A Hybrid Synthetic Pathway for Butanol Production by a Hyperthermophilic Microbe

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Abstract

Biologically produced alcohols are of great current interest for renewable solvents and liquid transportation fuels. While bioethanol is now produced on a massive scale, butanol has superior fuel characteristics and additional value as a solvent and chemical feedstock. Butanol production has been demonstrated at ambient temperatures in metabolically-engineered mesophilic organisms, but the ability to engineer a microbe for *in vivo* high-temperature production of commodity chemicals has several distinct advantages. These include reduced contamination risk, facilitated removal of volatile products, and a wide temperature range to modulate and balance both the engineered pathway and the host's metabolism. We describe a synthetic metabolic pathway assembled from genes obtained from three different sources for conversion of acetyl-CoA to 1-butanol, and 1-butanol generation from glucose was demonstrated near 70°C in a microorganism that grows optimally near 100°C. The module could also be used in thermophiles capable of degrading plant biomass.

Keywords

butanol, biofuels, hyperthermophile, module, hybrid, pathway

1. Introduction

Butanol as an advanced biofuel has several advantages over ethanol. It has a greater energy density, can be used in higher blend ratios, and is more compatible with the current infrastructure for transportation (Atsumi and Liao, 2008; Peralta-Yahya and Keasling, 2010; Peralta-Yahya et al., 2012; Zhang et al., 2011). For this reason, a significant amount of research has been directed toward microbial production of butanol and so-called ‘advanced biofuels’, by metabolically-engineered mesophilic organisms (Lan and Liao, 2013). High titers (30 g/L) of 1-butanol from glucose have been achieved by a heavily engineered strain of *Escherichia coli* with *in situ* product removal (Shen et al., 2011). Butanol and isobutanol have also been generated at ambient temperature using carbon dioxide as the carbon source in the mesophilic bacteria *Clostridium ljungdahlii* (Köpke et al., 2010), *Synechococcus elongates* (Lan and Liao, 2012) and *Ralstonia eutropha* (Li et al., 2012). While platform microorganisms that thrive at extreme temperatures offer potentially critical advantages for biofuel production (Abdel-Banat et al., 2010; Basen et al., 2012; Cripps et al., 2009; Keller et al., 2013), few thermophilic organisms have been engineered for butanol production: increased butanol in *Thermoanaerobacterium thermosaccharolyticum* (formerly *Clostridium thermosaccharolyticum*) at 55°C by overexpression of the native *bcs* operon (Bhandiwad et al., 2013), *Thermoanaerobacterium saccharolyticum* at 55°C by expression of the butanol pathway from the closely related *T. thermosaccharolyticum* (Bhandiwad et al., 2014), and isobutanol production from *Geobacillus thermoglucosidasius* at 50°C by expressing and optimizing an isobutanol pathway from multiple gene donors (Lin et al., 2014).

Metabolic engineering of thermophilic microorganisms for the production of a variety of useful chemicals such as polymer feedstocks and liquid transportation fuels has traditionally

been limited by the availability of genetically tractable model organisms. Within the last decade, however, genetic capabilities have been developed for several that grow at 85°C and above (Kim et al., 2010; Lipscomb et al., 2011; Sato et al., 2003). An advantage of using a thermophilic organism for metabolic engineering is the possibility of utilizing temperature as a way to modulate and balance both the engineered pathway and the host's metabolism. This novel concept has been demonstrated in the hyperthermophile *Pyrococcus furiosus*, which has an optimal growth temperature (T_{opt}) of 100°C, by the heterologous expression of lactate dehydrogenase from *Caldicellulosiruptor bescii* (T_{opt} 78°C) using a low temperature (72°C) shift to induce lactate formation (Basen et al., 2012). *P. furiosus* has also been engineered to utilize hydrogen and incorporate carbon dioxide into the valuable molecule 3-hydroxypropionate using a similar strategy for temperature-dependent product formation (Keller et al., 2013). Herein we demonstrate metabolic engineering of *P. furiosus* for the temperature-dependent production of the liquid fuel and chemical feedstock 1-butanol.

