2008).

The most widely studied *n*-butanol pathway is that found in *Clostridium acetobutylicum* and other related clostridia which have been used since the 1920s in the Weizmann process (Jones and Woods, 1986). The genes involved in this pathway have been cloned, proteins have been characterized (Boynton et al., 1996; Fischer et al., 1993; Petersen and Bennett, 1991; Stim-Herndon et al., 1995), and control mechanisms related to separate acidogenic and solventogenic stages have received considerable study (Gheshlaghi et al., 2009). In *C. acetobutylicum*, the primary genes responsible for *n*-butanol production are *thl, hbd, crt, bcd, etfA, etfAB, adhE2* and *adhE1* (Jones and Woods, 1986; Nölling et al., 2001) expressing enzymes thiolase, β-hydroxybutyryl CoA dehydrogenase, crotonase, butyryl CoA dehydrogenase, electron transfer flavoproteins subunit A and B and the bi-functional enzyme aldehyde–alcohol dehydrogenase that have activities to act on both substrates acetyl CoA and butyryl CoA for ethanol and *n*-butanol production, respectively. Many mesophiles including *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas putida* and *Lactobacillus brevis* have been engineered to produce *n*-butanol by heterologous expression of these genes from *C. acetobutylicum* (Atsumi et al., 2008; Berezina et al., 2010; Inui et al., 2008; Nielsen et al., 2009).

Use of thermophilic anaerobic bacteria in industrial biotechnological processes has long been thought to offer potential advantages over mesophiles, including but not limited to reduced risk of contamination, higher reaction rates, and lower differential costs for heating and cooling (Barnard et al., 2010; Zeikus, 1979).

Strain DSM 571 of *Thermoanaerobacterium thermosaccharolyticum* (formerly *Clostridium thermosaccharolyticum*) has been reported to produce *n*-butanol (Freierschroder et al., 1989) although not consistently (Demain et al., 2005; Hill et al., 1993; Mistry and Cooney, 1989). Moreover, the *n*-butanol pathway has not been characterized in this organism; however, putative genes responsible for producing *n*-butanol are present in the recently sequenced genome of *T. thermosaccharolyticum* DSM 571 (Hemme et al., 2010). It appears

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1096-7176/$ - see front matter © 2013 Published by Elsevier Inc. on behalf of International Metabolic Engineering Society.
http://dx.doi.org/10.1016/j.ymben.2013.10.012

that the genes (*crt, bcd, etfB, etfA, hbd* and *thl*) responsible for butyryl CoA formation occur as a multi-gene operon.

*Thermoanaerobacterium saccharolyticum* strain JW/SL-YS485, a closely related thermophile to *T. thermosaccharolyticum*, has been well characterized and engineered extensively. It is a gram positive thermophilic anaerobic bacterium isolated from Yellowstone National Park (Shao et al., 1995). This microorganism grows between temperatures of 45 and 65 °C and between pH 4.0 and 6.8 (Shaw et al., 2008a). *T. saccharolyticum* is capable of utilizing a variety of sugars found in cellulosic biomass including cellobiose, glucose, xylose, mannose, galactose, and arabinose. It can hydrolyze xylan, a major component of cellulosic biomass, as well as mannan, starch and pectin, and it is naturally competent, making it an attractive host for genetic manipulations (Shaw et al., 2010). It has also been engineered for the production of ethanol at high yields by deleting the genes involved in organic acid and $H_2$ production, directing the carbon and electron flux towards ethanol (Shaw et al., 2008b; Shaw et al., 2009).

In light of its substrate utilizing capabilities and the availability of well-developed genetic tools, *T. saccharolyticum* is an attractive host for heterologous *n*-butanol production. Here, we report engineering *T. saccharolyticum* to produce *n*-butanol at significant yield.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 1. For *T. saccharolyticum* transformation experiments all strains were grown at 55 °C in TSC1 medium (Shaw et al., 2011), containing per liter – 5 g of cellobiose, 1.85 g of $(NH_4)_2SO_4$, 0.05 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of $KH_2PO_4$, 1.0 g of $MgSO_4$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 2 g of trisodium citrate dihydrate, 8.5 g of yeast extract, 2 mg of resazurin, 0.5 g of L-cysteine–HCl. For solid medium, 12 g of agar was added. The pH was adjusted to 6.7 for selection on kanamycin (200 μg/mL). For growth comparisons all strains were grown in TSD medium (Shaw et al., 2012) at 55 °C, containing per liter – 1.85 g of $(NH_4)_2SO_4$, 0.05 g of $FeSO_4 \cdot 7H_2O$, 1.0 g of $KH_2PO_4$, 1.0 g of $MgSO_4$, 0.1 g of $CaCl_2 \cdot 2H_2O$, 2 g of trisodium citrate, 0.5 g of yeast extract, 2 mg *p*-amino benzoic acid, 2 mg thiamine–HCl, 0.01 mg vitamin B12, 0.12 g methionine. The carbon source was 10 g xylose and pH was adjusted to 6.1.

*E. coli* was grown in LB medium, supplemented with 1.5% agar and either 50 μg/mL kanamycin or 100 μg/mL ampicillin as appropriate. *Saccharomyces cerevisiae* was grown in YPD or SD-Ura medium under standard growth conditions (Shanks et al., 2006).

### 2.2. Reagents and chemicals

Chemicals were obtained from Sigma Aldrich (St. Louis, MO), Fisher Scientific (Pittsburgh, PA) or BD Difco (Franklin Lakes, NJ). Restriction enzymes and DNA polymerase (Phusion) were obtained from New England Biolabs (Ipswich, MA), and oligonucleotides were ordered from IDT (Coralville, IA)

### 2.3. Construction of plasmids

Standard yeast mediated cloning techniques (Shanks et al., 2006) were used for construction of plasmids expressing individual genes of the *n*-butanol pathway. The individual genes/gene cluster of interest encoding each enzymatic step *thl* (Tthe_1656), *hbd* (The_1657), *crt* (Tthe_1661), and *bcd* (Tthe_1660), *etfA* (The_1658), *etfB* (The_1659) from *T. thermosaccharolyticum* DSM571 and *adhE2*

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

| Strain/plasmid | Relevant genotype | Reference |
|---|---|---|
| ***Strains*** | | |
| *T. saccharolyticum* DSM 8691 | Type strain | DSMZ |
| *C. acetobutylicum* ATCC 824 | Type strain | ATCC |
| *T. thermosaccharolyticum* DSM571 | Type strain | DSMZ |
| InvSc1 | *MAT his3D1 leu2 trp1-289 ura3-52* | Invitrogen |
| M0355 | *T. saccharolyticum Δldh ΔptaΔack* | Shaw et al. (2011) |
| M0210 | *T. saccharolyticum Δldh, ermR* | Gift from Mascoma Corp. |
| M0350 | *T. saccharolyticum ΔptaΔack ΔpyrF* | Shaw et al. (2011) |
| Athl | M0355, *kanR, thl* (Tt) | This study |
| Ahbd | M0355, *kanR, hbd* (Tt) | This study |
| Acrt | M0355, *kanR, crt* (Tt) | This study |
| Abcd-etfAB | M0355, *kanR, bcd, etfA, etfB* (Tt) | This study |
| AadhE | M0355, *kanR, adhE2* (Ca) | This study |
| I2B | *T. saccharolyticum, thl* (Tt)*, hbd*(Tt), *crt*(Tt), *bcd*(Tt), *etfA*(Tt), *etfB*(Tt), *adhE*(Ca), *KanR* | This study |
| M0210-V | *M0210, thl* (Tt), *hbd*(Tt), *crt*(Tt), *bcd*(Tt), *etfA*(Tt), *etfB*(Tt), *adhE2*(Ca), *KanR* | This study |
| ***Plasmids*** | | |
| pYC2/CT | *S. cerevisiae-E. coli* cloning vector | Invitrogen |
| pMU131 | *T. saccharolyticum-E. coli* shuttle plasmid, *KanR, AmpR* | Shaw et al. (2010) |
| pMC500 | *T. saccharolyticum–E. coli* shuttle plasmid, *KanR* | Gift from Devin Currie |
| pTHL | pYC2/CT, *KanR, thl* (Tt)[a] | This study |
| pHBD | pYC2/CT, *KanR, hbd* (Tt)[b] | This study |
| pCRT | pYC2/CT, *KanR, crt* (Tt)[c] | This study |
| pBCD-etfAB | pYC2/CT, *KanR*, bcd(Tt)[d], *etfA* (Tt)[e], *etfB* (Tt)[f] | This study |
| pADHE | pYC2/CT, *KanR, adhE* (Ca)[g] | This study |
| pBu24 | pMC500, *thl* (Tt), *hbd*(Tt), *crt*(Tt), *bcd*(Tt), *etfA*(Tt), *etfB*(Tt), *adhE2*(Ca), *KanR* | |

