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# Isobutanol production at elevated temperatures in thermophilic *Geobacillus thermoglucosidasius*

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ABSTRACT

The potential advantages of biological production of chemicals or fuels from biomass at high temperatures include reduced enzyme loading for cellulose degradation, decreased chance of contamination, and lower product separation cost. In general, high temperature production of compounds that are not native to the thermophilic hosts is limited by enzyme stability and the lack of suitable expression systems. Further complications can arise when the pathway includes a volatile intermediate. Here we report the engineering of *Geobacillus thermoglucosidasius* to produce isobutanol at 50 °C. We prospected various enzymes in the isobutanol synthesis pathway and characterized their thermostabilities. We also constructed an expression system based on the lactate dehydrogenase promoter from *Geobacillus thermodenitrificans*. With the best enzyme combination and the expression system, 3.3 g/l of isobutanol was produced from glucose and 0.6 g/l of isobutanol from cellobiose in *G. thermoglucosidasius* within 48 h at 50 °C. This is the first demonstration of isobutanol production in recombinant bacteria at an elevated temperature.



## 1. Introduction

Microbial production of fuels and chemicals from plant biomass at elevated temperatures is desirable for multiple reasons. Since most cellulases have optimum temperatures of 50 to 55 °C (Liu and Xia, 2006; Lee et al., 2008; Ko et al., 2010; Balsan et al., 2012), fermentation in that temperature range can allow for simultaneous cellulose hydrolysis and fermentation and can reduce the loading of hydrolytic enzymes (Patel et al., 2005, 2006; Sun and Cheng, 2002; Brodeur et al., 2011). In addition, high temperature fermentation minimizes the chance of contamination and reduces energy consumption for product separation and fermenter cooling (Lynd, 1989). However, high temperature fuel and chemical production faces several challenges, including limited enzyme stability and availability of suitable expression systems, volatility of pathway intermediates, and increased product toxicity. To date, thermophilic ethanol production has been reported using *Geobacillus thermoglucosidasius* (Cripps et al., 2009), *Thermoanaerobacterium saccharolyticum* (Shaw et al., 2008), and *Clostridium thermocellum* (Argyros et al., 2011). In addition, *n*-butanol production by *T. saccharolyticum* has recently been demonstrated with a final titer of about 1.05 g/l from 10 g/l xylose (Bhandiwad et al., 2013; Bhandiwad et al.). Moreover, 3-hydroxypropionic acid has been produced from hydrogen gas and carbon dioxide using an engineered hyperthermophile, *Pyrococcus furiosus* (Keller et al., 2013). Here, we report the engineering of a thermophile, *G. thermoglucosidasius*, for production of isobutanol.

*G. thermoglucosidasius* is a facultative anaerobic, rod-shaped, Gram-positive and endospore-forming bacterium (Nazina et al., 2001). Species in the *Geobacillus* genus are capable of growth between 40 °C and 70 °C and can ferment hexose and pentose sugars to generate lactate, formate, acetate and ethanol as products. However, methods for genetic modification of most *Geobacillus* spp. are underdeveloped. An exception is *G. thermoglucosidasius*, for which transformation procedures enabling metabolic manipulation have been established (Cripps et al., 2009). *G. thermoglucosidasius* was therefore chosen as a platform for investigating isobutanol production at elevated temperatures.

High-flux ethanol and isobutanol biosynthesis (Atsumi et al., 2008) both utilize a keto acid decarboxylase (KDC) to decarboxylate a keto acid (pyruvate or 2-ketoisovalerate) to the corresponding aldehyde

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(acetaldehyde or isobutyraldehyde), which is then reduced to the alcohol by an alcohol dehydrogenase (ADH). The acetaldehyde intermediate in ethanol production has a boiling point of 20 °C and is highly volatile at the temperatures favored by thermophilic organisms. This may explain why pyruvate decarboxylase (PDC) has been reported only in mesophiles (Ingram et al., 1999). However, one exception is pyruvate-ferredoxin oxidoreductase from the hyperthermophile *P. furiosus,* which can produce acetaldehyde from pyruvate at 90 °C (Ma et al., 1997). If the aldehyde is not reduced to the alcohol fast enough, it may escape from the cell or exert significant toxic effects (Atsumi et al., 2009). Perhaps because of this, thermophilic ethanol production has only been demonstrated by a coenzyme A (CoA)-dependent pathway, using a bifunctional aldehyde/alcohol dehydrogenase, which minimizes the loss and toxicity of the volatile aldehyde by channeling it directly to alcohol form Cripps et al. (2009) engineered two strains of *G. thermoglucosidasius* by upregulating pyruvate dehydrogenase, which increased the carbon flux through acetyl-CoA and then to ethanol, the major fermentative product. Similarly, Shaw et al. (2008) engineered *T. saccharolyticum* to produce ethanol as the only detectable product by utilizing pyruvate-ferredoxin oxidoreductase and a putative bifunctional aldehyde/alcohol dehydrogenase.

Isobutanol biosynthesis (Atsumi et al., 2008) (Fig. 1) shares intermediates with the valine biosynthesis pathway, which exists in most microorganisms, including *G. thermoglucosidasius*. In the pathway assembled by Atsumi et al. (2008) in *Escherichia coli*, a non-native acetolactate synthase, AlsS from *Bacillus subtilis*, was overexpressed to replace the native enzyme, as it has specificity for pyruvate and is not end-product inhibited. In addition, the valine precursor 2-ketoisovalerate was decarboxylated using ketoisovalerate decarboxylase (Kivd) from *Lactococcus lactis*, which has 33% identity to *Zymomonas mobilis* pyruvate decarboxylase. The final step in the pathway is reduction of isobutyraldehyde by an ADH. Since isobutyraldehyde (bp 63 °C) is significantly less volatile than acetaldehyde, we reasoned that thermophilic isobutanol production should be feasible via this CoA-independent pathway using an appropriate KIVD and ADH.

