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Pathway Development and Enzyme Characterization for Microbial Production of Isobutanol and n-Butanol at Elevated Temperatures

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A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Chemical Engineering

By

Jennifer Lynn Takasumi

2015

ABSTRACT OF THE THESIS

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by

Jennifer Lynn Takasumi

Master of Science in Chemical Engineering

University of California, Los Angeles, 2015

Professor James C. Liao, Chair

Long-term energy stability and environmental concerns have driven development toward the production of transportation fuels from renewable feedstocks such as sugars or lignocellulosic biomass. *n*-Butanol and isobutanol have emerged as prominent advanced transportation biofuels because of their favorable fuel properties, compatibility with current infrastructure, and ability to serve as chemical feedstocks. Cellulolytic *Clostridium* species are among the most promising organisms to serve as hosts for consolidated bioprocessing (CBP) of cellulolytic butanol production. Here, the benefits of cellulolytic *Clostridia*, pathways for microbial *n*-butanol and isobutanol production, and strategies for achieving and improving cellulosic C-4 alcohol production will be described. In addition, a strategy for improving isobutanol production in *Clostridium thermocellum* was investigated. Although isobtuanol production has been realized in

the thermophile *C. thermocellum*, 2-ketoisovalerate (KIV) decarboxylase, or Kivd, is a limiting enzyme due to its poor thermostability. There are two promising strategies for improving thermophilic KIVD activity: 1) improving the thermostability of *Lactococcus lactis* Kivd or 2) improving the KIVD activity of a thermostable decarboxylase. Here, the latter strategy was investigated by identifying and characterizing the KIVD activity of enzymes similar to *L. lactis* Kivd from thermophilic hosts. Ultimately, the specific activities of the native thermophilic enzymes were found to be substantially lower than *L. lactis* Kivd and are not recommended for use in thermophilic isobutanol production. However, this work characterizes the KIVD activity for thermophilic decarboxylases have not been previously characterized.

The thesis of Jennifer Lynn Takasumi is approved.

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Table of Contents

Figures and Tables

Introduction

Long-term energy stability and environmental concerns have driven development toward the production of transportation fuels from renewable feedstocks such as sugars or lignocellulosic biomass. *n*-Butanol and isobutanol have emerged as prominent advanced transportation biofuels because of their favorable fuel properties, compatibility with current infrastructure, and ability to serve as chemical feedstocks. Microbial productions of such compounds from sugars have been demonstrated with reasonably encouraging yields and productivities, but high titer and yield production from cellulosic materials will be the ultimate goal. To this end, cellulolytic *Clostridium* species are among the most promising organisms to serve as hosts, as their native cellulose-degrading machinery combined with engineered biofuel pathways provides an opportunity for consolidated bioprocessing (CBP). Here, the benefits of cellulolytic *Clostridia*, pathways for microbial *n*-butanol and isobutanol production, and potential strategies for achieving and improving cellulosic C-4 alcohol production will be described.

Although thermophilic isobutanol production has been realized in the thermophile *Clostridium thermocellum*, 2-ketoisovalerate (KIV) decarboxylase, or Kivd, is a limiting enzyme due to its poor thermostability. There are two promising strategies for improving thermophilic KIVD activity: 1) improving the thermostability of *Lactococcus lactis* Kivd or 2) improving the KIVD activity of a thermostable decarboxylase. Here, the latter strategy was investigated by identifying and characterizing the KIVD activity of enzymes similar to *L. lactis* Kivd from thermophilic hosts. Ultimately, the absolute specific activities of the native thermophilic enzymes were found to be substantially lower than *L. lactis* Kivd and are not recommended for

use in thermophilic isobutanol production. However, this work characterizes the KIVD activity for thermophilic decarboxylases have not been previously characterized.

1. Current status and outlook of butanol production from cellulolytic strains of *Clostridia*

1.1 Motivation for butanol production by cellulolytic *Clostridia*

Lignocellulosic biomass is widely accepted as a desirable feedstock for biofuel production because it is an abundant, renewable, non-food carbon source that is an order of magnitude less expensive than simple sugars and starches[1]. However, industrialization of lignocellulose processing has been troubled by plant cell-wall recalcitrance, which necessitates expensive thermochemical pretreatment and the production of cellulolytic enzymes to release fermentable sugars[2]. Consolidated bioprocessing (CBP) offers as an economical alternative to current multistep processing, where the capacity for feedstock hydrolysis and fuel production are contained within a single microorganism. Cellulolytic *Clostridium* species are among the most promising organisms to serve as CBP hosts, as they possess robust lignocellulose-degrading machinery. Recent efforts have focused on overproduction of ethanol and molecular hydrogen from cellulolytic hosts, such as *Clostridium thermocellum*[3]. Furthermore, microbial productions of non-native products like *n*-butanol and isobutanol by CBP are of interest[4]. These C4 alcohols have emerged as prominent advanced biofuels because of their favorable fuel properties, compatibility with current infrastructure, and ability to serve as chemical feedstocks. Microbial productions of such compounds from sugars have been demonstrated with reasonably encouraging yields and productivities[5]. However, direct production of these compounds from cellulosic feedstocks is still in early development stages, despite the demonstration of feasibility

and bench-scale production[6]. Ultimately, production of butanol from cellulosic materials will be the goal.

1.2 Cellulolytic *Clostridia* and the cellulosome

Members of the genus *Clostridium* are strictly anaerobic, spore-forming bacteria. Cellulolytic *Clostridia*, in particular, are of interest for direct microbial conversion of biomass to liquid fuels due to their native, robust cellulolytic machinery--cellulosomes. Cellulosomes are lignocellulose-degrading multi-enzymatic complexes that have been found in anaerobic microorganisms, such as *Clostridia* and *Ruminococci*. They are composed of scaffoldins, cohesins, dockerins, carbohydrate binding modules (CBM) and catalytic, sugar-degrading subunits[7]. The flexible backbone of a cellulosome is formed by scaffoldin subunits, which are cohesin-containing moieties. Cohesins form highly specific, calcium-dependent bonds with dockerin domains of other subunits, such as catalytic domains and CBMs[7-8]. Catalytic domains can include cellulases, hemicellulases and other polysaccharide-degrading enzymes and vary between species. Finally, a carbohydrate binding module (CBM) allows the attachment of the cellulosome to the biomass substrate it is degrading.

These structural and catalytic features of a cellulosome provide many benefits for efficient biomass degradation. First, cellulosomes are typically bound to both the cell surface and the substrate which provides close proximity between cells and released cellodextrins, thus minimizing losses due to diffusion[8]. In addition, catalytic components of the cellulosome are thought to redistribute under different conditions, providing an adaptive structure[7, 9]. This concept of synergism among diverse hydrolytic enzymes has demonstrated improved efficiency of substrate utilization[8b, 10]. Furthermore, enzyme-microbe synergy has been observed, where cellulosome-cell attachment was found to improve cellulose degradation in *C. thermocellum* as

well as a synthetic minicellulosome displayed in *Bacillus subtilis*[11]. Because the biomass degradation capacity of cellulosomes is natively robust, cellulolytic *Clostridia* provide a promising platform for direct microbial conversion of biomass to fuels. Below, the cellulolytic and metabolic features of CBP candidates will be discussed, as well as the status of genetic techniques and examples of metabolic engineering.