[a] thl (Tt), thiolase from *T. thermosaccharolyticum* (Tthe_1656).
[b] hbd (Tt), β-hydroxybutyryl CoA dehydrogenase from *T. thermosaccharolyticum* (The_1657).
[c] crt (Tt), crotonase from *T. thermosaccharolyticum* (Tthe_1661).
[d] bcd (Tt), butyryl CoA dehydrogenase (Tthe_1660).
[e] etfA (Tt), electron transfer proteins subunit A (The_1658).
[f] etfB (Tt), electron transfer proteins subunit B (The_1659) from *T. thermosaccharolyticum*.
[g] adhE2 (Ca), aldehyde–alcohol dehydrogenase from *C. acetobutylicum* (CA_P0035).

(CA_P0035) from *C. acetobutylicum* ATCC824 were fused to the native *T. saccharolyticum pta-ack* promoter. The genes were individually cloned in plasmid pYC2/CT (Invitrogen, Grand Island, NY) flanked by upstream and downstream regions of the *pta-ack* locus of *T. saccharolyticum* for chromosomal insertion with selection with a kanamycin resistance gene cassette (Mai et al., 1997) (Fig. 1a). *T. saccharolyticum* non-replicative plasmid pBu24 was constructed in *E. coli Top10* (Invitrogen, Grand Island, NY) using Gibson assembly (Gibson et al., 2009) (Fig. 2a) for expression of the entire *n*-butanol pathway. *AdhE* (from *C. acetobutylicum* ATCC 824), gene cluster *crt, bcd, etfB* (from *T. thermosaccharolyticum* DSM571) fused to promoter *gapDH* (from *C. thermocellum* ATCC 27405), gene cluster *etfA, hbd, thl* (from *T. thermosaccharolyticum* DSM571) fused to promoter *cbp* (from *C. thermocellum* ATCC 27405) and kanamycin resistance gene cassette were cloned in plasmid pMC500, a derivative of pMC200 (Currie et al., 2013) containing upstream and downstream regions of *xynA*. The complete sequences for all the plasmids are available in Figs. S1–S6.

### 2.4. Construction of *T. saccharolyticum* strains

DNA for transformation was amplified via PCR from the plasmids pTHL, pHBD, pCRT, pBCD-etfAB, and pADHE. The PCR amplified regions included the upstream and downstream regions of the *pta-ack* locus that provided homology to *T. saccharolyticum*, the gene of interest (*thl* or *hbd* or *crt* or *bcd* and *etfA* and *etfB* or *adhE)* and the kanamycin resistance gene. Strain M0355 (Shaw et al., 2011) a previously constructed homo-ethanologenic strain of *T. saccharolyticum* was transformed with the PCR products to create strains Athl, Ahbd, Acrt, Abcd-etfAB and AadhE (Fig. 1b). Transformation of the non-replicative plasmid pBu24 was attempted in four strains of *T. saccharolyticum* for chromosomal integration (Fig. 2b) – wild type, M0210 (Δ*ldh*) (Gift from Mascoma Corp.), M0350 (Δ*pta* Δ*ack*) (Shaw et al., 2011), and M0355 (Δ*ldh* Δ*pta* Δ*ack*) (Shaw et al., 2011).

Transformation was carried out in an anaerobic chamber (Coy laboratories, Grass Lake MI) by inoculating 10 mL of TSC1 medium with 1 μL of frozen parent strain stocks. About 1 mL of the cultures were added to a tube containing approximately 500 ng of either linear DNA or plasmid DNA. The cultures were incubated for 15–18 h at 55 °C and dilutions were suspended in molten TSC1 agar at pH 6.7 and allowed to solidify before incubation at 55 °C (Shaw et al., 2010). Five kanamycin resistant *T. saccharolyticum* colonies per transformation experiment with colony formation were evaluated for chromosomal integration by PCR using primers external to the site of integration, followed by sequence verification.

### 2.5. Enzyme assays

*T. thermosaccharolyticum* and *T. saccharolyticum* strains were grown in TSC1 liquid medium at 55 °C under anaerobic conditions. About 100 mL media were inoculated with 1% (v/v) culture and grown for 24 h with 200 μg/mL kanamycin where appropriate.

Thiolase, β-hydroxybutyryl CoA dehydrogenase and crotonase assays were carried out aerobically, but anaerobic conditions were maintained for the butyryl CoA dehydrogenase, butyraldehyde dehydrogenase and butanol dehydrogenase assays. For preparation of the cell-free extracts, 100 mL cultures were centrifuged at 4 °C at 6000*g* in a Beckman Coulter Avanti J-25 centrifuge. The cell pellet was resuspended in 4 mL of appropriate buffer as required for the assay and sonicated using a Misonix Sonicator 4000 fitted with a micro-tip in a 10 mL glass beaker for 8 min with 10 s pulse on and 10 s pulse off at 50% of the max intensity. Crude cell-free extract was obtained by centrifugation at 14,000*g* for 25 min and

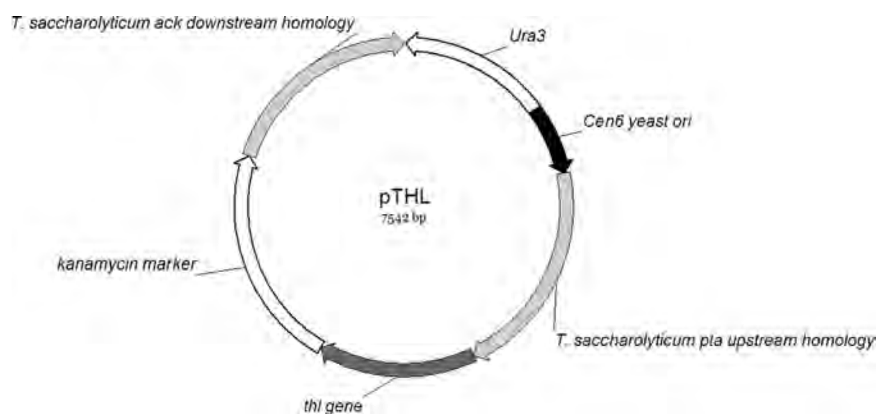


**Fig. 1.** Plasmid map for expressing individual genes of the *n*-butanol pathway. (plasmids pHBD, pCRT, pBCD-etfAB and pADHE were constructed similarly)