Here, we investigated the thermostabilities and activities of the mesophilic pathway enzymes *B. subtilis* AlsS and *L. lactis* Kivd as well as enzymes we identified as potential thermophilic homologs. We cloned, purified, and assayed native alcohol dehydrogenases from *G. thermoglucosidasius*, identifying both NADH- and NADPH-dependent isobutanol dehydrogenases. Furthermore, we prospected promoters and designed a plasmid overexpression system to express multiple combinations of isobutanol pathway genes. We report the first example of thermophilic isobutanol production with an engineered *G. thermoglucosidasius* strain capable of producing isobutanol from glucose or cellobiose.

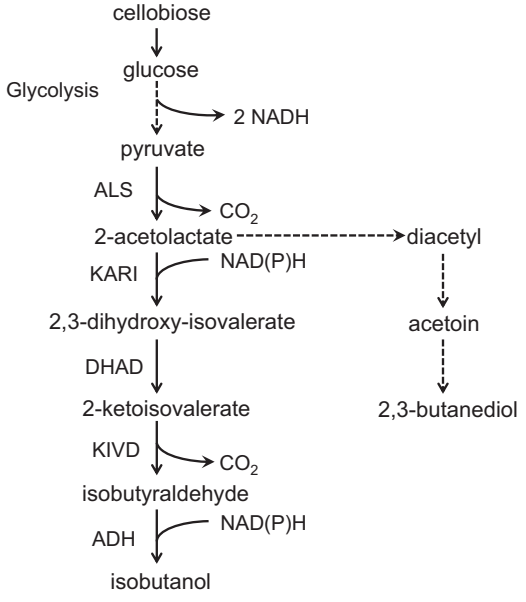


**Fig. 1.** Isobutanol pathway. Enzyme abbreviations are: ALS, acetolactate synthase; KARI, ketol-acid reductoisomerase; DHAD, dihydroxy acid dehydratase; KIVD, 2-ketoisovalerate decarboxylase; ADH, alcohol dehydrogenase.

## 2. Material and methods

### 2.1. Bacterial strains and plasmids

G. *thermoglucosidasius* DSM 2542$^T$ was used as the host for isobutanol production. *E. coli* XL1-Blue was used as the host for plasmid construction, and *E. coli* BL21 (DE3) was used for protein expression and purification. Strains and plasmids used in this study are listed in Table 1. Primers used in this study are listed in Table S1.

All plasmids were constructed by DNA assembly techniques with a modified ligation-independent cloning (LIC) protocol (Machado et al., 2012). In our modified LIC procedure, both vector and inserts (target genes) were amplified by PCR using Phusion High-Fidelity DNA polymerase. PCR products were purified by a PCR purification Kit (Zymo Research, Irvine, CA). Then, the vector and insert were mixed (1:1 mol ratio) in 10 µl 1X NEB Buffer 2 (New England Biolabs, Ipswich, MA) containing 1 unit of T4 DNA polymerase. This reaction was incubated at room temperature for 10 min and followed by the transformation procedures of Z-competent cells (Zymo Research). The presence of correctly cloned inserts was determined by colony PCR and DNA sequencing (Laragen, Culver City, CA).

### 2.2. Chemicals and reagents

All chemicals were acquired from Sigma-Aldrich (St. Louis, MO) or Thermo Scientific (Hudson, NH). Phusion High-Fidelity DNA polymerase, T4 polymerase and restriction enzymes were purchased from New England Biolabs.

### 2.3. Media and cultivation

All *E. coli* strains were grown in LB medium containing appropriate antibiotics at 37 °C on a rotary shaker (250 rpm). Antibiotics were used at the following concentrations: ampicillin, 200 µg/ml; kanamycin, 30 µg/ml; chloramphenicol, 20 µg/ml.

All *G. thermoglucosidasius* strains were grown in TGP medium (Cripps et al., 2009) at 50 °C at 250 rpm except for isobutanol production. TGP medium contains the following components: tryptone (17 g), soytone (3 g), glucose (2.5 g), NaCl (5 g), $K_2HPO_4$ (2.5 g), glycerol (4 ml) and sodium pyruvate (4 g) per liter of deionized water. 15 g of agar was added to 1 l of TGP medium prior to autoclaving for solid media. For *G. thermoglucosidasius* cultures, antibiotics were used at the following concentration: chloramphenicol, 15 µg/ml.

To examine isobutanol production, engineered *G. thermoglucosidasius* DSM 2542$^T$ was grown in modified ASYE medium (M9 medium, 0.2 M glucose or 0.1 M cellobiose, 0.5% yeast extract, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1000X dilution of Trace Metal Mix A5 (2.86 g $H_3BO_3$, 1.81 g $MnCl_2 \cdot 4H_2O$, 0.222 g $ZnSO_4 \cdot 7H_2O$, 0.39 g $Na_2MoO_4 \cdot 2H_2O$, 0.079 g $CuSO_4 \cdot 5H_2O$, 49.4 mg $Co(NO_3)_2 \cdot 6H_2O$ per liter water), 0.01 g/l thiamin, 2 mM citric acid, 100 µM $FeSO_4 \cdot 7H_2O$, 16.85 µM $NiCl_3 \cdot 6H_2O$, 12.5 µM biotin and 0.2 M HEPES buffer). Production of isobutanol was carried out in sealed 50 ml Falcon tubes with 10 ml of modified ASYE medium at pH 7.0, 50 °C with 250 rpm shaking. Cultures were inoculated by a 10% dilution