2. Material and methods

2.1. Strain construction.

The pathway genes for BuOH-1 (*thl*, TTE0549; *hbd*, TTE0548; *crt*, TTE0544; *ter*, Stherm_c16300; *bad*, Teth514_1942; *bdh*, Teth514_1935) and the genes for BuOH-2 (*thl*; *hbd*; *crt*; *ter*; *adhE*, Teth514_0627; *adhA*, Teth514_0564) were assembled into artificial operons (see **Fig. 1b**). The *ter* gene was codon optimized for *P. furiosus* (Genewiz, Inc., South Plainfield, NJ) and the sequence is given in Supplementary Table 1. The operon was driven by the promoter of the gene encoding the gamma subunit of pyruvate oxidoreductase (175 bp immediately upstream of PF0971, including its ribosomal binding site) and terminated with 19 bases of the 3' UTR of

the *hpyA1* gene (5'- aatcttttttagcactttt, locus PF1722). *P. furiosus* ribosomal binding sites (RBSs) consisting of 12–14 bp of sequence upstream of genes encoding highly expressed proteins were placed upstream of each internal gene using primer tails as follows: the RBS for highly expressed S-layer protein (5'-ggaggtggagaaaa) was placed in front of *ter* and *hbd* and the RBS for the highly expressed PEP synthase (5'-ggaggtttgaag) was placed in front of *bdh* or *adhA*, *thl* and *crt*. Gibson Assembly (Merryman and Gibson, 2012) was used to assemble the BuOH expression constructs into the vector pGL050 containing the *pyrF* genetic marker and flanking regions to delete ACS1 α (PF1540) (Thorgeresen et al., 2014), generating pGL053 and pGL056 (for BuOH-1 and BuOH-2, respectively **Supplementary Fig. 1**). Plasmids were sequence-verified and pGL056 was found to contain a single point mutation in the RBS in front of *adhA*, which may be inconsequential. Plasmids were linearized and used to transform *P. furiosus* COM1 according to previously described methods (Lipscomb et al., 2011). Transformants were colony-purified and strains were designated as follows: MW164 (pGL053 transformant) and MW196 (pGL056 transformant). These are herein referred to as the BuOH-1 and BuOH-2 strains, respectively (**Supplementary Table 2**).

2.2. Growth of *P. furiosus*.

Strains were cultured as previously described in a sea-water based medium containing 5 g/L maltose and 5 g/L yeast extract, 0.5 μ g/L riboflavin, and 20 μ M uracil (Adams et al., 2001; Basen et al., 2012). Cultures were grown at 98°C to $\sim 1 \times 10^8$ cells/mL and then transferred to a water bath calibrated to 50 to 70°C, which was maintained for up to 72 h. For growth in a 15 L fermenter, the culture was sparged with 10% CO₂/90% N₂, stirred, and the pH was maintained at 6.8 by addition of 10% NaHCO₃. The cultures were harvested by centrifugation at 6,000 x g for 10 min. Cell extracts were prepared anaerobically by sonicating the cell pellets in 100 mM

MOPS, pH 7.5, ultracentrifugation at 100,000 x *g* for 60 minutes, washing and re-concentrating three-times with a 10 kDa centrifugation filter and storing in sealed vials at -80°C.

2.3 RNA extraction and quantitative RT-PCR analyses.

RNA was extracted from 10 mL samples of culture using the Direct-zol™ RNA MiniPrep kit (Zymo Research). Genomic DNA was digested using TURBO DNase (Ambion) and further purified by phenol:chloroform extraction and precipitation. Synthesis of cDNA was performed with 1 µg purified RNA using the Affinity Script QPCR cDNA synthesis kit (Agilent). The Brilliant III SYBR® Green QPCR Master Mix (Agilent) was used for quantitative RT-PCR experiments using an MX3000P instrument (Stratagene). Primers were designed to amplify a ~150 b product within the target gene, including PF0971 (*P. furiosus* PORγ control gene), *bad*, *bdh*, *adhE*, *adhA*, codon-optimized *ter*, *thl*, *hbd*, and *crt*.