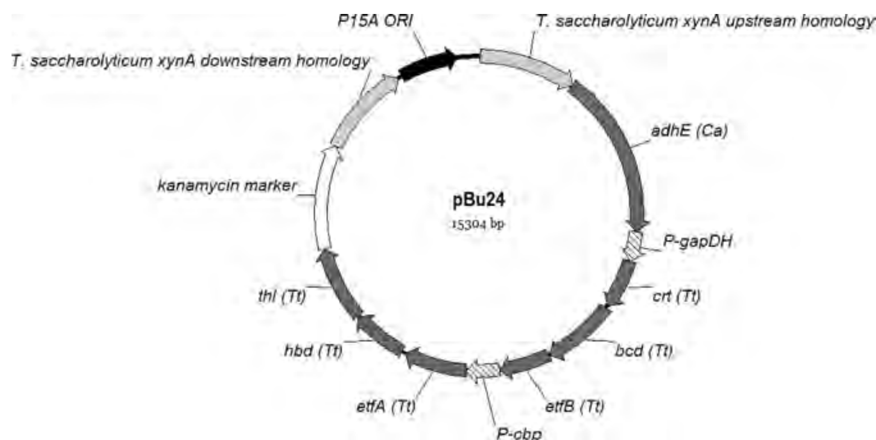


**Fig. 2.** Non-replicative plasmid construction for expressing heterologous *n*-butanol pathway.

removing the cell debris. Protein concentration was measured using Bradford reagent (Bio-rad, Hercules, CA). Absorbance measurements were done using an Agilent 8453 UV–vis spectrophotometer attached to a Peltier temperature controller. Assays were carried out at 55 °C. The protein concentrations of the crude cell-free extracts were in the range 1.5–3.6 mg/mL.

Thiolase (EC 2.3.1.9) was measured in the direction of acetoacetyl CoA cleavage at 303 nm. The reaction buffer contained 100 mM Tris–HCl, 1 mM DTT, 10 mM $MgCl_2$ at pH 8.0 (at 55 °C). The crude cell-free extract was added, blank measured, followed by the addition of substrate – 50 μM acetoacetyl CoA and 0.2 mM coenzyme A initiated the reaction (Hartmanis and Gatenbeck, 1984).

β-Hydroxybutyryl CoA dehydrogenase (EC 1.1.1.35) activity measured the NADH consumed at 340 nm. The reaction buffer contained 50 mM MOPS, 0.1 mM DTT at pH 7.0 (at 55 °C), to which the cell-free extract was added, followed by 0.2 mM co-factor NADH and the addition of the substrate – 75 μM acetoacetyl CoA started the reaction (Hartmanis and Gatenbeck, 1984).

Crotonase (EC 4.2.1.17) activity was determined by measuring the hydration of crotonyl CoA at 263 nm to form β-hydroxybutyryl CoA. The reaction buffer contained 100 mM Tris–HCl at pH 7.6 (at 55 °C) to which cell-free extract was added. The addition of crotonyl CoA initiated the reaction (Hartmanis and Gatenbeck, 1984).

Butyryl CoA dehydrogenase (with electron transfer flavoproteins AB) activity was measured by two different methods – the first method used a 50 mM MOPS reaction buffer at pH 7.0 (at 55 °C) to which cell-free extract and 0.4 mM crotonyl CoA were added. The mixture was equilibrated for 10 min followed by the addition of 0.2 mM ferrocenium ion. The decrease in absorbance at 300 nm was monitored for consumption of the ferrocenium (Inui et al., 2008). The second method used a 50 mM Tris–HCl, 2 mM DTT reaction buffer at pH 7.5 (at 55 °C) to which cell-free extract, 5 μM FAD, 20 μM ferredoxin (from *Spinacia oleracea*), and 0.1 mM NADH were added. Addition of 0.1 mM crotonyl CoA initiated the reaction. The NADH consumption was monitored at 340 nm (Li et al., 2008).

Butyraldehyde dehydrogenase (EC 1.2.1.57) activity was assayed based on NAD(P)H co-factor consumption monitored at 340 nm. The reaction buffer contained 67 mM Tris–HCl and 1 mM DTT at pH 6.0 (at 55 °C). Cell-free extract was added, followed by 0.27 mM NAD(P)H and 0.2 mM butyryl CoA initiated the reaction (Dürre et al., 1987).

Butanol dehydrogenase (EC 1.1.1.1) assay measured the NAD(P)H co-factor consumption at 340 nm. The reaction buffer contained 77 mM Tris–HCl and 1 mM DTT at pH 7.8 (at 55 °C). Cell-free extract was added, followed by 0.23 mM NAD(P)H and 11 mM butyraldehyde to initiate the reaction (Dürre et al., 1987).

### 2.6. Analytical methods

The fermentation products were measured by high-pressure liquid chromatography with an Aminex HPX-87 H column (Bio-rad Laboratories, Hercules, CA). The mobile phase consisted of 5 mM sulfuric acid at a flow rate of 0.6 mL/min. The detection of the metabolites was by refractive index using a refractometer (Waters 410) and the column temperature was 60 °C.

Hydrogen was measured by gas chromatography using a SRI 310C GC with thermal conductivity detector. A silica gel column was used, 60 °C isothermal with nitrogen carrier at 20 mL/min. The injection volume was 500 μL and the length of the run was 1 min.

### 2.7. Carbon and electron balance

Carbon balances were determined by calculating the ratio of moles of carbon in products to moles of carbon in substrate consumed, with carbon dioxide accounted for by the stoichiometric co-production with acetic acid, ethanol and butanol. The carbon in the dry cell weight was calculated empirically using the general formula for the cell composition ($CH_2N_{0.25}O_{0.5}$) (Shaw et al., 2008b). Electron balances were calculated a ratio of oxidized products to reduced products (O/R ratio) as a function of the available electrons per mole of the substrate and products (Moat et al., 2002; Papoutsakis, 2000).

## 3. Results

Individual enzymes of the *n*-butanol pathway were functionally expressed one at a time in the host organism *T. saccharolyticum* from the source organisms *T. thermosaccharolyticum* and *C. acetobutylicum*. After verification of individual enzyme activities, an entire *n*-butanol pathway was expressed in two strains of *T. saccharolyticum* for the demonstration of heterologous production of *n*-butanol.

### 3.1. Functional expression of individual enzymes of the n-butanol pathway in T. saccharolyticum

To demonstrate functionality of enzymes predicted to be involved in *n*-butanol production, genes encoding each step of the *n*-butanol pathway from the thermophile *T. thermosaccharolyticum* were individually heterologously expressed in *T. saccharolyticum*. The native *T. saccharolyticum pta-ack operon* promoter was used to express the individual genes of the butanol pathway. However, it was determined that replacing the native *pta* ribosomal binding site of *T. saccharolyticum* by a consensus sequence (AGGAGG) increased each of the activities 6–10-fold (data not shown); hence, the native Shine Dalgarno was replaced by the consensus sequence for each of the constructs.

