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

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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.

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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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).