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Metabolic Engineering of *Thermoanaerobacterium thermosaccharolyticum* for Increased n-Butanol Production

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ABSTRACT

Thermoanaerobacterium thermosaccharolyticum shows promise as a host for n-butanol production since it natively has the required genes involved in the n-butanol biosynthetic pathway. Overexpression of the natively occurring *bcs* operon containing the genes *thl*, *hbd*, *crt*, *bcd*, *etfA*, and *etfB* responsible for the formation of butyryl CoA increased the n-butanol production by 180% compared to the wild type from a n-butanol titer of 1.8 mM to 5.1 mM. The deletion of one of the six alcohol dehydrogenase genes confirmed that it was the primary gene responsible for ethanol and n-butanol production from acetyl CoA and butyryl CoA respectively.

Keywords: Biofuels; n-Butanol; Thermophile; Metabolic Engineering

1. Introduction

In recent years, cellulosic biofuels have gained much interest as an alternative to petroleum as transportation fuel. Besides ethanol, butanol is being considered as a potential next-generation biofuel. The four-carbon alcohol has been studied since 1920s as a product of clostridial fermentations [1]. The energy density and octane rating of n-butanol molecule is comparable to gasoline and can be blended with gasoline or used 100% as a fuel without engine modification. Additionally n-butanol is less corrosive and has a low enough vapor pressure that allows it to be transported via existing the infrastructure for petroleum [2]. There have been intensive ongoing efforts towards engineering a range of mesophilic organisms [3-6] including the native *Clostridial* n-butanol producers for increased yields and titers of n-butanol [7-11]. However, to date, engineering of thermophilic anaerobes for the production of n-butanol has not been reported. Thermophiles offer potential advantageous features compared to mesophiles for use in industrial processes [12].

T. thermosaccharolyticum (formerly called *Clostridiumthermosaccharolyticum*) was originally isolated in 1930s as spoilage from canned foods [13,14]. This microbe produces n-butanol at a yield of 0.03 mol/mol of glucose equivalent under non-pH controlled conditions besides producing lactic acid, acetic acid, butyric acid, and ethanol [15]. *T. thermosaccharolyticum*has been widely studied in the context of conversion of cellulosic biomass to ethanol. However, consistent n-butanol formation has not been reported [16-18].

The genome of *T. thermosaccharolyticum* DSM 571 became publicly available in 2010 [19]. Exploration of the genome indicated that the genes responsible for butyryl CoA production occur asa*bcs* operon comprising of the genesenoyl-CoA hydratase (*crt*) (Tthe_1661), acyl-CoA dehydrogenase (*bcd*) (Tthe_1660), electron transfer flavoprotein alpha/beta subunits(*etfA/B*) (Tthe_1658, Tthe_1659), 3-hydroxyacyl-CoA dehydrogenase (*hbd*) (Tthe_1657), and acetyl-CoA acetyltransferase (*thl*) (Tthe_1656). Deletion of competing pathways can increase the carbon flux towards n-butanol. Putative genes responsible for lactic acid production—*L-lactate dehydrogenase* (*ldh*) (Tthe_2412), and acetic acid production –*phosphate acetyltransferase* (*pta*) (Tthe_1502) and *acetate kinase* (*ack*) (Tthe_1501) have been identified as targets for deletion. There are six alcohol dehydrogenase genes identified on the genome, however only one (Tthe_2646) has sequence similarity to the conserved domains of the bi-functional aldehyde-alcohol dehydrogenase thought to be used for catabolic reduction of acetyl CoA to ethanol in *Thermoanaerobacterium sp.* and *Thermoanaerobacter sp.* [20]. The gene Tthe_2646 (*adhE*) is also assumed to be responsible for the butyryl CoA reduction to n-butanol. To confirm the functionality of this gene, it was targeted for deletion. In clostridia, the genes responsible for the formation of butyrate are *phosphotransbutyrylase* (*ptb*) and *butyrate kinase* (*bk*) [1,21-23]. However, similar genes do not appear to be

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present on the genome of *T. thermosaccharolyticum* DSM 571.

*T. thermosaccharolyticum*is naturally competent [24], utilizes hemicellulose sugars, and has temperature and pH optima compatible with cellulolytic thermophiles such as *C. thermocellum.* This study was undertaken with the objective of using metabolic engineering to increase n-butanol production.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

*T. thermosaccharolyticum*strain 571 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Germany). The bacterial strains and plasmids used are listed in **Table 1**. For transformation experiments all strains were grown in TSC1 medium [25] at 55˚C, 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 $Na_3C_6H_5O_7 \cdot 2H_2O$, 5 g of yeast extract, 0.002 g of resazurin, 0.5 g of L-cysteine-HCl; and for solid media 12 g of agarose used. The pH was adjusted to 6.7 for selection on kanamycin (200 μg/mL).