Table 2 presents activities of enzymes involved in *n*-butanol formation for wild-type *T. thermosaccharolyticum*, wild type and engineered strains of *T. saccharolyticum,* and values from the literature for *C. acetobutylicum*. The *T. saccharolyticum* parent strains – WT, M0355 and M0210 – did not show detectable activity for the first five enzymatic steps in the *n*-butanol pathway. The activities of heterologously expressed thiolase and β-hydroxybutyryl CoA dehydrogenase in *T. saccharolyticum* were approximately 1.5–3-fold lower than activities measured in wild type *T. thermosaccharolyticum* and literature reports for *C. acetobutylicum.* While the heterologous crotonase activities in *T. saccharolyticum* were nearly 2.5–5-fold lower than the activity in *T. thermosaccharolyticum*, they were approximately 50-fold lower than *C. acetobutylicum.*

The butyryl CoA dehydrogenase enzyme assays were performed in two different ways. The method described by Inui et al. did not yield any detectable activity for the catalysis of crotonyl CoA to butyryl CoA. However, the ferredoxin-dependent assay as described by Li et al. did demonstrate measurable activity. Negative control without the addition of ferredoxin did not yield measurable activity. The reaction catalyzed by butyryl CoA dehydrogenase enzyme and electron transfer proteins A and B highlights a possible metabolic pathway utilized by *T. thermosaccharolyticum*, suggesting that the exergonic reduction of crotonyl CoA to butyryl CoA is coupled to endergonic ferredoxin reduction (Li et al., 2008). Furthermore, the genetic comparison of the *T. thermosaccharolyticum* genes *bcd, etfA* and *etfB* (The_1660, The_1659, The_1658) shows a high homology to the *C. kluyveri* genes (CKL_0455, CKL_0456, CKL_0457) that catalyze a similar reaction from crotonyl CoA to butyryl CoA. However, additional enzymatic work is required for confirming the functionality of this enzyme complex (Li et al., 2008). The heterologous butyryl CoA dehydrogenase ferredoxin-dependent activities in *T. saccharolyticum* were 3-fold lower than the native activity of *T. thermosaccharolyticum*.

**Table 2**
Specific activities of the enzymes in the butanol pathway.

| Enzyme | Strain | Specific activity[a] (μmol/min/mg total protein) | Reference |
|---|---|---|---|
| Thiolase | *T.t.* | 5. 08 ± 0.74 | This study |
| | Athl | 2.47 ± 0.37 | This study |
| | I2B | 3.96 ± 0.82 | This study |
| | M0210-V | 4.06 ± 1.23 | This study |
| | M0355, M0210, *T.s.* | n.d.[b] | This study |
| | *C.a.* | 11 | Hartmanis and Gatenbeck (1984) |
| 3-Hydroxybutyryl CoA dehydrogenase | *T.t.* | 14.43 ± 0.65 | This study |
| | Ahbd | 6.81 ± 0.39 | This study |
| | I2B | 8.21 ± 0.68 | This study |
| | M0210-V | 8.72 ± 0.44 | This study |
| | M0355, M0210, *T.s* | n.d. | This study |
| | *C.a.* | 8 | Hartmanis and Gatenbeck (1984) |
| Crotonase | *T.t.* | 11.95 ± 1.15 | This study |
| | Acrt | 2.56 ± 0.19 | This study |
| | I2B | 4.81 ± 0.92 | This study |
| | M0210-V | 5.17 ± 0.79 | This study |
| | M0355, M0210, *T.s* | n.d. | This study |
| | *C.a.* | 135 | Hartmanis and Gatenbeck (1984) |
| Butyryl CoA dehydrogenase | *T.t.* | 0.67 ± 0.04[c] | This study |
| | Abcd-etfAB | 0.21 ± 0.05[c] | This study |
| | I2B | 0.27 ± 0.10[c] | This study |
| | M0210-V | 0.23 ± 0.09[c] | This study |
| | M0355, M0210, *T.s* | n.d.[c] | This study |
| | *C.k.* | 4[d] | Hartmanis and Gatenbeck (1984) |
| Butyraldehyde dehydrogenase (with NADH as co-factor) | AadhE | 0.04 ± 0.01 | This study |
| | I2B | 0.04 ± 0.06 | This study |
| | M0210-V | 0.05 ± 0.08 | This study |
| | M0355, M0210, *T.s* | n.d. | This study |
| | *C.a.* | 0.038 | Dürre et al. (1987) |
| Butyraldehyde dehydrogenase (with NADPH as co-factor) | AadhE | 0.02 ± 0.01 | This study |
| | I2B | 0.02 ± 0.07 | This study |
| | M0210-V | 0.02 ± 0.08 | This study |
| | M0355, M0210, *T.s* | n.d. | This study |
| | *C.a.* | 0.005 | Dürre et al. (1987) |
| Butanol dehydrogenase (with NADH as co-factor) | AadhE | 0.20 ± 0.01 | This study |
| | I2B | 0.22 ± 0.05 | This study |
| | M0210-V | 0.21 ± 0.08 | This study |
| | *M0355* | 0.04 ± 0.01 | This study |
| | *T.s* | 0.11 ± 0.04 | This study |
| | *M0210* | 0.07 ± 0.03 | This study |
| | *C.a.* | 0.02 | Dürre et al. (1987) |
| Butanol dehydrogenase (with NADPH as co-factor) | AadhE | 0.13 ± 0.01 | This study |
| | I2B | 0.18 ± 0.04 | This study |
| | M0210-V | 0.19 ± 0.02 | This study |
| | *T.s.* | 0.04 ± 0.04 | This study |
| | *M0210* | 0.06 ± 0.05 | This study |
| | *M0355* | 0.20 ± 0.01 | This study |
| | *C.a.* | 0.14 | Dürre et al. (1987) |

T.t, *Thermoanaerobacterium thermosaccharolyticum*; T.s., *Thermoanaerobacterium saccharolyticum*; C.a., *Clostridium acetobutylicum*; C.k., *Clostridium kluyveri*.

[a] Data shown in this study is an average of triplicate measurements. The specific activities measured are from crude cell-free extracts and expressed as total cellular protein concentration.

[b] Not detected.

[c] The specific activities reflected are using a ferredoxin coupled assay (Li et al., 2008).

[d] The reference measures specific activity for purified protein while the specific activities determined in this study are as a function of crude cell-free extract.

*T. saccharolyticum* encodes a bi-functional aldehyde/alcohol dehydrogenase (*adhE*; Accession no. EU313774) that is presumed to be responsible for the formation of ethanol in vivo. To determine whether the *T. saccharolyticum* AdhE or another native alcohol dehydrogenase could reduce butyraldehyde to *n*-butanol, butanol dehydrogenase activity was measured in all three parent strains – WT, M0355 and M0210. Interestingly, the strains are capable of reducing butyraldehyde (Table 2). While the parent strains of *T. saccharolyticum* demonstrated butanol dehydrogenase activity, there was no evidence of butyraldehyde dehydrogenase activity with butyryl-CoA as the substrate. The butyraldehyde dehydrogenase activities were detected in strains AadhE, I2B and M0210-V and were comparable to *C. acetobutylicum*. Upon heterologous expression of the *adhE* gene from *C. acetobutylicum* in strains of *T. saccharolyticum*,