**Table 1**
List of strains and plasmids used in this study.

| Strain or plasmid | Description[a] | Reference |
|---|---|---|
| **Strains** | | |
| *E. coli* XL1-Blue | F′ *pro*AB *lac*IqZΔM15 Tn10 $Tet^r$ | Stratagene |
| *E. coli* BL21(DE3) | *E. coli B dcm ompT hsdS*($r_B^- m_B^-$) *gal* | Invitrogen |
| *G. thermoglucosidasius* | DSM $2542^T$ | *Bacillus* Genetic Stock Center |
| *G. thermodenitrificans* | DSM $465^T$/ BGSC 94A1 | *Bacillus* Genetic Stock Center |
| **Plasmid** | | |
| pDL | Source of thermostable $lacZ_{GS}$ | *Bacillus* Genetic Stock Center |
| pNW33N | ColE1 and pBC1 *ori*; $Cm^R$; *E. coli-Bacillus* shuttle vector | *Bacillus* Genetic Stock Center |
| pHT01 | ColE1 and pBC1 *ori*; $Cm^R$; $lacZ_{GS}$ without promoter | This study |
| pHT13 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$alsS_{BS}$ | This study |
| pHT71 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$ilvC_{GT}$-$alsS_{BS}$ | This study |
| pHT77 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$ilvC_{GT}$-$alsS_{BS}$-$ilvD_{GT}$ | This study |
| pHT79 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$ | This study |
| pHT112 | Derivative of pET-26b (+) with putative *adh* (Geoth_3237) | This study |
| pHT114 | Derivative of pET-26b (+) with putative *adh* (Geoth_3823) | This study |
| pHT133 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$ilvC_{AA}$-$alsS_{BS}$ | This study |
| pHT134 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$ilvC_{AF}$-$alsS_{BS}$ | This study |
| pHT194 | Derivative of pET-26b (+) with putative *alsS* (Geoth_3495) | This study |
| pHT195 | Derivative of pET-26b (+) with putative *alsS* (Gtng_0348) | This study |
| pHT196 | Derivative of pET-26b (+) with putative *alsS* (Str0923) | This study |
| pHT208 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$ilvC_{GT}$-$alsS_{BS}$-$adhA_{GT}$ | This study |
| pHT209 | ColE1 and pBC1 *ori*; $Cm^R$; $P_{ldh}$::$kivd_{LL}$-$ilvC_{GT}$-$alsS_{ST}$ | This study |
| pKSR124 | Derivative of pET-22b (+) with Gtng_0348 | This study |
| pSA65 | Source of $kivd_{LL}$ | Atsumi et al. (2008) |
| pSA69 | Source of $alsS_{BS}$ | Atsumi et al. (2008) |
| pSA159 | Derivative of pETDuet-1 with $alsS_{BS}$ | Atsumi et al. (2009) |

[a] In plasmid descriptions, subscripts indicate the source of the gene as follows: GS, *Geobacillus stearothermophilus*; LL, *Lactococcus lactis*; BS, *Bacillus subtilis*; GT, *Geobacillus thermoglucosidasius*; AA, *Alicyclobacillus acidocaldarius*; AF, *Anoxybacillus flavithermus*; ST, *Streptococcus thermophilus*;GD, *Geobacillus thermodenitrificans*.

of aerobically grown cultures (in TGP medium) which have an optical density (600 nm) between 1.0 and 1.6.

Stock cultures of *G. thermoglucosidasius* were maintained at −80 °C in 13% (v/v) glycerol.

### 2.4. G. thermoglucosidasius transformation

*G. thermoglucosidasius* transformation was conducted as described (Cripps et al., 2009). Briefly, *G. thermoglucosidasius* DSM $2542^T$ strains were grown in 50 ml of TGP medium at 50 °C and 250 rpm with a 1% inoculation from an overnight culture to exponential phase ($OD_{600}$, 1.0 to 1.6). The culture was chilled on ice for 10 min, and cells were collected by centrifugation in 50 ml Falcon tubes at 4 °C and 4000 rpm in a Beckman Coulter Allegra X-14 centrifuge for 15 min. The resulting pellets were washed twice with 25 ml of cold Milli-Q water and once with 25 ml of cold electroporation buffer (0.5 M mannitol, 0.5 M sorbitol and 10% (v/v) glycerol), with centrifugation at 4 °C and 4000 rpm for 10 min. After the supernatant was decanted, the pellets were resuspended in 2 ml of electroporation buffer, aliquoted and stored at −80 °C for future use.

For each transformation, 60 µl of the competent cells were mixed with about 200 ng of DNA. In 1-mm-gap electroporation cuvettes (Molecular BioProducts, San Diego, CA), the cells and plasmid DNA were electroporated (2.5 kV, 25 µF, and 48 Ω) with a Bio-Rad gene pulser apparatus (Bio-Rad Laboratories, Richmond, CA). The electroporated cells were transferred to 1 ml of fresh TGP medium. The cells were rescued for 2 h at 50 °C; then, the cell pellets were spread on TPG agar plates containing 15 µg/ml of chloramphenicol. The plates were incubated at 55 °C overnight to isolate single colonies.

### 2.5. Acetolactate synthase purification and thermostability assay

To purify potential acetolactate synthase (ALS) enzymes, *B. subtilis alsS* and three putative *alsS* genes (Geoth_3495 from *G. thermoglucosidasius* DSM $2542^T$, Gtng_0348 from *G. thermodenitrificans* and Str0923 from *Streptococcus thermophilus*) were cloned into pETDuet-1 and pET-26b (+) with an N-terminal polyhisidine-tag to make plasmids pSA159, pHT194, pHT195, pHT196 (Table 1, Table S1). The *E. coli* BL21 Star™ (DE3) strains transformed with these plasmids were grown to $OD_{600}$ of 0.4 to 0.6 in 50 ml LB medium at 37 °C at 250 rpm, and induced with 1 mM IPTG. Protein overexpression was performed at room temperature for 4 h. The cells were centrifuged, resuspended in 2 ml of His-binding buffer (His-Spin Protein Miniprep™ Kit, Zymo Research) and lysed at 30 Hz for 6 min by a Tissue Lyser II (Qiagen, Valencia, CA). To separate soluble and insoluble proteins, the samples were centrifuged for 20 min (15,000 rpm, 4 °C). The putative enzymes were purified using a His-Spin Protein Miniprep™ Kit. Purified protein concentrations were measured by Pierce BCA Protein Assay (Thermo Scientific).