2.2. Construction of Recombinant Strains of *T. thermosaccharolyticum*

The native genes of *T. thermosaccharolyticum* were over expressed on a replicative plasmid pBu-bcs. The expression of the genes *crt*, *bcd*, *etfB*, *etfA*, *hbd* and *thl* was mediated by the glyceraldehyde-3-phosphate dehydrogenase (gapDH) promoter from *C. thermocellum*. Non-replicative plasmids pTTLAKO, pTTAAKO and pTTADHEKO were constructed in order to disrupt the lactate, acetate and alcohol production pathways respectively. All the plasmids were constructed using Gibson Assembly using *E. coli* cells [26] in which each fragment in the construction that was amplified using PCR had an overlap region of 35 bp with the adjacent fragments. The flanking upstream and downstream regions of homology contained a kanamycin resistance gene [27] that replaced the genes *ldh*; *pta* and *ack*; and *adhE.* 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. 1 mL of the cultures were added to a tube containing approximately 500 ng of plasmid DNA. The cultures were incubated for 15 - 18 hours at 55˚C and dilutions were suspended in molten TSC1 agar at pH 6.7 and allowed to solidify before incubation at 55˚C [24]. Selection of the positive clones was done by growing on 200 μg/mL kanamycin supplemented media. Five such positive clones were selected for further analysis.

Table 1. Strains and plasmids used.

Strain/Plasmid	Relevant genotype	Reference
Strains		
T. thermosaccharolyticum DSM571	Type strain	DSMZ
TT-bcs	*T. thermosaccharolyticum*, pBu-bcs	This study
Δldh	*T. thermosaccharolyticum*, *Δldh*, *kanR*	This study
ΔadhE	*ΔadhE*, *kanR*	This study
Plasmids		
pMU158	*T. thermosaccharolyticum-S. cerevisiae-E.coli* shuttle plasmid, AmpR	Mascoma
pBu-bcs	pMU158, KanR, *bcs* operon(Tt)	This study
pTTLAKO	*T. thermosaccharolyticum* suicide plasmid, *kanR*	This study
pTTAAKO	*T. thermosaccharolyticum* suicide plasmid, *kanR*	This study
pTTADHEKO	*T. thermosaccharolyticum* suicide plasmid, *kanR*	This study

2.3. Transcript Analysis

Cells were harvested in mid-log phase and stabilized with RNA protect. The RNA extraction was performed using RNeasy Kit (Qiagen, Valencia CA) along with the on-column DNAse treatment. The qPCR was conducted using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-rad, Hercules CA) . Primers were designed for targeting 150 - 200 bp internal regions of the genes of interest.

2.4. End Product Determination

The fermentation end 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).

2.5. Growth Comparisons

The growth comparisons between the strains constructed was carried out using a plate reader (BioTek, Winooski VT) placed inside an anaerobic chamber. 200 μL TSC1 media was inoculated with 10 μL of an overnight grown culture. The cultures were allowed to grow at 55˚C until each strain reached stationary phase of growth. Each measurement was carried out in triplicate.

2.6. Statistical Analysis

A one-tailed statistical test was applied to examine the significance of end product formation results from the different *T. thermosaccharolyticum* strains constructed. A critical value of significance was considered a p-value less than 0.05. The Graph Pad software was used to analyze and calculate the p-values.

3. Results

3.1. Construction of *T. thermosaccharolyticum* Recombinant Strains

Transformation of *T. thermosaccharolyticum* strain DSM 571 with the replicative plasmid pBu-bcs containing the genes in the *bcs* operon driven by *gapDH* promoter (**Figure 1**) resulted successful transformants with an efficiency of 10^3 transformants/μg plasmid DNA. Five transformants were selected for verification of plasmid DNA and n-butanol production. One strain designated as TT-bcs was selected for further characterization.

Transformation of *T. thermosaccharolyticum* strain DSM 571 with non-replicative plasmids pTTLAKO (**Figure 2**) and pTTADHEKO resulted in successful transformants with similar efficiency of 10^3 transformants/μg plasmid DNA. After verification of chromosomal integration at the desired locus, one strain each for *ldh* deletion and *adhE* deletion was selected and designnated Δ*ldh* and Δ*adhE* respectively. Multiple efforts to delete *pta* and *ack* were unsuccessful in achieving a Δ*pta*Δ*ack* strain.