1.2.1 *Clostridium thermocellum*

C. thermocellum is one of the most investigated cellulosome-expressing bacteria and is the model thermophilic cellulolytic *Clostridium*, growing optimally around 60 °C[3a]. It has one of the fastest growth rates on cellulose [8b] and its cellulosomes are more complex (based on cellulosome size) than mesophilic *Clostridia* such as *C. cellulolyticum* [12]. Furthermore, the temperature for optimal cellulosomal activity corresponds to the host's growth temperature and the cellulosome has demonstrated resistance to inhibitors and fermentative products[13]. Its thermophilic nature minimizes the chance of contamination and facilitates product recovery[3a]. *C. thermocellum* produces hydrogen, ethanol, and acetate as major fermentative products, and the effects of end product accumulation on metabolism have been investigated[14]. Metabolic and cellulolytic features have been examined by microarray analysis to help elucidate some novel features of this organism[15]. For example, *C. thermocellum* uses an atypical pathway for synthesizing the central metabolite pyruvate. Instead of pyruvate kinase, which produces pyruvate and ATP from phosphoenolpyruvate, it uses the malate shunt, aka. transhydrogenase-malate pathway, which directs flux to oxaloacetate and malate, and is dependent on different cofactors[16].

In addition to studies on *C. thermocellum* metabolism, development of genetic techniques and examples of metabolic engineering provide a background for introducing and optimizing

butanol production pathways. Tyurin et al. demonstrated uptake of plasmid DNA by electrotransformation and Guss et al. improved the transformation efficiency to strain DSM 1313 by preparing plasmid DNA with a dam+ dcm- *Escherichia coli* strain[17]. Gene deletions and heterologous expression via chromosomal insertion in *C. thermocellum* have also been achieved[16b, 18]. Efforts in metabolic engineering have focused on improving bioethanol production. For example, Deng et al. overexpressed pyruvate kinase from *Thermoanaerobacterium saccharolyticum*, which, combined with a lactate dehydrogenase deletion, improved ethanol production by 3.25 fold[16b]. Argyros et al. was also successful in redirecting carbon flux from biomass by deleting genes from competing fermentative pathways: lactate dehydrogenase and phosphotransacetylase. Evolving the strain over 2000 hours resulted in improving ethanol titers and selectivity[19]. Genetic engineering tools have significantly aided progress toward *C. thermocellum* as a CBP host, but additional technologies will be necessary to achieve goals of industrialization. Since *C. thermocellum* is not a native butanol-overproducer, expression of heterologous genes will be necessary. Additional development of a dependable multi-gene expression system remains a major challenge moving forward, as well as strategies for achieving high titers and yields in this organism.

1.2.2 *Clostridium cellulolyticum*

C. cellulolyticum is a model mesophilic cellulolytic *Clostridium*, with a growth temperature of 34 °C. In addition to its ability to degrade cellulose, the cellulosomes of *C. cellulolyticum* also contain components for degrading hemicelluloses and pectin[12]. Techniques for DNA transfer and gene deletions have been established[20] and have been utilized for biofuel production in this organism. Interestingly, it secretes the central metabolite, pyruvate, in nutrient-rich conditions, suggesting an imbalance carbon flow at this node[21]. Guedon et al.

overexpressed two genes from the ethanol-producer, *Zymomonas mobilis*, pyruvate decarboxylase and alcohol dehydrogenase, to metabolize accumulated pyruvate. This improved both cellulose consumption and ethanol production[22]. Alternatively, Li et al. deleted lactate dehydrogenase and malate dehydrogenase of competing pathways to improve the ethanol production 8.5 fold from crystalline cellulose[20b]. Higashide et al. reported the first instance of cellulose to isobutanol by a CBP organism. A recombinant strain of *C. cellulolyticum* containing five heterologous genes produced 660mg/L isobutanol from crystalline cellulose in 7 to 9 days[6a]. This demonstrative work is encouraging for continuing research on CBP of cellulose to non-native products.

1.2.3 Other cellulolytic hosts

In addition to *C. thermocellum* and *C. cellulolyticum*, other organisms merit consideration as native cellulolytic CBP hosts. For example, *C. cellulovorans* is a mesophilic, cellulosome-expressing *Clostridium* that can degrade a broad range of substrates such as cellulose, xylan and pectin. It also ferments acetate and butyrate, in addition to ethanol, lactate, hydrogen, formate and CO_2[23]. *C. phytofermentans* is another interesting mesophilic cellulolytic *Clostridium*. Unlike other species discussed here, there is no evidence of cellulosome expression, as it lacks scaffolding and dockerin domains, but some catalytic enzymes do adhere to the substrate via CBMs[24]. *C. phytofermentans* is an attractive host because it contains the highest number of genes for lignocellulose-degradation among sequenced *Clostridia*[25] and has a broad range of carbon substrates, which include diverse polysaccharides, oligosaccharides and monosaccharides[26].

The industrial *n*-butanol-producer, *C. acetobutylicum*, has also been considered as a CBP host. Although it is unable to grow on cellulose, it contains 11 cellulosomal components[27] and

secretes hemicellulose-degrading enzymes[28]. Modifying this inactive system to enable cellulolytic capacity presents another promising strategy for butanol CBP. Finally, thermophilic cellulolytic species of the genus *Caldicellulosiruptor*, such as *C. bescii*, are promising hosts because they can efficiently degrade plant substrates that have not undergone chemical pretreatments[3c, 29]. Eliminating the biomass pretreatment step in lignocellulose processing is an economical benefit that has led to continued research in organisms of this genus. Their cellulolytic systems are composed of non-cellulosomal, multi-domain cellulases[30]. Furthermore, the recent ability to transform DNA to *C. bescii* may soon enable metabolic engineering for biofuel production[31].

1.3 Microbial n-butanol and isobutanol-producing pathways

Microbial production of higher alcohols has become of increasing interest in the past decade for use as both transportation fuels and chemical precursors[4b]. Native and synthetic pathways have been constructed in microorganisms to utilize central metabolites such as pyruvate and acetyl-coenzyme A (CoA) and direct flux to desired chemical products. *n*-Butanol and isobutanol are desirable substitutes for liquid transportation fuels due to their comparable octane number and heating value to gasoline[4a]. In addition, their low hygroscopicity makes them compatible fuels for storage and distribution[5a]. This section will introduce enzymatic pathways for producing either *n*-butanol or isobutanol, present metabolic engineering of these pathways into desirable hosts, and outline general and species-specific challenges leading to industrialization of such microbial processes.