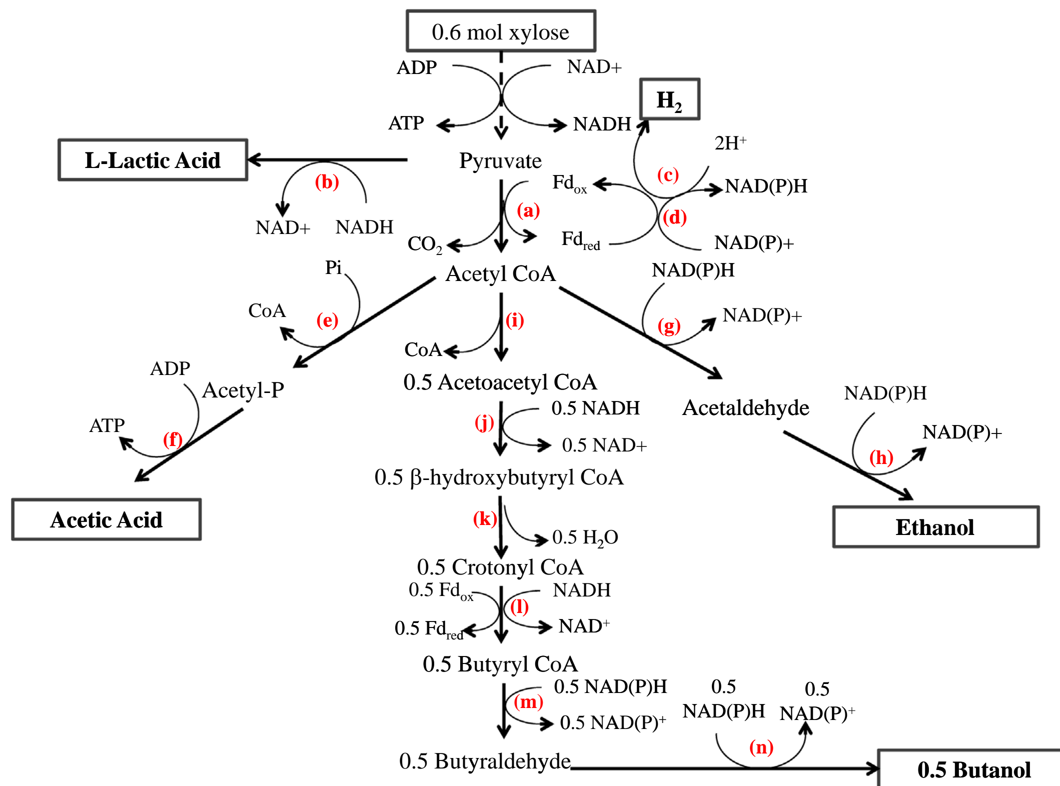


**Fig. 3.** Metabolic pathway for heterologous *n*-butanol production in *T. saccharolyticum*.

there was change in co-factor specificity in some of the recombinant strains but the overall activity of all the three engineered strains – Aadhe, I2B and M0210-V – were enhanced in comparison to the parent strains – M0355, WT and M0210.

### 3.2. Construction of *T. saccharolyticum* strains expressing a n-butanol pathway

Having verified functional expression of the individual enzymes for the production of *n*-butanol from acetyl CoA, a synthetic pathway (Fig. 3) was constructed for introduction into *T. saccharolyticum* for heterologous production of *n*-butanol. The genes responsible for the complete *n*-butanol pathway were harbored on a non-replicative plasmid pBu24 for integration into the chromosome of *T. saccharolyticum. AdhE* from *C. acetobutylicum* was placed under the catabolite repressed *xynA* promoter of *T. saccharolyticum*. The *xynA* promoter was previously shown to work as an inducible system in *T. saccharolyticum* (Currie et al., 2013) and hence chosen to avoid toxicity issues that could arise from overexpression of *adhE* from *C. acetobutylicum* that is similar to the constitutive *adhE* from *T. saccharolyticum*. Expression of the *crt, bcd* and *etfB* genes from *T. thermosaccharolyticum* was mediated by the glyceraldehyde-3-phosphate dehydrogenase (*gapDH*) promoter from *C. thermocellum* ATCC 27405 and expression of *etfA, hbd* and *thl* from *T. thermosaccharolyticum* was mediated by the cellobiose phosphorylase (*cbp*) promoter from *C. thermocellum* ATCC 27405 (Shaw et al., 2012).

Transformation was undertaken with four *T. saccharolyticum* strains – WT, *Δldh*, *Δpta Δack* and *Δldh Δpta Δack* – with the non-replicating plasmid pBu24. While approximately $10^3$ transformants/μg of DNA were obtained for the WT and *Δldh* strains, no transformants were obtained for the strains with a *Δpta Δack* and *Δldh Δpta Δack* genotype despite simultaneous successful transformations with a positive control plasmid pMC500.

### 3.3. Characterization of butanol production from engineered *T. saccharolyticum* strains

Five transformants of the WT and *Δldh* were verified by sequencing the *xynA* region of the chromosome, which now contained the butanol production pathway. One clone of each was chosen for further characterization, designated as I2B and M0210-V, respectively. WT, I2B, *Δldh*, and M0210-V were grown in defined media with xylose as the sole carbon source to induce expression of *adhE* (Ca) (Fig. 4).

Strain I2B produced up to 0.85 g/L butanol from 10 g/L xylose, corresponding to a molar yield of 0.17 mol butanol/mol of xylose and 21% of the maximum possible theoretical yield. Strain M0210-V produced up to 1.05 g/L butanol from 10 g/L xylose, corresponding to a molar yield of 0.21 mol butanol/mol of xylose and 26% of the maximum possible theoretical yield. On the other hand, in WT and *Δldh* strains, no butanol was detected (limit of detection 0.01 g/L). Shifts were observed in the fermentation products in strains I2B and M0210-V with butanol being produced (Table 3). Since both the butanol-producing strains possess the WT *adhE* gene that uses acetyl CoA as substrate, ethanol production is not abolished. However, the ethanol production is reduced by approximately 50% in response to butanol production. Interestingly, acetic acid production increases by 46% and 67% in strains I2B and M0210-V, respectively. The WT strain of *T. thermosaccharolyticum* DSM 571 was grown under similar growth conditions and observed to produce only 0.1 g/L *n*-butanol (data not shown), indicating an 8–10-fold increase in butanol production in the engineered *T. saccharolyticum* strain relative to the native host. Since the synthetic operon cloned into *T. saccharolyticum* was under a tightly regulated promoter the recombinant strains were able to produce butanol only when grown on xylose but were unable to produce detectable amounts of butanol when grown on glucose.

The growth characteristics of the engineered strains of *T. saccharolyticum* were distinctly different with respect to the wild