2.4. NADH dependent enzyme assays.

All reactions were carried out in sealed anaerobic cuvettes at 60°C (unless otherwise indicated) containing 25 mM MOPS pH 7.5. Extract was added to a final concentration of 0.2 mg/mL and NADH to A₃₄₀ ~ 1.0. After addition of the relevant substrate, NADH oxidation was measured at 340 nm. The relevant substrates for the Thl+Hbd, Hbd, Ter, Bad, and Bdh assays are acetyl-CoA, acetoacetyl-CoA, crotonyl-CoA, butyryl-CoA, and butyraldehyde respectively.

2.5. *In vitro* Butanol production.

All reactions were carried out in sealed anaerobic vials at 60°C (unless otherwise indicated) containing 100 mM MOPS pH 7.5 and 5 mM MgCl₂. Extract was added to a final concentration of 2.0 mg/mL. NADH and the indicated substrates were added to a butanol theoretical yield of 1 mM. Acetyl-CoA was added to assay the entire synthetic pathway, butyryl-CoA was added to

assay Bad+Bdh, and butyraldehyde was added to assay Bdh. Samples were acidified to 8% formic acid and analyzed via gas chromatography flame ionization detection.

2.6. In vivo butanol production.

P. furiosus strains COM1, MW164, and MW196 were grown in 1 L cultures in sealed bottles at 98°C until the cell density reached 8×10^7 cells/mL. They were then incubated at 60°C for 72 h for enzyme expression. After the enzyme expression, the cultures were harvested and suspended in sea-water based salts with the addition of 100 mM MOPS (pH 7.5) and 50 g/L maltose. Cells from each 1 L culture were suspended in 5 mL so that the cell density was concentrated to ~ 200x the original culture density. The suspensions were then incubated at 60°C for product formation. Aliquots of 250 μ L were centrifuged, and the supernatants were acidified to pH 2 using formic acid and analyzed via gas chromatography flame ionization detection. Maltose consumption was estimated by the production of acetate, ethanol and butanol assuming stoichiometric ratios of 1:4, 1:4 and 1:2, respectively.

3. Results and Discussion

3.1. Design of the hybrid synthetic pathways

P. furiosus is a genetically tractable marine anaerobe that has an optimal growth temperature (T_{opt}) of 100°C and ferments carbohydrates and peptides (Fiala and Stetter, 1986; Lipscomb et al., 2011). The highest temperature at which engineered butanol formation has been reported is 55°C (Bhandiwad et al., 2013; Bhandiwad et al., 2014). To increase this temperature limit for butanol production in *P. furiosus*, genes were selected from three different organisms to assemble an artificial pathway that combines steps from a butyrate-forming fermentation pathway with aldehyde and alcohol dehydrogenases (**Fig. 1a**). The first three enzymes of the pathway convert acetyl-CoA to crotonyl-CoA and are derived from the fermentative bacterium