1.3.1 Microbial *n*-butanol pathways

1.3.1.1 CoA-dependent n-butanol production in solventogenic Clostridia

n-Butanol production by solventogenic *Clostridia* was industrialized in the early 1900s and has recently regained attention[32]. This group of *Clostridia* natively produces acetone, *n*-butanol, and ethanol in what is known as ABE fermentation. Growth occurs in two phases: acidogenesis (Figure 1), where organic acids such as acetate and butyrate are produced with ATP, and then solventogenesis (Figure 2), where the acids are reassimilated to produce acetone, butanol, and ethanol at a ratio of 3:6:1, respectively[33]. Recent efforts in optimizing native butanol production have aimed at better understanding key gene regulation, as well as improving titers and butanol selectivity against other fermentative products[32].

The CoA-dependent butanol pathway proceeds in six steps from acetyl-CoA. First, two molecules of acetyl-CoA are converted to butyryl-CoA by *thiL*, *hbd*, *crt*, and *bcd*/*etfA*/*etfB* in a pathway analogous to fatty acid biosynthesis. Butyryl-CoA is then converted to butanol by an aldehyde dehydrogenase and alcohol dehydrogenase, such as the bifunctional aldhehyde-CoA/alcohol dehydrogenases encoded by *adhE* or *adhE1*, which catalyze both reactions[32, 34]. Alternatively, butyrate can be converted to *n*-butanol by CtfAB and AdhE1, while producing the acetone precursor, acetoacetate[35].

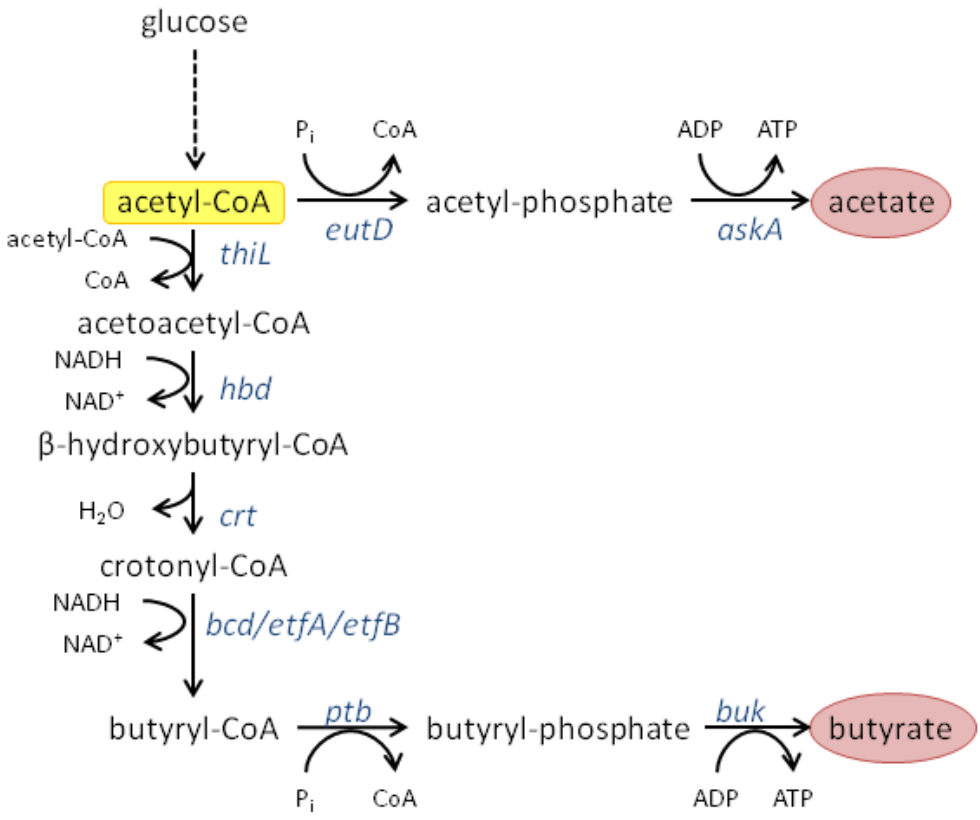


Figure 1. Acidogenic pathways of C. acetobutylicum

Schematic of acidogenic fermentative pathways. *thiL*: acetyl-CoA acetyltransferase; *hbd*: 3-hydroxybutyryl-CoA dehydrogenase; *crt*: 3-hydroxybutyryl-CoA dehydratase; *bcd*: butyryl-CoA dehydrogenase; *etfAB*: electron transfer flavoprotein; *eutD*: phosphotransacetylase; *askA*: acetate kinase; *ptb*: phosphate butyryltransferase; *buk*: butyrate kinase.