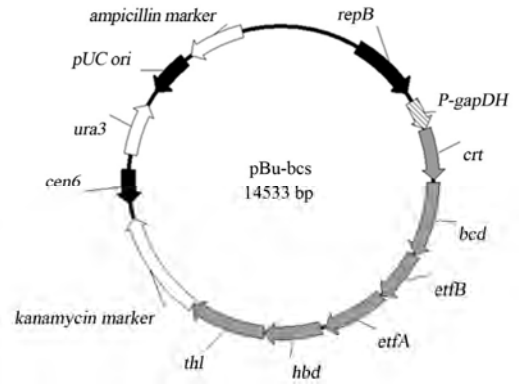

Figure 1. Construction of replicative plasmid pBu-bcs.

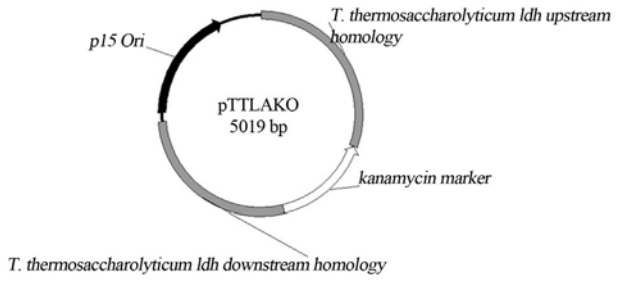

Figure 2. Non-replicative plasmid construction plasmid for lactate deletion (pTTAAKO and pTTADHEKO were constructed similarly).

3.2. Transcript Analysis

The transcription levels of each of the genes in strain TT-bcs—*thl*, *hbd*, *crt*, *bcd*, *etfA* and *etfB* were 3 - 4 times higher than the WT strain (**Figure 3**). Although *adhE* was not overexpressed in TT-bcs, the transcript levels were seen to be elevated almost 28 fold higher than the WT strain.

The quantitative reverse transcriptase PCR data confirmed that the *ldh* gene was not transcribed in strain Δ*ldh*. The transcript levels of genes *thl*, *hbd*, *crt*, *bcd*, *etfA*, *etfB* and *adhE* increased approximately 2-fold compared to the WT strain.

The qPCR data confirmed that *adhE* was not transcribed in strain Δ*adhE*. Interestingly, *ldh* transcript levels in strain Δ*adhE* were 32-fold higher than the WT strain.

3.3. End Product Analysis

n-Butanol production by strain TT-bcs increased by 180%, from 1.8 mM to 5.1 mM compared to the WT strain. Statistical analysis illustrated that this was a significant increase with a p-value of 0.003. A cetate production increased in strain TT-bcs by 20% from 24.8 mM to 30 mM compared to the WT strain (statistically significant with a p-value of 0.03). Further, butyric acid production decreased by 20% in TT-bcs compared to WT 34.8 mM to 27.8 mM (statistically significant with a p-value of 0.002) (**Table 2**).

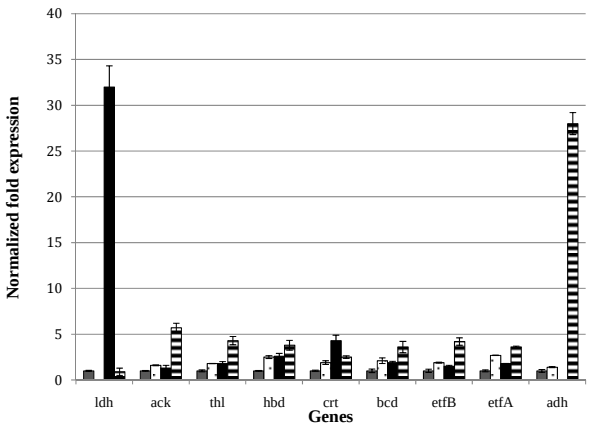

Figure 3. Normalized expression levels of genes *ldh, ack, thl, hbd, crt, bcd, etfB, etfA* and *adhE* in four strains of *T. thermosaccharolyticum* relative to 16S expression WT (solid grey), Δ*ldh* (black dots), Δ*adhE* (solid black) and TT-bcs (black stripes). Data represents an average of triplicates with error bars.

In strain Δ*ldh*, lactate production was not detected confirming the functionality of the *ldh* gene. The end product spectrum shifted to allow an increase of 8% in butanol production, although statistically this was insignificant (p-value of 0.38).