Thermoanaerobacter tengcongensis, (T_{opt} 75°C) (Xue et al., 2001). The reduction of crotonyl-CoA to butyryl-CoA is carried out by the enzyme trans-2-enoyl-CoA reductase (Ter). *T. tengcongensis* contains a homolog of this enzyme, but it is a complex electron-bifurcating enzyme that requires two equivalents of NADH and produces reduced ferredoxin in addition to reducing crotonyl-CoA (Li et al., 2008; Shen et al., 2011). We, therefore, chose the enzyme Ter from *Spirochaeta thermophila*, a fermentative bacterium that grows optimally at 65°C (Aksenova et al., 1992), as it requires only one equivalent of NADH as substrate and has the added benefit of being irreversible (Shen et al., 2011). For the conversion of butyryl-CoA to butanol we chose to follow two strategies. For one pathway, butyraldehyde dehydrogenase (Bad) and butanol dehydrogenase (Bdh) were selected from *Thermoanaerobacter* sp. X514 (T_{opt} 65°C) (Roh et al., 2002). These are both previously uncharacterized from this organism but together should reduce the CoA derivative to the alcohol, equivalent to the bi-functional enzyme alcohol dehydrogenase E (AdhE) (Extance et al., 2013). Bad and Bdh have homology to the two domains of AdhE, which are an aldehyde dehydrogenase and an iron containing alcohol dehydrogenase. The pathway containing Bad and Bdh is designated as the BuOH-1 pathway. For comparison, we also constructed a pathway, termed BuOH-2, containing the bi-functional AdhE along with alcohol dehydrogenase A (AdhA), both from *Thermoanaerobacter* sp. X514 (**Fig. 1a**).

The pathway genes were assembled into synthetic operons, driven by the strong constitutive promoter of the gene encoding the γ subunit of pyruvate oxidoreductase (POR) of *P. furiosus* (**Fig. 1b**). Ribosomal binding sites of highly expressed *P. furiosus* proteins were included between each gene for optimal protein production, along with a terminator sequence from *P. furiosus* at the end of the operon. A codon-optimized version of the Ter gene was used

because the GC content of *S. thermophila* (61.9%) was significantly higher than *P. furiosus* (40.8%).

3.2. Modifying native *P. furiosus* metabolism

The butanol pathway expression constructs were inserted into the *P. furiosus* chromosome to simultaneously delete the alpha subunit of acetyl-CoA synthase I (ACSI), generating the BuOH-1 and BuOH-2 strains (**Supplementary Table 2**). ACSI is one of two major enzymes responsible for conversion of acetyl-CoA to acetate (Mai and Adams, 1996); therefore, the BuOH strains are designed to redirect acetyl-CoA away from acetate production and into the butanol pathway. While the effect of this knockout on butanol production was not determined here, a knockout of ACSI α was previously shown to improve production of 3-hydroxypropionate in an engineered *P. furiosus* strain (Thorgersen et al., 2014). This is an analogous strategy as both the butanol and 3-hydroxypropionate pathways utilize acetyl-CoA as their primary carbon substrate.

3.3. Expression strategy for the fuel pathway

We performed quantitative PCR to determine expression levels of the BuOH-1 and BuOH-2 pathways at 98, 65 and 60°C (**Supplementary Figure 2**). The expression levels of the genes in the synthetic operons are similar between the two strains, and they are within an order of magnitude of the native host gene encoding the gamma subunit of the glycolytic enzyme pyruvate ferredoxin oxidoreductase, whose promoter was used to drive expression of the pathways.

Expression of active heterologous enzymes that yield a functional butanol pathway in *P. furiosus* is dependent on growth temperature as the genes are derived from organisms with lower optimal growth temperatures than the host. At 98°C they are all expected to be inactive, hence

growth of the *P. furiosus* BuOH-1 strain at this temperature results in wild type metabolism and the production of acetate (via other ACS homologs), carbon dioxide and hydrogen from sugar (the disaccharide, maltose). A shift to a lower temperature would lead to the production of active forms of the butanol pathway enzymes that together should divert acetyl-CoA to butanol. The net result is one molecule of glucose yielding two molecules of carbon dioxide and one molecule of butanol (**Supplementary Fig. 3**).