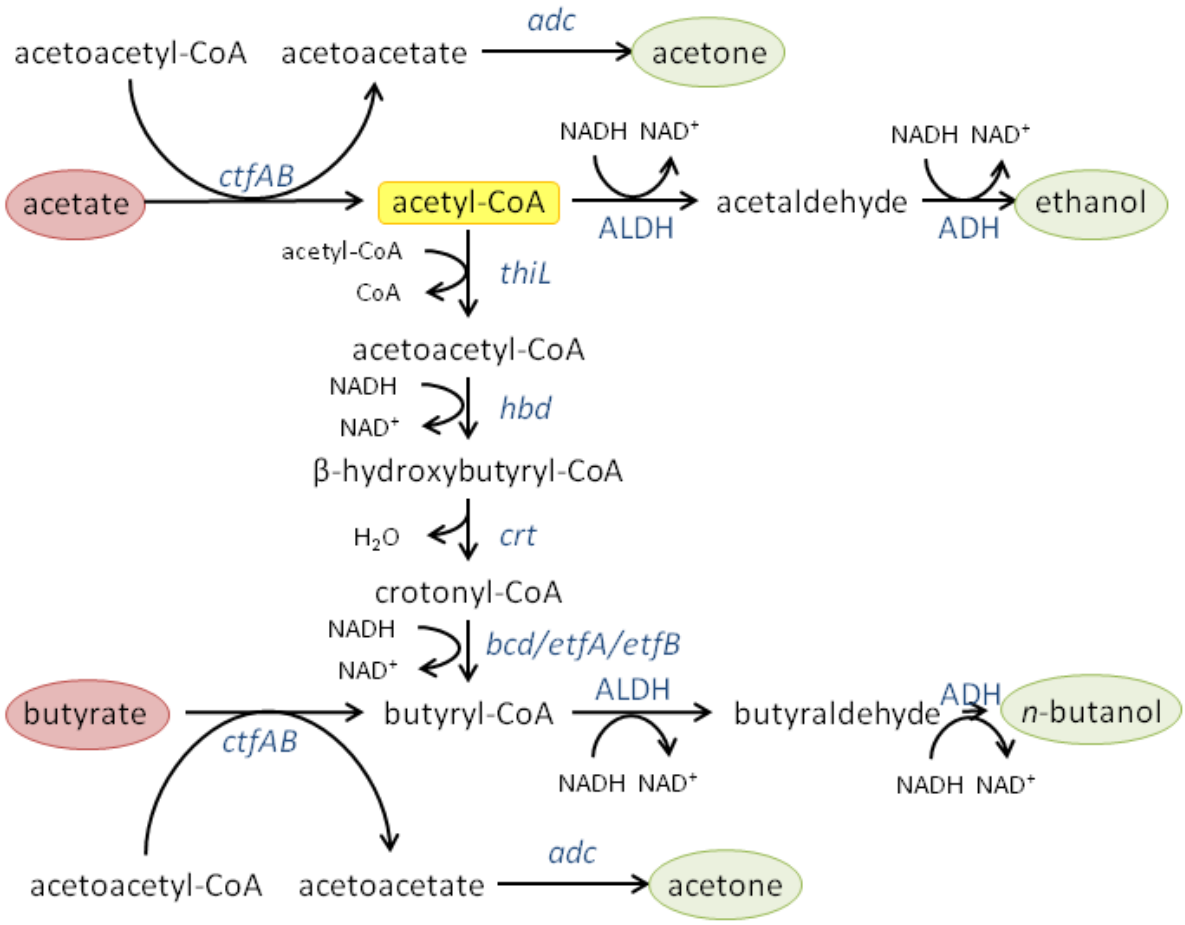


Figure 2. Solventogenic pathways of C. acetobutylicum

Schematic of solventogenic pathways. *ctfAB*: butyrate-acetoacetate CoA-transferase; *adc*: acetoacetate decarboxylase; ALDH: aldehyde dehydrogenase; ADH: alcohol dehydrogenase.

Butanol titers exceeding 15g/L have been achieved in *C. acetobutylicum*[36], *C. beijerinckii*[5c, 37], and *C. saccharoperbutylacetonicum*[38]. Genetic manipulations have focused on *C. acetobutylicum* with strategies such as knocking out competing pathway[36a, 39] and overexpressing butanol production genes[35-36, 39]. In addition, Nair et al. demonstrated a successful regulatory strategy. They identified a repressor, SolR, that acts on the *sol* locus to down regulate expression of solventogenic genes (*aad*, *ctfA*, *ctfB* and *adc*). Inactivating the *solR* gene led to significant improvement in butanol and acetone titers[36b]. It is worth noting that all of these productions

occurred in acidic conditions, as it is a trigger for solventogenesis[33]. Although much has been revealed about the metabolism of solventogenic *Clostridia* and effective manipulation strategies, research continues in improving genetic techniques, understanding biphasic regulation, and improving solvent tolerance[33, 40].

1.3.1.2 n-butanol production in non-native hosts

In the past five years, heterologous expression of clostridial CoA-dependent pathways has enabled butanol production in many non-native hosts. Strategies, such as deleting competing pathways and increasing expression of pathway genes have been implemented, as well as investigating homologous enzymes and utilizing host-specific driving forces have helped improve industrial feasibility.

As a highly investigated bacterial host with well-developed genetic techniques, *E. coli* serves as an attractive host for heterologous butanol production. Atsumi et al. were the first to produce *n*-butanol in *E. coli* with this pathway. They expressed *C. acetobutylicum* butanol genes (*hbd*, *crt*, *bcd*/*etfA*/*etfB*, *adhE2*), but replaced *thiL* with a native *E. coli* thiolase gene, *atoB*. To improve butanol production, they knocked out genes from competing fermentative pathways (Δ*adhE*, Δ*ldhA*, Δ*frdBC*, Δ*pta*) and the anaerobic regulator, Fnr (Figure 3). The best strain produced 550mg/L butanol from 20g/L glucose in semi-aerobic conditions[41]. Inui et al. also introduced the *C. acetobutylicum* butanol pathway, including *thiL*, to produce 1.2g/L of butanol from 40g/L glucose anaerobically[34]. The most successful examples of high titer, high yield butanol productions were achieved by replacing *bcd*/*etfA*/*etfB* with *ter* from *Treponema denticola*[42] and increasing pathway flux with NADH and acetyl-CoA driving forces[42b]. Shen et al. knocked out fermentative pathways that consume acetyl-CoA and NADH (Δ*pta*, Δ*adhE*, Δ*ldhA*, Δ*frdBC*) and overexpressed a formate dehydrogenase from *Candida boidinii* to direct carbon flux from acetyl-

CoA through the butanol pathway. Anaerobic growth on glucose generates NADH, a product of glycolysis, which could not be recycled to NAD^+ because all the native fermentative pathways in the host were deleted. The synthetic pathway, which requires four NADH-consuming reactions, allowed the strain to regenerate the NAD^+ necessary for continued glucose consumption. The deletion of the acetyl-CoA-consuming pathway mediated by *pta* further boosted the production. Utilization of these driving forces enabled production of 15 g/L anaerobically, or 30g/L with continuous product removal, yielding 70-88% of the theoretical maximum[5b].