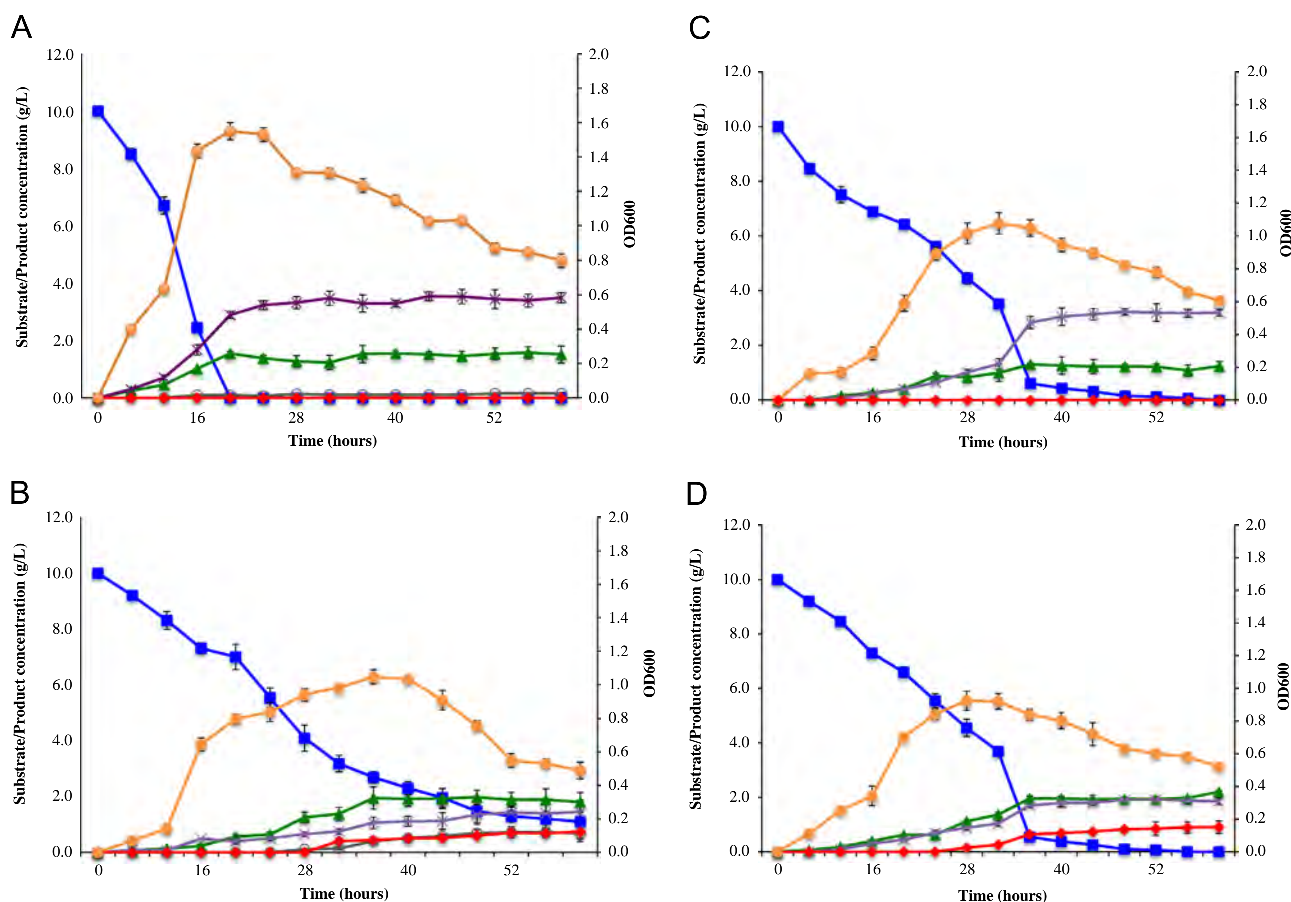


**Fig. 4.** Growth curves depicting the end product fermentations of *T. saccharolyticum* strains up to 60 h (A) WT, (B) I2B, (C) M0210, and (D) M0210-V showing residual substrate – xylose (filled blue squares), lactic acid (open gray circles), acetic acid (filled green triangles), ethanol (purple cross), butanol (filled red diamonds) and $OD_{600}$ (filled orange circle).

**Table 3**
End product profile for all strains of *T. saccharolyticum*.

| Strain | Residual xylose (mM) | Butanol (mM) | Ethanol (mM) | Acetic acid (mM) | Lactic acid (mM) | Hydrogen (mM) | Dry cell weight (mM) | Carbon recover (%) | O/R ratio |
|---|---|---|---|---|---|---|---|---|---|
| **WT** | 0.0 | 0.0 | 78.3 ± 3.11 | 27.3 ± 2.28 | 3.6 ± 0.91 | 22.9 ± 0.09 | 23.5 ± 0.08 | 104.9 | 1.09 |
| **I2B** | 0.7 ± 0.07 | 11.5 ± 0.21 | 38.0 ± 1.22 | 33.0 ± 4.15 | 13.3 ± 1.87 | 31.8 ± 1.21 | 19.6 ± 0.39 | 103.1 | 1.15 |
| **M0210** | 0.3 ± 0.04 | 0.0 | 85.7 ± 2.21 | 22.5 ± 1.90 | 0.0 | 25.2 ± 3.22 | 19.6 ± 0.51 | 103.3 | 1.05 |
| **M0210-V** | 0.3 ± 0.02 | 14.2 ± 0.53 | 38.7 ± 1.23 | 38.7 ± 3.82 | 0.0 | 38.5 ± 1.74 | 19.6 ± 0.75 | 101.2 | 1.16 |

Data represents 60 h fermentation with an initial 67 mM xylose. Fermentation data in triplicate.

type strain. The butanol producing strain I2B had a 30% lower specific growth rate of 0.15 $h^{-1}$ compared to the specific growth rate of the WT strain of 0.21 $h^{-1}$. Similarly the butanol producing strain M0210-V had a lowered specific growth rate of 0.11 $h^{-1}$ compared to its parent strain M0210 at 0.18 $h^{-1}$. The cell yield of strains M0210 (0.074 g/g xylose), I2B (0.07 g/g xylose) and M0210-V (0.058 g/g xylose) decreased 25%, 30% and 42% compared to the WT cell yield of 0.1 g/g xylose. One possibility for the lower cell yield of strain M0210 is the decreased ATP generation resulting from loss of acetate kinase activity (Shaw et al., 2008b). The substrate utilization rates of the WT, I2B, M0210 and M0210-V strains were 0.49 g/L/h, 0.21 g/L/h, 0.35 g/L/h and 0.19 g/L/h, respectively. The data suggests that the butanol producing strains of *T. saccharolyticum* were not effective at tolerating the heterologous production of *n*-butanol that was being produced. However, additional work that was performed to determine the butanol tolerance of strains of *T. saccharolyticum* suggested a tolerance level at least 4 g/L (data not shown). A reduction in optical density was observed for all the strains in stationary phase (Fig. 4), likely due to the onset of cell lysis.

## 4. Discussion

In this study, we demonstrated heterologous production of *n*-butanol in a thermophilic host *T. saccharolyticum*. The source for genes of the *n*-butanol pathway were derived from a related thermophile *T. thermosaccharolyticum* and the well characterized *n*-butanol producer *C. acetobutylicum*. While previous reports have successfully utilized the entire *n*-butanol pathway from *C. acetobutylicum* for the heterologous production of *n*-butanol, not all enzymes in the pathway are thermostable at 55 °C, so enzymes

from the thermophile *T. thermosaccharolyticum* were also evaluated. Production of *n*-butanol via a synthetic metabolic pathway was achieved in WT and $\Delta ldh$ strains of *T. saccharolyticum*, but we were unable to demonstrate this in strains deficient in acetate production. The apparent lethality of *n*-butanol production without an acetic acid pathway was surprising, but it suggests that acetate production is essential for accommodating butanol production within the native metabolism of *T. saccharolyticum*. This phenomenon has been demonstrated in several other butyrate-producing bacterial species (Hino et al., 1991; Thauer et al., 1977). Acetate production also increased in the two butanol producing strains I2B and M0210-V.

The fermentative pathway in *C. acetobutylicum* producing *n*-butanol from glucose was predicted to require two Acetyl CoA molecules, two NADH molecules and two NAD(P)H molecules for each molecule of *n*-butanol produced (Fig. 3). However, Li et al. (2008) demonstrated that the exergonic reduction of crotonyl CoA to butyryl CoA is coupled to the endergonic reduction of ferredoxin, requiring an additional NADH as electron donors. The enzymatic work performed in this study demonstrates a similar ferredoxin-coupled reaction for the catalysis of crotonyl CoA to butyryl CoA. Thus, there is a requirement of three molecules of NADH to catalyze the conversion from the carbohydrate substrate to butyryl CoA, with generation of one molecule of reduced ferredoxin (Fig. 3). An additional two molecules of NADH or NADPH are required to produce *n*-butanol, depending on the co-factor specificities of butyraldehyde dehydrogenase and *n*-butanol dehydrogenase. In contrast to the five reduced nicotinamide co-factors required for production of one molecule of *n*-butanol, only two NADH are produced from glycolysis per six carbons of sugar, along with two reduced ferredoxin molecules during oxidation of pyruvate. If reduced ferredoxin can be directly converted to NADH, this co-factor imbalance would be rectified; however, a search of the *T. saccharolyticum* genome indicated that the only known ferredoxin:nicotinamide oxidoreductase is encoded by the *nfnAB* genes for the enzyme NADH-dependent reduced ferredoxin: $NADP^+$ oxidoreductase (Wang et al., 2010), which reversibly transfers electrons from one NADH and one reduced ferredoxin to two $NADP^+$ to generate two NADPH.