Ethanol and n-butanol production was not detected in strain Δ*adhE*. This was accompanied by a 17-fold increase in lactic acid production compared to WT strain from 1.8 mM to 31.1 mM. Moreover, no acetate or butyrate production was detected in strain Δ*adhE*.

3.4. Growth Comparisons

The growth rate of the TT-bcs strain was comparable to the WT (**Figure 4**). However, the maximum cell density reached was compromised by approximately 10%. The specific growth rate of the Δ*ldh* strain (0.23 h^{-1}) decreased by 20% compared to the wild type (0.29 h^{-1}). The Δ*adhE* strain grew very poorly, with a specific growth rate of 0.033 h^{-1} compared to 0.29 h^{-1} for the wild type strain.

4. Discussion

In order to make *T. thermosaccharolyticum* aviable host for n-butanol production, multiple efforts need to be undertaken to channel the maximum possible carbon and electron flux through the n-butanol pathway. To understand this concept more thoroughly, we studied the effect of overexpressing the *bcs* operon containing the gene products for n-butanol production, as well as targeting acetate, lactate, and alcohol pathways for deletion.

Overexpression of the genes responsible for butyryl CoA formation not only led to elevated transcription levels of *thl*, *hbd*, *crt*, *bcd*, *etfA*, and *etfB*, but also increased transcription of *adhE*. Although the over expression of the *bcs* operon genes increased n-butanol production by 180% compared to WT levels, this translated to only 9% of the maximum theoretical yield.

The phenotype of strain Δ*adhE* confirmed that the targeted gene *adhE* out of the six identified alcohol dehydrogenase genes, is responsible for both ethanol and butanol production since neither of the alcohols (ethanol or n-butanol) were detected by HPLC in the Δ*adhE* strain. Deletion of *adhE* caused increased lactate production, to roughly 1600% WT levels. The carbon flow is diverted primarily to the lactic acid pathway.

However in this study, targeted gene deletions of the primary acetate pathway involving the genes *pta* and *ack* proved ineffective, and additional effort needs to be applied in order to effectively eliminate acetate production.

Although this study has demonstrated some improvements over the WT for n-butanol production, additional work is required to realize the application of *T. thermosaccharolyticum* as a feasible host for n-butanol production in an industrial process.

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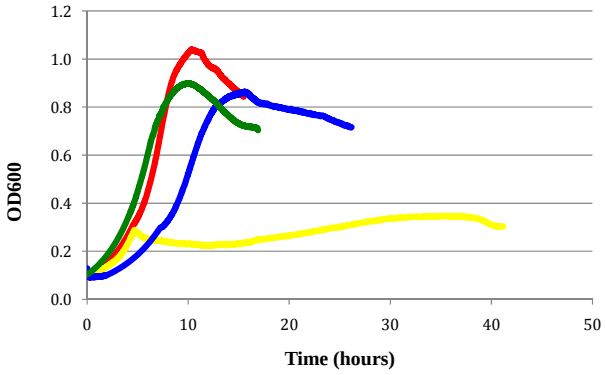

Figure 4. Growth comparison between strains of *T. thermosaccharolyticum* WT (red), TT-bcs (green), Δ*ldh* (blue), Δ*adhE* (yellow). Data represents an average of triplicate experiments performed at 55˚C in a plate reader.

Table 2. End product profiles of *T. thermosaccharolyticum* strains.

	mM					Product yield (mol/mol glucose equivalent)				
Strain	**Lactic acid**	**Acetic acid**	**Butyric acid**	**Ethanol**	**Butanol**	Y_{LA}	Y_{AA}	Y_{BA}	Y_{Et}	Y_{Bu}
WT	1.8 ± 0.2	24.8 ± 4.2	34.8 ± 1.6	18.3 ± 2.3	1.8 ± 0.4	0.03	0.43	0.60	0.31	0.03
TT-bcs	1.7 ± 0.3	30 ± 3.8	27.8 ± 3.3	18.7 ± 3.7	5.1 ± 0.5	0.03	0.51	0.48	0.32	0.09
Δ*ldh*	0	25.7 ± 2.9	30.7 ± 3.7	18.5 ± 2.2	1.9 ± 0.2	0.00	0.44	0.52	0.32	0.03
Δ*adhE*	31.1 ± 2.3	0	0	0	0	1.90	0.00	0.00	0.00	0.00

Data represents batch fermentations in triplicates with 10 g initial cellobiose/l.

to the analytical methods.

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