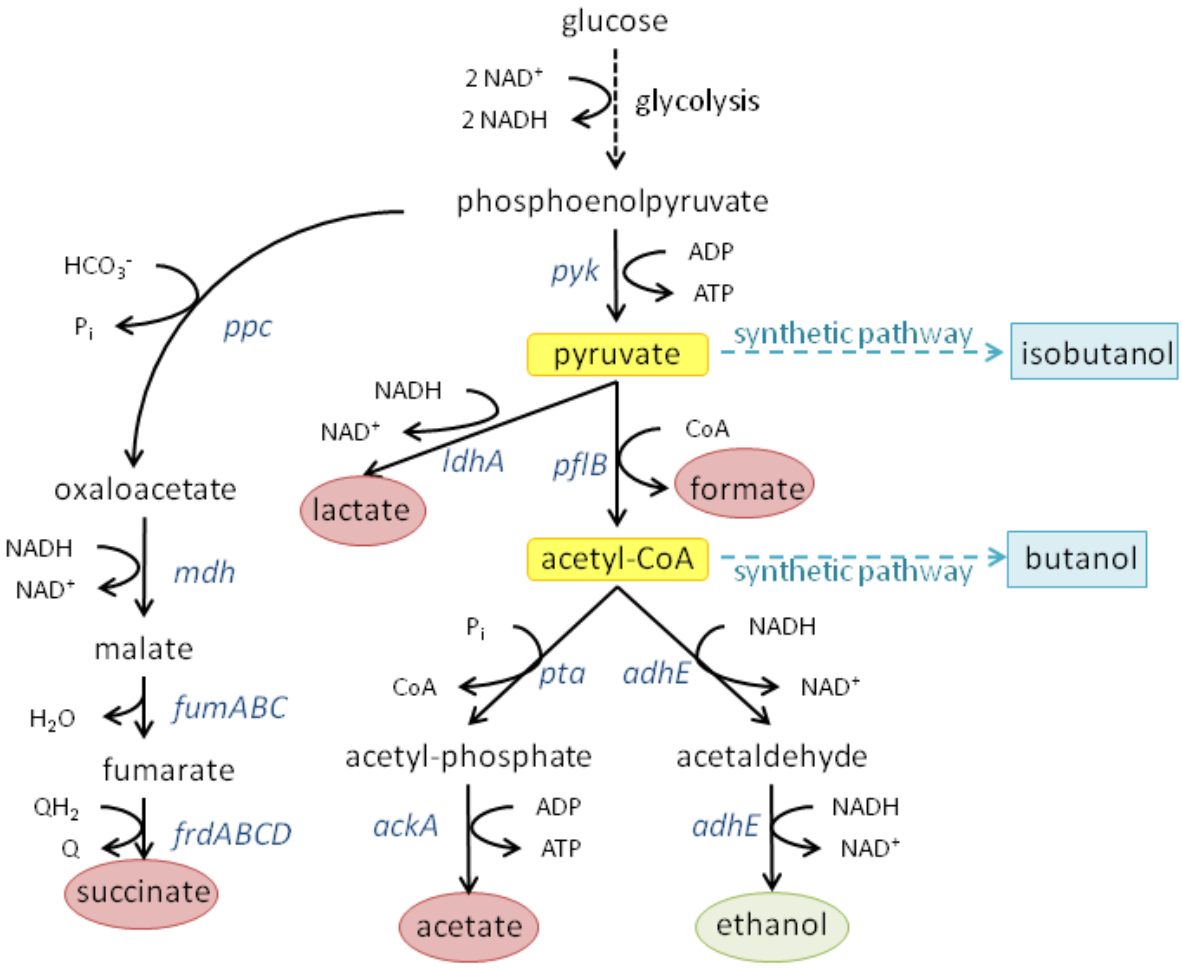


Figure 3. Fermentative pathways of E. coli

Schematic of fermentative pathways. *ppc*: phosphoenolpyruvate carboxylase; *mdh*: malate dehydrogenase; *fumABC*: fumarase; *frdABCD*: fumarate reductase; *pyk*: pyruvate kinase; *pflB*: pyruvate formate-lyase; *ldhA*: lactate dehydrogenase; *pta*: phosphate acetyltransferase; *ackA*: acetate kinase; *adhE*: acetaldehyde/alcohol dehydrogenase; *ldhA*: lactate dehydrogenase. ppc: phosphoenolpyruvate carboxylase; aspC: aspartate aminotransferase; thrA: aspartate kinase; thrB: homoserine kinase; thrC: threonine synthase; ilvA: threonine deaminase; leuA: 2-isopropylmalate synthase; leuCD: isopropylmalate isomerase; leuB: 3-isopropylmalate dehydrogenase; ilvE: branched-chain amino-acid aminotransferase; pyk: pyruvate kinase; ilvIH: acetolactate synthase I; ilvC: acetohydroxy acid isomeroreductase; ilvD: dihydroxy acid dehydratase.

The clostridial CoA-dependent butanol pathway has been introduced to other hosts including *Saccharomyces cerevisiae*[43], *Pseudomonas putida*[44], *Bacillus subtilis*[44], *Lactobacillus brevis*[45],

and *Synechococcus elongatus*, a cyanobacterium[46]; however, production in these organisms lags behind native *Clostridia* and *E. coli*. In many of these cases, metabolic engineering principles were applied to improve butanol production from initial pathway introduction, such as using host enzymes or other homologs to replace activities of the clostridial enzymes or by designing host-specific driving forces. Berezina et al. selected the lactic acid bacterium, *L. brevis*, for its butanol tolerance and native expression of a thiolase, aldehyde and alcohol dehydrogenase. Recombinant introduction of five clostridial genes (*hbd*, *crt*, and *bcd*/*etfA*/*etfB*) resulted in production of 300mg/L butanol[45]. Lan and Liao demonstrated a four-fold improvement in photosynthetic butanol production in *S. elongatus* by requiring irreversible ATP-consuming reactions and selecting enzymes that used the cofactor NADPH instead of NADH[46b]. These instances of heterologous butanol pathways demonstrate promise for use in cellulolytic *Clostridia*, but also illustrate the common challenge of increasing production to industrial levels.

In addition to CoA-dependent synthesis, *n*-butanol production has also been successful from 2-keto acid intermediates of amino acid biosynthesis. Atsumi et al.[5a] demonstrated that these metabolites can be utilized for alcohol production via two steps: decarboxylation and reduction. The expression of a keto acid decarboxylase (KDC) and alcohol dehydrogenase (ADH) in *E. coli* enabled microbial production of *n*-butanol, and other alcohols such as *n*-propanol, 2-methyl-1-butanol, and isobutanol, which will be discussed later.