To assess the thermostability of different ALS enzymes, purified proteins were incubated at varying temperatures (30 °C to 90 °C) for 10 min. The heat-treated ALS enzymes were assayed as described (Yang et al., 2000), with the exception that the reaction mixture contained 20 mM pyruvate, 100 mM MOPS buffer (pH=7.0), 1 mM $MgCl_2$ and 100 µM TPP. *B. subtilis* AlsS was assayed at 37 °C and ALS from *G. thermoglucosidasius*, *G. thermodenitrificans* and *S. thermophilus* were assayed at 50 °C. The concentration of acetoin produced was determined by a standard curve created using pure acetoin.

### 2.6. Kivd purification and thermostability assay

To purify *L. lactis* Kivd, two Kivd variants (CAG34226 and LLKF_1386) were cloned into pET-22b (+) (Table 1, Table S1; Invitrogen, Carlsbad, CA). These enzymes were overexpressed in *E. coli* BL21 Star™ (DE3) strains (Invitrogen). 10 ml of an overnight culture grown at 37 °C in LB containing 100 µg/ml of ampicilin were used to inoculate a 1 l culture. After 4 h of growth at 37 °C and 250 rpm, the temperature was decreased to 25 °C and protein production was

induced with 0.1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). After 16 h of induction, the cells were harvested by centrifugation at 5000*g* and 4 °C for 20 min. The cell pellets were resuspended in 20 mM $Na_3PO_4$ (pH7.5), 500 mM NaCl, 20 mM imidazole and frozen at −20 °C overnight. The cell pellets were then thawed, 700 μg/ml of lysozyme and 25 μg/ml of DNaseI were added, and the cells were incubated for 30 min at 37 °C and then lysed by sonication. The lysate was cleared by centrifugation at 25,000*g* for 30 min and filtering through a 0.2 μm filter. Since the enzymes were polyhistidine-tagged, the soluble fraction of the cell lysate was passed through a Ni-NTA column and washed with 20 mM $Na_3PO_4$ (pH=7.5), 500 mM NaCl, 20 mM imidazole. The proteins were eluted from the column using a linear gradient running up to 20 mM $Na_3PO_4$ (pH=7.5), 500 mM NaCl, 500 mM imidazole. The fractions containing protein were pooled and the buffer was exchanged to 10 mM Tris pH7.5, 10 mM NaCl, 2.5 mM $MgSO_4$ and 0.1 mM thiamine pyrophosphate (TPP). The protein concentration was determined by the Bradford assay and purity was confirmed by SDS-PAGE. The proteins were then aliquoted, frozen on dry ice and stored at −80 °C until further use.

To test enzyme thermostability, 600 ng of *L. lactis* Kivd was incubated at different temperatures with 100 μl of 10 mM Tris (pH=7.5), 10 mM NaCl, 2.5 mM $MgSO_4$, 0.1 mM TPP, 1 μM Aaci_0153 (thermostable ADH), 500 μM NADH and 10 mM 2-ketoisovalerate in a 96-well PCR plate for 20 min. The PCR plate was placed on ice and 50 μl of the reaction mixture were transferred to a multi-well plate and the reaction was stopped by addition of guanidinium hydrochloride up to an end concentration of 3.5 M to a total volume of 100 μl. The NADH consumption was determined by fluorescence (excitation 340 nm, emission 440 nm) using a Tecan Infinite M200 PRO.

### 2.7. ADH purification, characterization and thermostability assay

To purify ADH enzymes from *G. thermoglucosidasius* DSM 2542$^T$, fourteen putative *adh* genes were cloned into pET-26b (+) (Invitrogen) with C-terminal polyhistidine-tags to make plasmids pHT109 to pHT126 (Table 1, Table S1). Enzyme overexpression and protein purification procedures were as described in the acetolactate synthase purification and thermostability assay section.

Activity (isobutyraldehyde formation) was determined by monitoring the oxidation of NADH by a decrease in absorbance at 340 nm. The assay mixture contained 50 mM MOPS buffer (pH=7.0), 0.25 mM NADH and varying concentrations of isobutyraldehyde (0.05 mM to 20 mM). The 600 μl of samples were incubated at 50 °C for 10 min, the reaction was initiated by adding 2 μg to 5 μg purified enzymes, and the reaction was monitored using a Beckman Coulter DU800 spectrophotometer.

To assess the thermostability of ADH from *G. thermoglucosidasius* DSM 2542$^T$, 500 ng/ml of protein was incubated at different temperature (30 °C to 90 °C) with 100 μl of 50 mM MOPS buffer (pH=7.0) for 10 min. After heat treatment, the ADH enzyme was assayed with 20 mM isobutyraldehyde, 50 mM MOPS buffer (pH=7.0) and 0.25 mM NADH (final volume is 200 μl) at 50 °C using a BioTek PowerWave HT microplate spectrophotometer.

### 2.8. Promoter identification

To establish an efficient expression system, the thermostable *lacZ* gene from *Geobacillus stearothermophilus* was used as a reporter gene for analysis of promoter strength in *G. thermoglucosidasius*. Different promoters from *G. thermoglucosidasius* and *G. thermodenitrificans* were cloned to drive the expression of thermostable β-galactosidase. The recombinants were grown in TGP medium with antibiotic. Promoter strength was measured by β-galactosidase activity of whole *G. thermoglucosidasius* cells as reported previously (Guarente, 1983), except that the incubation temperature of the enzyme was adjusted to 50 °C.

## 3. Results

### 3.1. Prospecting thermostable ALS

The synthesis of acetolactate from two pyruvate molecules can be catalyzed by either an acetohydroxy acid synthase (AHAS) or an ALS. AHAS enzymes are used in branched-chain amino acid biosynthesis; the heterotetrameric enzyme contains a large catalytic subunit and a small regulatory subunit and is typically regulated by end products (Gollop et al., 1989; Weinstock et al., 1992). ALS is a single subunit enzyme belonging to the acetoin biosynthesis pathway. Gollop et al. (1990) reported that the catabolic *B. subtilis* AlsS is highly specific for acetolactate formation. Atsumi et al. (2008) used the *B. subtilis* AlsS instead of the native biosynthetic enzymes to improve the isobutanol titer in *E. coli*. Unlike most AHAS enzymes, ALS, is not regulated by feedback inhibition. However, ALS-like enzymes from thermophiles have not yet been reported. After searching for homologs of *B. subtilis alsS* in thermophiles, we cloned and purified three putative enzymes: Geoth_3495 from *G. thermoglucosidasius*, Gtng_0348 from *G. thermodenitrificans*, and Str0923 from *S. thermophilus*. Of these, only *S. thermophilus* Str0923 showed ALS activity, which was 3% that of the *B. subtilis* AlsS at 37 °C.