3.4. *In vitro* characterization of BuOH-1 and BuOH-2

The butanol pathways were assembled from three different gene donors with optimal growth temperatures ranging from 60-75°C. To determine if active forms of the heterologously-expressed enzymes were produced in *P. furiosus*, the BuOH-1 strain was grown to mid-log phase at 98°C, near the optimal temperature of the host, and then shifted to temperatures ranging from 50°C to 70°C. The cytosolic extracts were assayed to determine if butanol could be produced *in vitro* at 60°C, the lowest optimal growth temperature of the three gene donors. As shown in **Fig. 2a**, butanol was produced from extracts of cells shifted to all temperatures tested for BuOH-1, but activity was extremely low in BuOH-2. The highest specific activity for butanol formation was in the 60 to 65°C range. To compare C4 specificity in the BuOH-1 versus BuOH-2 strain (**Fig. 1b**), cytosolic extracts from both strains were prepared following heterologous expression at 60°C. Along with parental strain COM1, cell-extracts were prepared and were assayed at 60°C for NADH dependent activity on crotonyl-CoA (Ter), butyryl-CoA (Bad), and butyraldehyde (Bdh) (**Fig. 2b**). While the Ter activities for both recombinant strains are nearly identical, extracts of the strain BuOH-1 shows higher activity on the C-4 substrates, particularly in the final step that converts butyraldehyde to butanol. Hence, in the following we focus on *P. furiosus* strain BuOH-1 for more in depth characterization.

Specific activities were determined for individual enzymes or sets of enzymes in the BuOH-1 strain at various assay temperatures using cell extracts prepared from cells shifted to 60°C (**Fig. 3a-f**). In *P. furiosus* extracts, the Thl and Hbd enzymes from *T. tengcongensis* (**Fig. 3a, b**) exhibited maximal activity at or above 80°C, which is not surprising as this is the gene donor with the highest optimal growth temperature (75°C). Temperatures above 80°C were not assayed because of the instability of the substrate. The Ter enzyme showed maximal activity at 60°C (**Fig. 3c**), consistent with the optimal growth temperature of *S. thermophila* (60-65°C). The Bad and Bdh enzymes exhibited broader temperature ranges for activity, and background Bdh activity was observed from the *P. furiosus* parent strain at the higher assay temperatures (**Fig. 3d, e**). For the complete pathway, the *in vitro* conversion of acetyl-CoA and NADH to butanol was optimal at 60°C (**Fig. 3f**), and this is most likely limited by the optimal temperature for activity of either the Ter or Bdh enzyme.

3.5. *In vivo* demonstration of BuOH-1 and BuOH-2

To demonstrate *in vivo* butanol production in *P. furiosus* and to further compare the use of Bad and Bdh in the BuOH-1 strain versus AdhE and AdhA in the BuOH-2 strain, concentrated cell suspensions ($\geq 10^{10}$ cells/mL) of both strains, along with the parental strain COM1, were prepared in the presence of maltose and incubated at 60°C. The cell suspensions were analyzed for butanol, acetate, and ethanol at various time points up to 48 h (**Fig. 4**). In strain BuOH-1 extracellular butanol (**Fig. 4a**) production reached a maximum of ~70 mg/L after 48 h, which corresponds to 5% of theoretical yield based on the maltose that was consumed (**Fig. 4b**). The strain BuOH-2 had much lower productivity as it produced 15 mg/L butanol after 48 hrs, which corresponds to 1% of the maximal theoretical yield (**Fig. 4b**). In addition, production of ethanol and acetate were analyzed (**Fig. 4c-d**). While no difference was observed in acetate

formation, ethanol production was tripled from 500 mg/L to 1500 mg/L in the AdhE and AdhA containing strain BuOH-2. The strain BuOH-1 showed little to no increase in ethanol production compared to the parental strain COM-1. These results, along with the *in vitro* data presented in Figure 2, demonstrates that the use of the *bad* and *bdh* genes for butanol production is superior to the use of AdhE and AdhA.