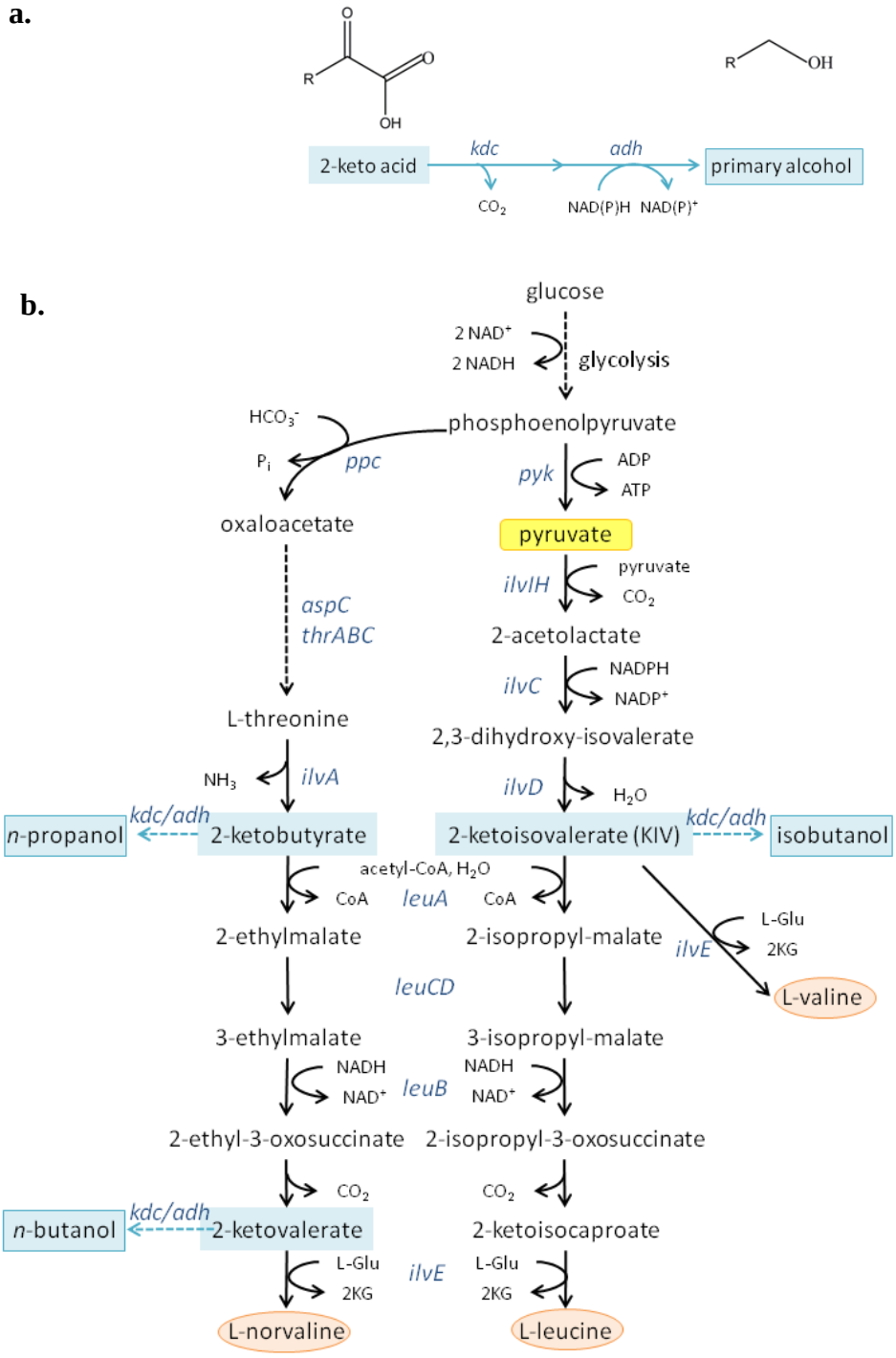


Figure 4. Keto acid pathways for n-butanol and isobutanol production

(a) Production of a primary alcohol from a 2-keto acid intermediate by *kdc*: keto acid decarboxylase and *adh*: alcohol dehydrogenase. (b)Schematic of keto acid pathways for *n*-butanol and isobutanol production.

Butanol production by the keto acid pathway, aka. amino acid pathway, stems from synthesis of the unnatural amino acid, norvaline. From threonine, a deaminase (*ilvA*) produces 2-ketobutryate, which is catalyzed by the leucine pathway (*leuABCD*) to form the keto acid precursor, 2-ketovalerate. 2-ketovalerate is then catalyzed by exogeneous Kdc and Adh to produce butanol (Figure 4). To improve the titer, *ilvA* and the leucine pathway were overexpressed and *ilvD* was deleted to minimize competitive substrates and flux toward leucine biosynthesis[5a]. In another study, Shen and Liao co-produced *n*-propanol and butanol in *E. coli* at about 1g/L each. This was achieved by a combination of pathway overexpression (*ilvA*, *leuABCD*, and a feedback resistant *thrA* of leucine biosynthesis, and *kivd* and *ADH2* of the keto acid pathway) and competing pathway deletion (Δ*metA*, Δ*tdh*, Δ*ilvB*, Δ*ilvI*, Δ*adhE*)[42b]. Currently, butanol production via this pathway lags behind the clostridial pathway, but its promise remains.

1.3.2 Microbial isobutanol pathways

Production of isobutanol by the amino acid pathway uses the valine biosynthesis pathway to produce the intermediate, 2-ketoisovalerate (KIV). Two pyruvate molecules undergo condensation and decarboxylation to form 2-acetolactate by an acetohydroxyacid synthase (AHAS). Then, reduction and dehydration by IlvC and IlvD yields KIV, the substrate for isobutanol production by KDC and ADH.

Atsumi et al. overexpressed the valine pathway (*ilvIHCD*) along with *kivd* and *ADH2* in *E. coli*, and removed competing pathways (Δ*adhE*, Δ*ldhA*, Δ*frdAB*, Δ*fnr*, Δ*pta*) to produce 2.3g/L isobutanol[5a]. Exchanging the AHAS, encoded by *ilvIH*, with a catabolic enzyme from *Bacillus subtilis*, AlsS, and deleting *pflB* led to production of 22g/L microaerobially[5a]. AlsS has a significantly higher affinity for pyruvate than IlvIH, which enhanced flux from the pyruvate

node through the isobutanol pathway. Moving production to a bioreactor with gas stripping enabled titers to exceed 50g/L[47]. Alternatively, an evolutionary approach was used to develop an *E. coli* strain capable of similar isobutanol titers. Multiple rounds of random mutagenesis and selection were conducted to evolve strains resistant to norvaline, a branched chain amino acid analog that becomes toxic at high concentrations. Introduction of isobutanol pathway genes (*alsS, ilvC, ilvD, kivd, adhA*) to the evolved strain enabled production of 21.2g/L isobutanol[48]. (Figure 4)

Isobutanol has also been produced by the keto acid pathway in *Corynebacterium glutamicum*[49], *B. subtilis*[50], and *S. cerevisiae*[51]. Furthermore, using renewable resources such as CO_2 in *S. elongatus*[52], electricity in *Ralstonia eutropha*[53], and waste proteins in *E. coli*[54] highlight the potential for renewable advanced biofuels. Similar metabolic engineering strategies were employed to improve isobutanol production following initial introduction of the pathway. For instance, expressing genes to increase pyruvate and KIV pools was employed in multiple hosts, as was deleting competing pathways.

Improving enzymes is another strategy for enhancing microbial fuel production. Bastian et al. used *in vitro* enzyme evolution to improve isobutanol yields in *E. coli*[55]. They improved the balance of the cofactor, NADH, which is produced during glycolysis and oxidized during isobutanol production, by engineering IlvC to prefer NADH as an electron donor over NADPH, the preferred substrate of the wild-type enzyme. They also overexpressed a transhydrogenase to transfer electrons between NADH and $NADP^+$. Finally, they improved the affinity of AdhA for isobutryaldehyde to achieve 100% of the theoretical yield from glucose anaerobically[55]. Another approach was used by Matsuda et al. who engineered *S. cerevisiae* to express pathway enzymes in the cytosol instead of the mitochondrion, where it is normally expressed. This

resulted in doubling production and demonstrates the role compartmentalization may play in microbial chemical production[51d].

As is the case for clostridial pathway *n*-butanol production, engineering high titer, high yield isobutanol production has been most successful in *E. coli*. While work in other hosts has demonstrated improvements in titer and yield, a more comprehensive understanding of how to manipulate metabolism will be necessary to reach industrial levels. Thus, continuing research on the level of basic metabolism will be necessary to understand relevant cell regulations and enable the design of host-specific driving forces.