The *S. thermophilus* (*ST*) Str0923 was tested for thermostability along with *B. subtilis* AlsS. Fig. 2 shows the ALS specific activities of the two enzymes after heat treatment at different temperatures for 10 min. These two enzymes showed similar thermostabilities, but the specific activity of the *B. subtilis* AlsS was higher than the thermophilic enzyme. We therefore chose AlsS from *B. subtilis* for thermophilic isobutanol production.

### 3.2. Prospecting thermostable KIVD enzymes

The decarboxylation of 2-ketoisovalerate to isobutyraldehyde is the key enzymatic step to divert flux from the native valine biosynthesis pathway to isobutanol production. To identify a suitable KIVD, we

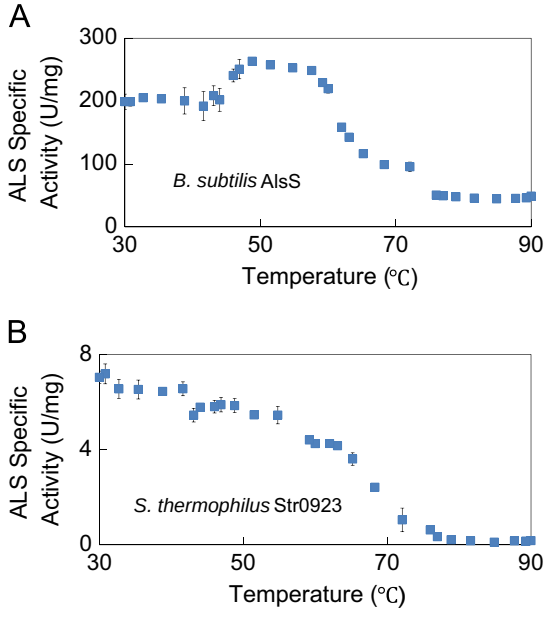


**Fig. 2.** ALS specific activity of (A) *B. subtilis* AlsS and (B) *S. thermophilus* Str0923 after a 10 min heat treatment at different temperatures. Enzyme unit, U, is defined as the amount of the enzyme that catalyzes the conversion of 1 micromole of pyruvate per minute. Error bars represent standard deviation, $n=3$.

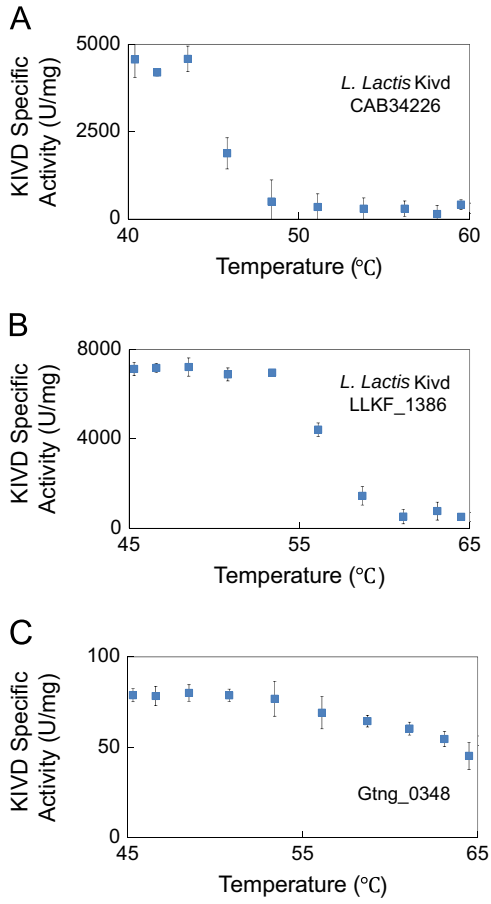


**Fig. 3.** KIVD specific activity of (A) *L. lactis* Kivd (CAB34226) (B) *L. lactis* Kivd (LLKF_1386) and (C) Gtng_0348 after a 20 min heat treatment at different temperatures. Enzyme unit, U, is defined as the amount of the enzyme that catalyzes the conversion of 1 micromole of 2-ketoisovalerate per minute. Error bars represent standard deviation, $n=3$.

first determined the thermostabilities of two *L. lactis* Kivd variants that were previously characterized for the decarboxylation of 2-ketoisovalerate to isobutyraldehyde (named according to their accession numbers, CAG34226 and LLKF_1386) (Plaza et al., 2004; Atsumi et al., 2010). Although the two proteins differ by only seven amino acids, their $T_{50}$ (the temperature at which the enzyme loses half of its activity upon a 20-min. incubation) are 45 °C for CAB34226 and 57 °C for LLKF_1386 (Fig. 3A and B). The more thermostable Kivd, LLKF_1386, has been used previously for isobutanol biosynthesis (Atsumi et al., 2008, 2009; Smith et al., 2010; Higashide et al., 2011; Li et al., 2012).

Since acetolactate synthase (encoded by *alsS*) from *B. subtilis* has been shown to also catalyze the decarboxylation of 2-ketoisovalerate to isobutyraldehyde (Atsumi et al., 2009), we also analyzed homologous genes that have been annotated *als* in different *Geobacillus* species. We excluded putative biosynthetic-type genes, based on the presence of a small regulatory subunit gene downstream, as these enzymes are usually highly regulated. The remaining enzymes (Table S2) were cloned and purified with an N-terminal (Geoth_3495, Gtng_0348 and Gtng_0651) or a C-terminal (Gtng_1810 and Gtng_1891) his-tag attached. Two purified enzymes displayed Kivd activity at 60 °C *in vitro*: *G. thermoglucosidasius* Geoth_3495 and *G. thermodenitrificans* Gtng_0348, which share 67% similarity in amino acid sequence. The corresponding enzymes exhibit respective $k_{cat}$ of 1.1 $s^{-1}$ and 0.4 $s^{-1}$. However, the $k_{cat}$ of *L. lactis* Kivd was reported by Plaza et al. (2004) as 120 $s^{-1}$, which is at least 100 fold higher. We also assayed the KIVD specific activity following a 20 min heat-treatment for Gtng_0348 (Fig. 3C). The KIVD specific activity of LLKF_1386 is 87-fold higher than Gtng_0348 when pre-incubated at 50 °C (Fig. 3B and C). Thus, we chose the *L. lactis* Kivd, LLKF_1386, for thermophilic isobutanol production.