One potential supply of surplus NADH is through production of acetate, in which NADH generated during glycolysis is not re-assimilated on a carbon containing end product. Stoichiometric coupling of acetate and *n*-butanol production would allow co-factor balancing, where excess NADH produced during acetate generation would be consumed by *n*-butanol generation, and excess reduced ferredoxin produced by both pathways would be converted to hydrogen. Depending on the co-factor specificities of butyraldehyde dehydrogenase and *n*-butanol dehydrogenase (and the participation of *nfnAB* in the NADPH generating direction), an acetate to *n*-butanol molecule ratio of 3:1 to 2:1 would be required to balance co-factors. The acetate to *n*-butanol ratios during fermentation of xylose with strains 12B and M0210-V were 2.9:1 and 2.7:1, respectively (Table 3).

As a first report on heterologous *n*-butanol production in a thermophile, the titers obtained are comparable to some of the initial work done with mesophilic non-butanol producing hosts engineered to produce *n*-butanol – *E. coli* (0.4–1.2 g/L) (Atsumi et al., 2008; Inui et al., 2008), *P. putida* (0.12 g/L), *B. subtilis* (0.024 g/L) (Nielsen et al., 2009), *S. cerevisiae* (0.0025 g/L) (Steen et al., 2008) and *L. brevis* (0.3 g/L) (Berezina et al., 2010). However, to achieve maximum yield and titer, it is beneficial to direct all carbon and electron flux to butanol. Future engineering efforts to increase *n*-butanol yield will require shifting metabolism from ethanol to butanol, and will likely require replacement of the native ethanol producing genes in *T. saccharolyticum* with proteins that have greater specificity for butanol production (Lehmann et al., 2012; Sillers et al., 2009). Further, if *n*-butanol production is stoichiometrically coupled to $H_2$ and acetate production, then expressing enzymes that make *n*-butanol production stoichiometrically independent should increase butanol yield. One such solution could be heterologous expression of the Rnf enzyme complex (Buckel and Thauer, 2012; Müller et al., 2008), which directly transfers electrons from reduced ferredoxin to $NAD^+$ in a process coupled to energy conservation via creation of a membrane gradient. By providing a route for electrons from reduced ferredoxin to NAD+, we may allow engineering of a butanologenic $\Delta ldh\ \Delta pta\Delta ack$ strain. Alternatively, expression of a Trans-enoyl CoA reductase (Ter) (Bond-Watts et al., 2011; Shen et al., 2011) to catalyze the reduction of crotonyl-CoA to butyryl-CoA independently of ferredoxin and flavoproteins could allow for high yield *n*-butanol production, although it would need to withstand thermophilic temperatures for functional expression in *T. saccharolyticum*.

This study has demonstrated the portability of the thermophilic *n*-butanol production system. Similar efforts could be applied in the future towards engineering cellulolytic thermophilic organisms such as *C. thermocellum* for production of *n*-butanol via consolidated biomass processing (Lynd et al., 2005; Olson et al., 2012).

## Acknowledgments

This work was supported by Mascoma Corporation and Bio-Energy Science Center. We would like to thank Dan Olson and Elizabeth Mearls for critically reviewing the paper. We would also like to thank Phil Thorne for contributing to the analytical methods.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.10.012.

## References

Atsumi, S., Cann, A.F., Connor, M.R., Shen, C.R., Smith, K.M., Brynildsen, M.P., Chou, K.J., Hanai, T., Liao, J.C., 2008. Metabolic engineering of *Escherichia coli* for 1-butanol production. Metab. Eng. 10, 305–311.

Barnard, D., Casanueva, A., Tuffin, M., Cowan, D., 2010. Extremophiles in biofuel synthesis. Environ. Technol. 31, 871–888.

Berezina, O.V., Zakharova, N.V., Brandt, A., Yarotsky, S.V., Schwarz, W.H., Zverlov, V.V., 2010. Reconstructing the clostridial *n*-butanol metabolic pathway in *Lactobacillus brevis*. Appl. Microbiol. Biotechnol. 87, 635–646.

Bond-Watts, B.B., Bellerose, R.J., Chang, M.C., 2011. Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. Nat. Chem. Biol. 7, 222–227.

Boynton, Z.L., Bennet, G.N., Rudolph, F.B., 1996. Cloning, sequencing, and expression of clustered genes encoding beta-hydroxybutyryl-coenzyme A (CoA) dehydrogenase, crotonase, and butyryl-CoA dehydrogenase from *Clostridium acetobutylicum* ATCC 824. J. Bacteriol. 178, 3015–3024.

Buckel, W., Thauer, R. K., 2012. Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. Biochim. Biophys. Acta.

Currie, D.H., Herring, C.D., Guss, A.M., Olson, D.G., Hogsett, D.A., Lynd, L.R., 2013. Functional heterologous expression of an engineered full length CipA from *Clostridium thermocellum* in *Thermoanaerobacterium saccharolyticum*. Biotechnol. Biofuels 6, 32.

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

Dong, H., Tao, W., Dai, Z., Yang, L., Gong, F., Zhang, Y., Li, Y., 2012. Biobutanol. Adv. Biochem. Eng./Biotechnol. 128, 85–100.

Dürre, P., 2008. Fermentative butanol production: bulk chemical and biofuel. Ann. N.Y. Acad. Sci. 1125, 353–362.

Dürre, P., Kuhn, A., Gottwald, M., Gottschalk, G., 1987. Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. Appl. Microbiol. Biotechnol. 26, 268–272.

Fischer, R.J., Helms, J., Durre, P., 1993. Cloning, sequencing, and molecular analysis of the sol operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175, 6959–6969.