Some thermophilic and hyperthermophilic archaea also possess enzymes that offer an alternative route to isobutanol from KIV. 2-Ketoisovalerate ferredoxin oxidoreductase (VOR) is a class of enzymes capable of CoA-dependent decarboxylation of 2-keto acids, analogous to the reaction of the more common enzyme, pyruvate ferredoxin oxidoreductase (PFOR). VORs have been identified and characterized in *Thermococcus litoralis* and *Pyrococcus sp.*[56], *Methanobacterium thermoautotrophicum*[57], and *Thermococcus profundus*[58]. These multimeric enzymes are composed of either three or four subunits and are often oxygen-sensitive. VORs are most relevant for isobutanol CBP in the thermophile, *C. thermocellum* due to their activity at high temperatures. Biochemical assays have demonstrated their activity *in vitro* but heterologous expression has not been reported in literature. Lin et al. measured VOR (aka. KOR) activity in *C. thermocellum* lysate and demonstrated its participation in isobutanol production; however, heterologously expressed *kivd* was found to be the major contributor to isobutanol production in this system[6b].

1.4 Progress toward butanol CBP in cellulolytic *Clostridia*

Examples of native butanol production in *Clostridia* and heterologous C4 alcohol production in other microbes elucidate a general strategy for enabling and improving production of these chemicals. The first step is overexpressing pathway genes in the selected host. Alternative enzymes should also be considered for host compatibility, preferable cofactor usage[55], or superior activity characteristics, such as the pyruvate specificity of *B. subtilis* AlsS[5a] or the irreversibility of *T. denticola* Ter[5b]. Once the pathway is functional, production can be improved by deleting competing pathways, overexpressing additional genes to improve pathway flux, or disrupting unproductive regulation[36b]. Finally, optimizing production conditions, developing host-specific driving forces, and strain evolution have demonstrated success for achieving goals of high titer and yield. In solventogenic *Clostridia*, controlling pH and the switch from acidogenesis to solventogenesis was essential for improved titers[5c]. Balancing cofactors and providing effective driving forces are essential for synthetic pathways. For example, anaerobic NADH and acetyl-CoA accumulation in the specific *E. coli* knockout strain provided the necessary driving forces for CoA-dependent butanol production[5b]. In addition, continuous product removal may be used to reduce product toxicity and improve titers[47]. Cellulolytic *Clostridia* are still in the early stages of strain development, but this section will outline three promising strategies for developing *n*-butanol or isobutanol CBP strains and their current status.

1.4.1 Isobutanol CBP in *Clostridium cellulolyticum*

CBP of crystalline cellulose to isobutanol in *C. cellulolyticum* was the first demonstration of isobutanol CBP. Five genes from the *E. coli* pathway were expressed on a plasmid downstream of a ferredoxin promoter to produce 660mg/L isobutanol. Challenges and unexpected results that occurred in engineering *C. cellulolyticum* will help inform future engineering of cellulolytic

microbes for isobutanol CBP. For example, transformants containing wild-type *alsS* directly downstream of a constitutive promoter could not be obtained. Enzyme toxicity was believed to be the cause. However, moving the gene to the third position in the operon enabled successful transformation and isobutanol production thereafter. Additionally, *in vitro* assays indicated that recombinant strains did not have statistically improved activities for IlvC, IlvD, or alcohol dehydrogenase. Thus, native enzymes may be sufficient to manage the pathway flux at these nodes. On the other hand, negative controls demonstrated the overexpressing AlsS and Kivd is necessary for detecting *in vitro* activity, implying that these may be the most important enzymes for isobutanol CBP in cellulolytic *Clostridia*[6a].

1.4.2 Isobutanol CBP in *Clostridium thermocellum*

C. thermocellum is one of the most promising hosts for cellulolytic CBP due to the microbe's rapid growth on cellulose and the favorable properties of high temperature anaerobic bioprocessing[3a, 59]. Lin et al. engineering a heterologous pathway in this thermophilic organism, overcoming challenges such as increased chemical toxicity. High temperatures are believed to increase chemical toxicity, which may include unnatural pathway products and intermediates[60]. Notably, aldehydes have increased volatility, such as the isobutanol precursor isobutyraldehyde with a boiling point of 63 °C (pure aldehyde). By overexpressing *L. lactis kivd* and the valine biosynthesis genes (*ilvBNC*, and *ilvD*) on an integrated plasmid, 5.4 g/L isobutanol was produced from cellulose in 75 hours[6b].

1.4.3 Toward n-butanol CBP in *Clostridium acetobutylicum*

As mentioned earlier, *C. acetobutylicum* secretes hemicelluloses-degradation enzymes and possesses cellulosomal genes even though it cannot grow on crystalline cellulose. Combined with native *n*-butanol fermentation, the development of a chimeric cellulosomal system in this

organism offers another encouraging route to *n*-butanol CBP. Fierobe et al. demonstrated that functional chimeric cellulosomes could be produced by mixing dockerin and catalytic domains from *C. thermocellum* and *C. cellulolyticum*[10] with the previous knowledge that cohesin-dockerin interactions are species-specific[61]. Perret et al. then demonstrated that these heterologous miniscaffoldins could be expressed and secreted by *C. acetobutylicum* in mature and active forms[62]. Recently, minicellulosomes containing *C. cellulolyticum* catalytic subunits, including a mannose and some cellulases, were functionally expressed[28, 63]. Unfortunately, expression of larger catalytic modules was unsuccessful because of secretion problems. The identification of an additional chaperone protein for proper secretion of larger proteins may be necessary for enabling CBP in *C. acetobutylicum*[28]. Although activity of the minicellulosomes was demonstrated *in vitro*, CBP capacity has yet to be reported.

1.5 Summary

Butanol CBP has emerged as a promising route for producing renewable advanced fuels, although the ultimate role butanol CBP will play among diverse biofuel production strategies remains unclear. A number of challenges such as the improvement of heterologous expression systems, limited understanding of metabolic regulation, and toxicity of feedstocks, intermediates and products still face this field. Nonetheless, the diversity of research and significant progress that has been made over the past 20 years supports a bright future. The various strategies discussed here will not only contribute to developing a butanol CBP organism, but will also help shape general design principles used in the broader field of metabolic engineering. The following two chapters will describe efforts made in characterizing and engineering enzymes for high temperature KDC activity, as well as investigating enzymes and proposing a strategy to expand the types of branched-chain alcohols that can be biologically produced.

2. Characterization of thermophilic AHASs for KIVD activity

2.1 Introduction

Although high temperature isobutanol production has been demonstrated in both *G. thermoglucosidasius* and *C. thermocellum, in vitro* heat treatment enzyme assays indicate that *L. lactis* KIVD activity may be weak if production temperatures are raised to 55 or 60 °C[64]. With limited thermostability of the model enzyme and no reported homologs in thermophilic hosts, engineering a thermostable enzyme with high KIVD activity is of interest to improve the high temperature isobutanol pathway. There are two promising strategies for realizing this goal: 1) improving the thermostability of *L. lactis* Kivd or 2) improving the KIVD activity of a thermostable decarboxylase. Here, the latter strategy was investigated by identifying and characterizing the KIVD activity of enzymes similar to *L. lactis* Kivd from thermophilic hosts. Ultimately, the absolute specific activities of the native thermophilic enzymes were found to be substantially lower than *L. lactis* Kivd and are not recommended for use in thermophilic isobutanol production.