### 3.3. Identification of an isobutanol dehydrogenase from *G. thermoglucosidasius*

The last step in the isobutanol production pathway is the conversion of isobutyraldehyde to isobutanol catalyzed by an

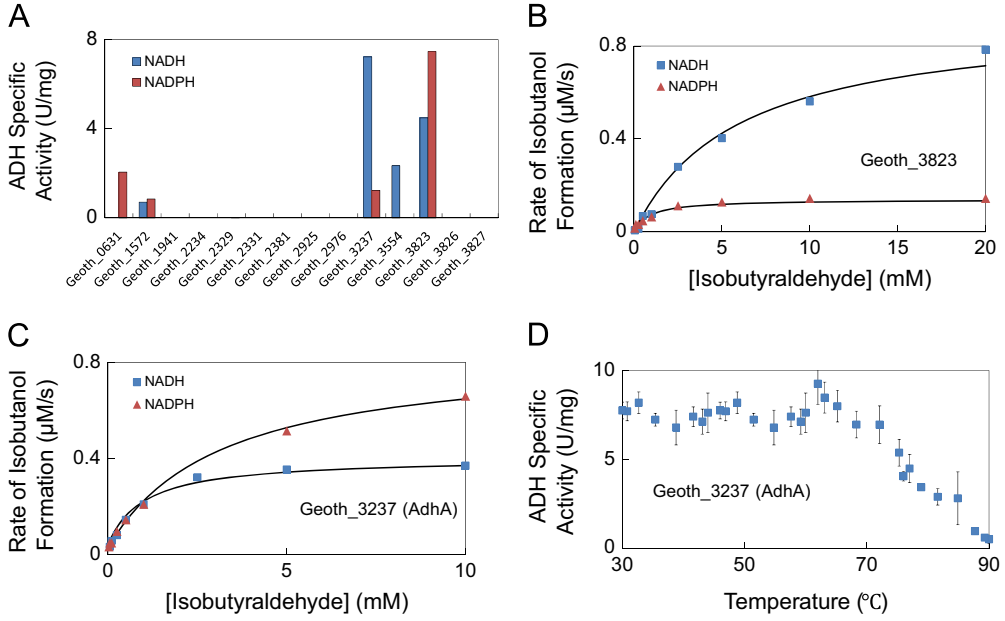


**Fig. 4.** (A) ADH specific activity for isobutyraldehyde reduction of putative alcohol dehydrogenases from *G. thermoglucosidasius*. Blue bars represent the enzyme activity using NADH as the cofactor. Red bars represent the enzyme activity using NADPH as the cofactor. Enzyme unit, U, is defined as the amount of the enzyme that catalyzes the conversion of 1 micromole of isobutyraldehyde per minute. Michaelis–Menten plots of (B) *G. thermoglucosidasius* AdhA (Geoth_3237) and (C) *G. thermoglucosidasius* Geoth_3823 using NADH or NADPH as cofactor at room temperature. (D) ADH specific activity of *G. thermoglucosidasius* AdhA after a 10 min treatment at different temperatures. Error bars represent standard deviation, $n=3$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

isobutanol dehydrogenase. Isobutanol was detected in the culture medium of wild-type *G. thermoglucosidasius* when it was supplemented with isobutyraldehyde (results not shown), indicating that an isobutanol-producing ADH exists in this organism. Since the genome sequence of this strain, *G. thermoglucosidasius* DSM 2542$^T$, is not available, we used the genome sequence of a related strain, *G. thermoglucosidasius* C56-YS93, to search for potential isobutanol dehydrogenases. Seventeen putative *adh* genes are annotated in *G. thermoglucosidasius* C56-YS93, but none had been previously characterized for isobutanol dehydrogenase activity. Among the 17 putative enzymes, one (Geoth_3897) is a potential bifunctional aldehyde/alcohol dehydrogenase, and was excluded. Two *adh* genes (Geoth_1917 and Geoth_3108) could not be PCR-amplified from the *G. thermoglucosidasius* DSM 2542$^T$ genomic DNA. The remaining 14 enzymes were cloned and overexpressed in *E. coli* with a His-tag and purified.

Among these ADH enzymes, Geoth_3237, Geoth_3554 and Geoth_3823 showed significant NADH-dependent activity for converting isobutyraldehyde to isobutanol (Fig. 4A). In addition, Geoth_0611 and Geoth_3823 showed significant NADPH-dependent isobutanol dehydrogenase activity (Fig. 4A). We rename Geoth_3237, *adhA*. This enzyme showed weak activity on isobutyraldehyde with NADPH as a cofactor. The preference for NADH was consistent with the presence of the Gly-X-Gly-X-X-Gly (where X is any amino acid) sequence that is the highly conserved in many NADH-binding domains (Scrutton et al., 1990). Using purified enzyme, we determined the kinetic parameters of *G. thermoglucosidasius* AdhA: $K_M$=5.9 mM, $k_{cat}$=9.5 $s^{-1}$ with NADH; $K_M$=0.87 mM, $k_{cat}$=0.81 $s^{-1}$ with NADPH (Fig. 4B) at room temperature, comparable to the *L. lactis* AdhA ($K_M$=9.1 mM, $k_{cat}$=6.6 $s^{-1}$) (Atsumi et al., 2010). We also characterized Geoth_3823. Fig. 4C shows the kinetic parameters: $K_M$=0.89 mM, $k_{cat}$=7.0 $s^{-1}$ with NADH; $K_M$=3.0 mM, $k_{cat}$=12 $s^{-1}$ with NADPH.