4. Conclusion

We have demonstrated butanol production in a hyperthermophilic archaeon, using an artificial pathway assembled from three gene donors having moderate differences in growth temperature optima. Furthermore, the BuOH-1 pathway can utilize acetyl-CoA from the host metabolism, which is operating at close to 40°C below its optimal temperature. A fuel module such as this can be combined with a more advanced strategy in which H₂ and CO₂ can be used to supply acetyl-CoA in place of host sugar metabolism (Hawkins et al., 2011; Keller et al., 2013). The module may also be used in other genetically tractable thermophilic microbes, in particular, those that have slightly lower growth temperature optima than *P. furiosus* and may be more suited for expression of a pathway at 60°C (Atomi et al., 2004), or ones that are capable of degrading plant biomass (Basen et al., 2014). Such a strategy would couple the artificial fuel pathway to the native metabolism of the host and would have applications for consolidated bioprocessing of renewable biomass to a readily useable liquid transportation fuel.

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Author Contributions.

R.M.K. and M.W.W.A. conceived of and managed the research; A.J.L. and G.L.L. performed operon design and construction, DNA cloning, and transformation of *P. furiosus*; M.W.K. and G.J.S. performed physiological and enzymatic analyses; M.W.K., G.L.L., R.M.K. and M.W.W.A. wrote the manuscript.

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Figure Legends

Figure 1. The hybrid synthetic butanol pathway. **(a)** Diagram of the BuOH-1 and BuOH-2 pathways which use acetyl-CoA generated from the host's glycolytic pathway along with NADH to produce butanol. The pathways differ following the production of butyryl-CoA by the enzyme Ter. **(b)** Schematic diagram of the six gene synthetic operons for expressing the BuOH-1 and BuOH-2 pathways, under control of the *P. furiosus* POR γ promoter (bent arrow).

Figure 2. Comparison of the BuOH-1 and BuOH-2 pathways and optimal temperature for the expression of the BuOH-1 pathway. **(a)** The rate of butanol formation from acetyl-CoA and NADH (nmol butanol/min/mg) measured in cytosolic extracts prepared from BuOH-1 (blue) and BuOH-2 (red) grown to 1×10^8 cells/mL at 98°C and shifted to the indicated temperature and

incubated for 72 hours. **(b)** The NADH dependent activity ($\mu\text{mol}/\text{min}/\text{mg}$) on crotonyl-CoA (Ter), butyryl-CoA (Bad), and butyraldehyde (Bdh) are compared in the strains BuOH-1 (blue), BuOH-2 (red), and the parental strain COM1 (orange).

Figure 3. Optimal temperatures for activities of the BuOH-1 pathway enzymes. **(a-e)** Enzyme activities for pathway enzymes and reactions (shown in **Fig. 1a**) in the BuOH-1 strain (blue) compared to the COM1 control strain (orange), assayed at the indicated temperatures. The strains were grown at 98°C to 1×10^8 cells/mL and incubated at 60°C for 72 h in a 15L pH controlled fermenter. The individual enzyme rates (μmol NADH oxidation/min/mg) were measured by the addition of acetyl-CoA for Thl+Hbd **(a)**, acetoacetyl-CoA for Hbd **(b)**, crotonyl-CoA for Ter **(c)**, butyryl-CoA for Bad **(d)**, and butyraldehyde for Bdh **(e)**. **(f)** Specific butanol production from acetyl-CoA and NADH (nmol butanol/min/mg) for the complete pathway was measured in cytosolic extracts at the indicated temperatures.

Figure 4. *In vivo* butanol (a-b), acetate (c), and ethanol (d) production from maltose in BuOH-1 (blue), BuOH-2 (red), and COM1 (orange) strains in concentrated cell suspensions. The strains were grown at 98°C and then incubated at 60°C for 72 h after which a 200x concentrated cell suspension ($\geq 10^{10}$ cells/mL) was prepared in the presence of maltose and incubated at 60°C to assess product formation.

Figures

Figure a

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Figure 2

Figure 3

Figure 4