Freierschroder, D., Wiegel, J., Gottschalk, G., 1989. Butanol formation by *Clostridium thermosaccharolyticum* at neutral Ph. Biotechnol. Lett. 11, 831–836.
Gheshlaghi, R., Scharer, J.M., Moo-Young, M., Chou, C.P., 2009. Metabolic pathways of clostridia for producing butanol. Biotechnol. Adv. 27, 764–781.
Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., Smith, H.O., 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345.
Grayson, M., 2011. Biofuels. Nature 474, S1.
Hartmanis, M.G., Gatenbeck, S., 1984. Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. Appl. Environ. Microbiol. 47, 1277–1283.
Hemme, C.L., Mouttaki, H., Lee, Y.J., Zhang, G., Goodwin, L., Lucas, S., Copeland, A., Lapidus, A., Glavina del Rio, T., Tice, H., Saunders, E., Brettin, T., Detter, J.C., Han, C.S., Pitluck, S., Land, M.L., Hauser, L.J., Kyrpides, N., Mikhailova, N., He, Z., Wu, L., Van Nostrand, J.D., Henrissat, B., He, Q., Lawson, P.A., Tanner, R.S., Lynd, L.R., Wiegel, J., Fields, M.W., Arkin, A.P., Schadt, C.W., Stevenson, B.S., McInerney, M. J., Yang, Y., Dong, H., Xing, D., Ren, N., Wang, A., Huhnke, R.L., Mielenz, J.R., Ding, S.Y., Himmel, M.E., Taghavi, S., van der Lelie, D., Rubin, E.M., Zhou, J., 2010. Sequencing of multiple clostridial genomes related to biomass conversion and biofuel production. J. Bacteriol. 192, 6494–6496.
Hill, P.W., Klapatch, T.R., Lynd, L.R., 1993. Bioenergetics and end-product regulation of *Clostridium thermosaccharolyticum* in response to nutrient limitation. Biotechnol. Bioeng. 42, 873–883.
Hino, T., Miyazaki, K., Kuroda, S., 1991. Role of extracellular acetate in the fermentation of glucose by a ruminal bacterium, *Megasphaera elsdenii*. J. Gen. Appl. Microbiol. 37, 121–129.
Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H., Toda, H., Yamamoto, S., Okino, S., Suzuki, N., Yukawa, H., 2008. Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. Appl. Microbiol. Biotechnol. 77, 1305–1316.
Jones, D.T., Woods, D.R., 1986. Acetone–butanol fermentation revisited. Microbiol. Rev. 50, 484–524.
Lehmann, D., Honicke, D., Ehrenreich, A., Schmidt, M., Weuster-Botz, D., Bahl, H., Lutke-Eversloh, T., 2012. Modifying the product pattern of *Clostridium acetobutylicum*: physiological effects of disrupting the acetate and acetone formation pathways. Appl. Microbiol. Biotechnol. 94, 743–754.
Li, F., Hinderberger, J., Seedorf, H., Zhang, J., Buckel, W., Thauer, R.K., 2008. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. J. Bacteriol. 190, 843–850.
Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J., Wyman, C.E., 2008. How biotech can transform biofuels. Nat. Biotechnol. 26, 169–172.
Lynd, L.R., van Zyl, W.H., McBride, J.E., Laser, M., 2005. Consolidated bioprocessing of cellulosic biomass: an update. Curr. Opin. Biotechnol. 16, 577–583.
Mai, V., Lorenz, W.W., Wiegel, J., 1997. Transformation of Thermoanaerobacterium sp. strain JW/SL-YS485 with plasmid pIKM1 conferring kanamycin resistance. FEMS Microbiol. Lett. 148, 163–167.
Mistry, F.R., Cooney, C.L., 1989. Production of ethanol by *Clostridium thermosaccharolyticum*: II. A quantitative model describing product distributions. Biotechnol. Bioeng., 34, pp. 1305–1320.
Moat, A.G., Foster, J.W., Spector, M.P., 2002. Wiley InterScience (Online Service), 2002. Microbial Physiology. Wiley-Liss, New York. (pp. xx, 714 p. ill. 26 cm).
Müller, V., Imkamp, F., Biegel, E., Schmidt, S., Dilling, S., 2008. Discovery of a ferredoxin:NAD+-oxidoreductase (Rnf) in *Acetobacterium woodii*: a novel potential coupling site in acetogens. Ann. N.Y. Acad. Sci. 1125, 137–146.
Nielsen, D.R., Leonard, E., Yoon, S.H., Tseng, H.C., Yuan, C., Prather, K.L., 2009. Engineering alternative butanol production platforms in heterologous bacteria. Metab. Eng. 11, 262–273.
Nölling, J., Breton, G., Omelchenko, M.V., Makarova, K.S., Zeng, Q., Gibson, R., Lee, H.M., Dubois, J., Qiu, D., Hitti, J., Wolf, Y.I., Tatusov, R.L., Sabathe, F., Doucette-Stamm, D.R., Soucaille, P., Daly, M.J., Bennett, G.N., Koonin, E.V., Smith, D.R., 2001. Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J. Bacteriol. 183, 4823–4838.
Olson, D.G., McBride, J.E., Shaw, A.J., Lynd, L.R., 2012. Recent progress in consolidated bioprocessing. Curr. Opin. Biotechnol. 23, 396–405.
Papoutsakis, E.T., 2000. Equations and calculations for fermentations of butyric acid bacteria. Reprinted from Biotechnol. Bioeng. XXVI, 174–187 (1984). Biotechnol. Bioeng. 67, 813–826.
Petersen, D.J., Bennett, G.N., 1991. Cloning of the *Clostridium acetobutylicum* ATCC 824 acetyl coenzyme A acetyltransferase (thiolase; EC 2.3.1.9) gene. Appl. Environ. Microbiol. 57, 2735–2741.
Shanks, R.M., Caiazza, N.C., Hinsa, S.M., Toutain, C.M., O'Toole, G.A., 2006. *Saccharomyces cerevisiae*-based molecular tool kit for manipulation of genes from gram-negative bacteria. Appl. Environ. Microbiol. 72, 5027–5036.
Shao, W., Deblois, S., Wiegel, J., 1995. A high-molecular-weight, cell-associated xylanase isolated from exponentially growing Thermoanaerobacterium sp. strain JW/SL-YS485. Appl. Environ. Microbiol. 61, 937–940.
Shaw, A., Covalla, S.F., Miller, B.B., Firliet, B.T., Hogsett, D.A., Herring, C.D., 2012. Urease expression in a *Thermoanaerobacterium saccharolyticum* ethanologen allows high titer ethanol production. Metab. Eng. 14, 528–532.
Shaw, A.J., Covalla, S.F., Hogsett, D.A., Herring, C.D., 2011. Marker removal system for *Thermoanaerobacterium saccharolyticum* and development of a markerless ethanologen. Appl. Environ. Microbiol. 77, 2534–2536.
Shaw, A.J., Hogsett, D.A., Lynd, L.R., 2009. Identification of the [FeFe]-hydrogenase responsible for hydrogen generation in *Thermoanaerobacterium saccharolyticum* and demonstration of increased ethanol yield via hydrogenase knockout. J. Bacteriol. 191, 6457–6464.
Shaw, A.J., Hogsett, D.A., Lynd, L.R., 2010. Natural competence in Thermoanaerobacter and Thermoanaerobacterium species. Appl. Environ. Microbiol. 76, 4713–4719.
Shaw, A.J., Jenney, F.E., Adams, M.W.W., Lynd, L.R., 2008a. End-product pathways in the xylose fermenting bacterium, *Thermoanaerobacterium saccharolyticum*. Enzyme Microb. Tech. 42, 453–458.
Shaw, A.J., Podkaminer, K.K., Desai, S.G., Bardsley, J.S., Rogers, S.R., Thorne, P.G., Hogsett, D.A., Lynd, L.R., 2008b. Metabolic engineering of a thermophilic bacterium to produce ethanol at high yield. Proc. Natl. Acad. Sci. USA 105, 13769–13774.
Shen, C.R., Lan, E.I., Dekishima, Y., Baez, A., Cho, K.M., Liao, J.C., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. Appl. Environ. Microbiol. 77, 2905–2915.
Sillers, R., Al-Hinai, M.A., Papoutsakis, E.T., 2009. Aldehyde-alcohol dehydrogenase and/or thiolase overexpression coupled with CoA transferase downregulation lead to higher alcohol titers and selectivity in *Clostridium acetobutylicum* fermentations. Biotechnol. Bioeng. 102, 38–49.
Smith, W.K., Cleveland, C.C., Reed, S.C., Miller, N.L., Running, S.W, 2012. Bioenergy potential of the United States constrained by satellite observations of existing productivity. Environ. Sci. Technol. 46, 3536–3544.
Steen, E.J., Chan, R., Prasad, N., Myers, S., Petzold, C.J., Redding, A., Ouellet, M., Keasling, J.D., 2008. Metabolic engineering of *Saccharomyces cerevisiae* for the production of *n*-butanol. Microb. Cell Fact. 7, 36.
Stim-Herndon, K.P., Petersen, D.J., Bennett, G.N., 1995. Characterization of an acetyl-CoA C-acetyltransferase (thiolase) gene from *Clostridium acetobutylicum* ATCC 824. Gene 154, 81–85.
Thauer, R.K., Jungermann, K., Decker, K., 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41, 100–180.
Wang, S., Huang, H., Moll, J., Thauer, R.K., 2010. NADP+ reduction with reduced ferredoxin and NADP+ reduction with NADH are coupled via an electron-bifurcating enzyme complex in *Clostridium kluyveri*. J. Bacteriol. 192, 5115–5123.
Zeikus, J.G., 1979. Thermophilic bacteria – ecology, physiology and technology. Enzyme Microb. Tech. 1, 243–252.