To test the thermostability of AdhA from *G. thermoglucosidasius*, purified enzyme was pre-incubated at different temperatures and assayed at 50 °C as described in Materials and Methods. Fig. 4D shows that this enzyme has a $T_{50}$ of about 72 °C.

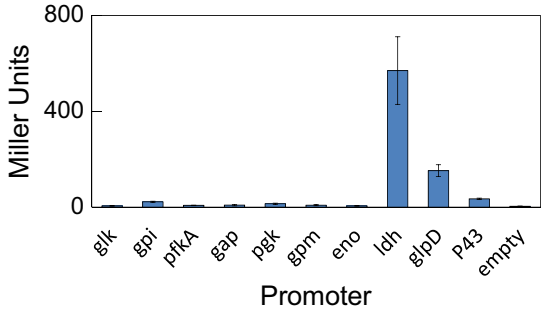


**Fig. 5.** Promoter characterization. Promoters were PCR-amplified and used to drive the expression of the thermostable *G. stearothermophilus lacZ* gene. *G. thermoglucosidasius* strains harboring these plasmids were assayed for promoter strength with a β-galactosidase assay at 50 °C. Promoter abbreviations are: *glk*, glucose kinase; *gpi*, glucose phosphate isomerase; *pfkA*, phosphofructokinase; *gap*, glyceraldehyde phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *gpm*, phosphoglycerate mutase; *eno*, enolase; *ldh*, lactate dehydrogenase; *glpD*, glycerol-3-phosphate dehydrogenase. Error bars represent standard deviation, $n$=3.

### 3.4. Promoter prospecting

Having identified thermostable enzymes for the isobutanol pathway, we needed a strong promoter to express the genes in *G. thermoglucosidasius*. We employed a thermostable *lacZ* gene isolated from *G. stearothermophilus* as a reporter for testing different promoters. Several promoters, including most of the native glycolytic promoters (*glk, pgi pfkA, gap, pgk, gpm, eno*), were selected to drive this *lacZ* gene. We also included *ldh* (Gtng_0487) and *glpD* (Gtng_2098) promoters from *G. thermodenitrificans* and the P43 promoter from *B. subtilis*, which is a constitutive promoter. The DNA fragments (200–300 b.p.) upstream of the start codon of the corresponding gene were PCR-amplified from genomic DNA and assembled in plasmids to express the thermostable *lacZ* gene. Fig. 5 shows that the *ldh* promoter from *G. thermodenitrificans* was the strongest among all tested. Thus, we selected the *ldh* promoter to drive the expression of pathway genes for isobutanol production in *G. thermoglucosidasius*.

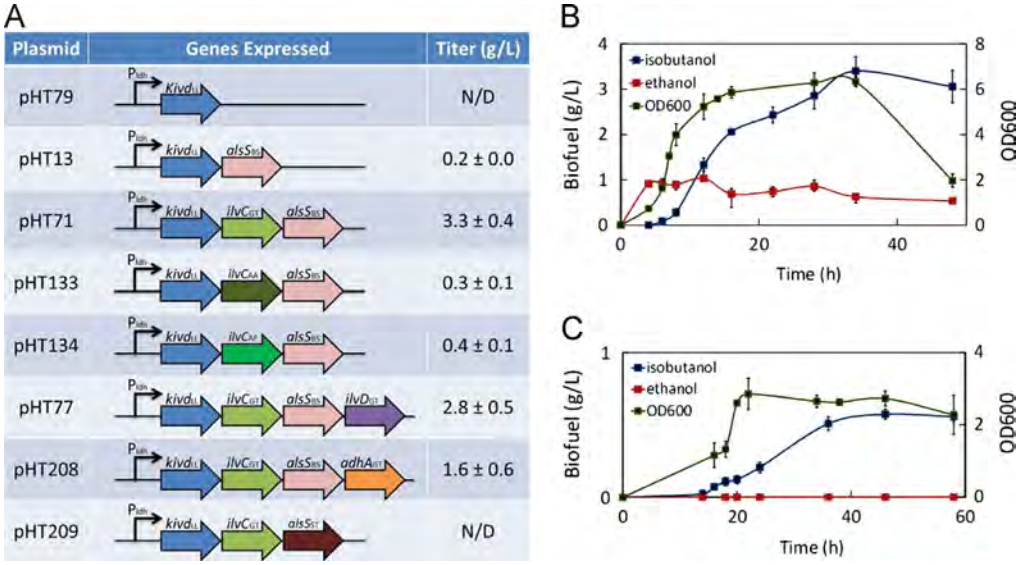


**Fig. 6.** (A) Plasmid constructs and isobutanol production titers at 50 °C from glucose over two days; In gene symbols, subscripts indicate the source of the gene: GS, *Geobacillus stearothermophilus*; LL, *Lactococcus lactis*; BS, *Bacillus subtilis*; GT, *Geobacillus thermoglucosidasius*; AA, *Alicyclobacillus acidocaldarius*; AF, *Anoxybacillus flavithermus;* ST, *Streptococcus thermophiles*; GD, *Geobacillus thermodenitrificans*. Biofuel production and optical density of pHT71 in *G. thermoglucosidasius* from (B) glucose (C) cellobiose. Error bars represent standard deviation, $n$=3.

### 3.5. Isobutanol production

With appropriate thermostable enzymes identified and the expression system established, we proceeded to establish isobutanol production in *G. thermoglucosidasius*. We first cloned *kivd* from *L. lactis* under the control of the *ldh* promoter from *G. thermodenitrificans* into the pNW33N (Table 1) backbone, resulting in plasmid pHT79 (Fig. 6A). Isobutanol was not detected when only Kivd was overexpressed *in G. thermoglucosidasius* using ASYE media. However, when 2-ketoisovalerate was added to the medium, isobutanol was detected, indicating that Kivd was expressed and functional. This result suggested that the native metabolic flux from pyruvate to 2-ketoisovalerate was insufficient to produce isobutanol in *G. thermoglcosidasius* at 50 °C.

When *B. subtilis alsS* was overexpressed along with *kivd* (Fig. 6A, pHT13), 0.2 g/l of isobutanol was produced in two days at 50 °C from glucose. However, 0.4 g/l of 2,3-butanediol was also produced. 2,3-butanediol is a reduced form of acetoin (Fig. 1), which could be produced from acetolactate through spontaneous cleavage at elevated temperature (Xiao et al., 2012). This result indicates that the flux from acetolactate to 2,3-dihydroxy-isovalerate is insufficient, and suggests that overexpressing KARI may help isobutanol production. We thus cloned and overexpressed *kivd* and *alsS* with each of the three thermophilic *ilvC* genes: Geoth_0987 from *G. thermoglucosidasius* (Fig. 6A, pHT71), Aaci_2227 from *A. acidocaldarius* (Fig. 6A, pHT133) and Aflv_0593 from *A. flavithermus* (Fig. 6A, pHT134). Among these, the strain with *G. thermoglucosidasius ilvC* overexpressed (pHT71) achieved the highest isobutanol production level: 3.3 g/l of isobutanol in two days at 50 °C (Fig. 6A and B).

We also overexpressed *G. thermoglucosidasius ilvD* in the same operon downstream of *kivd*, *ilvC* and *alsS* (Fig. 6A, pHT77). Interestingly, overexpression of *ilvD* in addition to the *kivd*, *ilvC* and *alsS* had no significant effect on isobutanol production. It is possible that *ilvD* expression reduced expression of the other three enzymes due to limited transcription/translation machinery.

To test whether increasing intracellular isobutanol dehydrogenase concentration could improve the isobutanol titer, *G. thermoglucosidasius adhA* was overexpressed with *kivd*, *ilvC*, *alsS* (Fig. 6A, pHT208). Surprisingly, overexpression of AdhA reduced isobutanol production (Fig. 6A). This result indicates that chromosomal AdhA expression was sufficient to support isobutanol production. We hypothesize that the transcription/translation machinery may be saturated with the strong *ldh* promoter from *G. thermodenitrificans*.

To test the utility of other thermostable enzymes for isobutanol production, we also tested the alternative ALS, *ST* Str0923, which was substituted for *B. subtilis* AlsS (Fig. 6A, pHT209). Unfortunately, no isobutanol was detected when pHT209 was used, indicating that the *ST* Str0923 activity was not enough to support isobutanol production from glucose. This is consistent with the *in vitro* enzyme assay that showed the *ST* Str0923 was significantly less active than *B. subtilis* AlsS (Fig. 2).

Using the best production strain (pHT71), we also tested isobutanol production from the glucose disaccharide cellobiose, which is a primary product of cellulose hydrolysis. However, only a small amount of cellobiose is consumed by *G. thermglucosidasius* in the modified ASYE medium with 0.5% yeast extract, which led us to remove yeast extract from the medium. Ultimately, 0.6 g/l of isobutanol was produced from cellobiose (without yeast extract) (Fig. 6C) at 50 °C.

## 4. Discussion

In this work, we have demonstrated production of isobutanol in a thermophilic organism and established *G. thermoglucosidasius* as a platform for biofuel production. Although this organism has an established transformation protocol and an integration vector system for gene replacements (Cripps et al., 2009), no expression system has been established. Here, we identified a robust promoter to drive gene expression from a plasmid, and we characterized enzymes for isobutanol biosynthesis at elevated temperature. With the best combination of enzymes (*L. lactis* Kivd, *G. thermoglucosidasius* KARI and *B. subtilis* AlsS) and our expression system, we achieved titers of 3.3 g/l isobutanol from glucose and 0.6 g/l isobutanol from cellobiose at 50 °C. The success of thermophilic isobutanol production at 50 °C demonstrated that isobutyraldehyde volatility is not a major issue at this temperature, although we could not rule out this issue at 55 °C or higher temperatures.

Previously, Atsumi et al. (2008) showed that *E. coli* can be metabolically engineered to produce isobutanol by diverting the keto acid intermediate in the valine biosynthesis pathway toward isobutanol production. Two important foreign enzymes, *L. lactis* Kivd and *B. subtilis* AlsS, were used to establish this heterologous pathway. The same key enzymes have been used for isobutanol production in other mesophiles (Atsumi et al., 2009; Smith et al., 2010; Higashide et al., 2011; Li et al., 2012).

To produce isobutanol in a thermophile, we identified Gtng_0348 from *G. thermodenitrificans* for high temperature Kivd activity. We also identified several ALS enzymes from thermophiles. Interestingly, the mesophilic *L. lactis* Kivd and *B. subtilis* AlsS were found to be the most active enzymes at 50 °C.

The system also produced isobutanol at 55 °C, but inconsistently and at lower titers, possibly due to the instability of Kivd, which has a $T_{50}$ value of about 56.7 °C (Fig. 3B). A highly thermostable Kivd has not been reported. Also, because Kivd-homologs have not been found in thermophiles, either new enzyme identification or protein engineering may be required for production above 55 °C. Although Gtng_0348 showed Kivd activity *in vitro*, the activity is about 100 times lower than *L. lactis* Kivd.

One of the key features of high titer fuel production is growth-independent production (Atsumi et al., 2008). However, the *G. thermoglucosidasius* cells enter the death phase (lysis) under micro-aerobic conditions after 34 h (i.e., the $OD_{600}$ dropped significantly due to cell lysis and sometimes aggregation). Therefore, the isobutanol production stopped at 3.3 g/l in this system. Tang et al. (2009) also reported a similar phenomenon. Increasing production titers may be achieved by extending the stationary phase of the *G. thermoglucosidasius* cells.

With our established thermophilic isobutanol production strain, we have demonstrated the feasibility of producing higher-density liquid fuels at a high temperature, essential for producing next-generation cellulosic biofuels using simultaneous saccharification and fermentation.

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## 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.2014.03.006.

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