2.2 Materials and methods

2.2.1 Plasmid construction

Plasmids were constructed using isothermal assembly as described by Gibson et al[65]. Table 1 describes the plasmids and strains used in this study. pHT211 was constructed from a shuttle vector for *E. coli* and *Geobacillus spp*. with an lactate dehydrogenase (*ldh*) promoter, pHT71, described in Lin et al[64]. The *ilvB* gene from *C. thermocellum DSM1313* was amplified from genomic DNA and the vector backbone was amplified from pHT71. Both DNA fragments were amplified using polymerase chain reaction (PCR) by primers designed with overlapping regions

of complementarity. An N-terminal hexahistidine sequence was incorporated into the primers to tag *ilvB* for protein purification.

Plasmids pRW09-13 were also constructed by Gibson isothermal assembly, but using an *E. coli* expression vector backbone amplified from pTW49. pTW49 was a gift from Tung-Yun Wu of James Liao's laboratory at UCLA. Gene inserts were amplified from genomic DNA based on sequences obtained through the National Center for Biotechnology Information (NCBI). All plasmids sequences were verified by Sanger sequencing run by Laragen.

Table 1. List of strains and plasmids

Name	Description	His-tag location
Strains		
G. thermoglucosidasius	DSM 2542	
E. coli BL21(DE3)	*E. coli B dcm ompT hsdS* ($r_B^-mB_B^-$) gal	
Plasmids		
pHT71	P_{ldh}:: *kivd ilvC alsS* Cm^R ColE1 RepB	
pTW49	P_{T7}:: *L. lactis kivd* Amp^R ColE1	N-terminus
pHT211	P_{ldh}:: *C. thermocellum ilvB* Cm^R ColE1 RepB	C-terminus
pRW09	P_{T7}:: *A. flavithermus ilvB* Amp^R ColE1	N-terminus
pRW10	P_{T7}:: *C. bescii ilvB ilvN* Amp^R ColE1	N-terminus of *ilvB*
pRW11	P_{T7}: *C. saccharolyticus* 0837 Amp^R ColE1	N-terminus
pRW12	P_{T7}:: *C. saccharolyticus* 1142 Amp^R ColE1	N-terminus
pRW13	P_{T7}:: *T. thermophilus ilvB ilvH* Amp^R ColE1	N-terminus of *ilvB*

2.2.2 Competent cell preparation and transformation

G. thermoglucosidasius competent cells were prepared as described in Lin et al., except cells were grown at 55 °C instead of 50 °C[64]. *E. coli* electrocompetent cells were prepared from mid- to late-log cells grown at room temperature, washed and concentrated in 10% glycerol solution at 4 °C. All competent cells were stored at -80 °C and were thawed on ice before electroporation.

60 µl of *G. thermoglucosidasius* competent cells were transformed with 1 µl of purified pHT211 plasmid using 1 mm-gap electroporation cuvettes at 2.5 kV, 25 µF, and 48 Ω using a Bio-Rad gene pulser apparatus. *E. coli* competent cells were transformed using the same cuvette and apparatus at 1.8 kV.

After electroporation, *G. thermoglucosidasius* cells were rescued in 1 ml of TGP media for 2 hours at 50 °C. TGP medium was described in Cripps et al[66]. Cells were spread on TGP agar plates containing 15 µg/ml chloramphenicol and incubated at 55 °C overnight. *E. coli* cells were rescued in 400 µl of LB media 1 hour at 37 °C. Cells were spread on LB agar plates containing 100 µg/ml ampicillin.

2.2.3 Protein expression

Single colonies were picked from agar plates and inoculated into TGP media (for *G. thermoglucosidasius*) or LB media (for *E. coli*), containing the same concentration of antibiotics as in the agar plates. These 4 ml starter cultures were grown overnight in 14ml culture tubes at 50 °C or 37 °C, respectively.

G. thermoglucosidasius/pHT211 was overexpressed four 500ml baffled flasks, each containing 350 ml cultures of TGP media with chloramphenicol. Cultures were inoculated from the starter culture and grown at 50 °C for 24 hours. Cells were harvested by centrifugation at final OD600~ 4.5.

Similarly, *E. coli* strains were overexpressed in 500 ml baffled flasks with 350 ml of LB with ampicillin at 37 °C. At OD600~0.6 -0.8, cultures were induced with 1mM IPTG and shaken at room temperature for ~18 hours. Cells were harvested by centrifugation at final OD600~2.5-4. All pellets were stored at -80 °C until lysis.

2.2.4 Protein purification

Proteins were purified using his-tag purification with Ni-NTA beads. First, cell pellets were resuspended in 30 ml of a sodium phosphate binding buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM NaCl, 10 mM imidazole, 0.03% Triton X-100, based on protocol from Zymo Research). Cells were sonicated on ice for five cycles of 25 Amps for 30 seconds, separated by 40 second breaks. Sonicated cells were then centrifuged at 8000 rpm for 30-60 minutes at 4 °C. The supernatant was retained for his-tag purification.

All thermophilic proteins were heat-treated at 50 °C for 10 minutes to denature undesired protein. Samples were then centrifuged again for 15 minutes to isolate the soluble fraction. 1 ml of Qiagen Ni-NTA agarose beads were added to a gravity column for each protein sample. The bead storage fluid was removed by gravity filtration and washed with 2 ml binding buffer. The beads were then resuspended in the lysate and incubated for 10 minutes at 4 °C with continuous inversion.

The bead-containing lysate was flown through the gravity column, isolating the agarose beads. ~20 ml of wash buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM NaCl, 50 mM imidazole, 0.03% Triton X-100) was flown through each column, and checked for residual protein using the Bradford assay.

Finally, purified protein was eluted with an elution buffer (50 mM sodium phosphate buffer pH 7.7, 300 mM NaCl, 250 mM imidazole) in 1 ml fractions until all protein was isolated. Using Millipore Amicon filter tubes, proteins were concentrated and the buffer was exchanged to 50 mM potassium phosphate pH 7.0, 2.5 mM $MgCl_2$, 0.1 mM thiamin diphosphate (ThDP), and 0.1 mM Flavin adenine dinucleotide (FAD). Purified protein was stored in equal volume glycerol at 4 °C until assayed and concentration was measured by Bradford assay.

2.2.5 Enzyme assays

Decarboxylase assays were conducted using a coupled assay with an NADH-consuming horse liver alcohol dehydrogenase (ADH) purchased from Sigma-Aldrich. Enzyme assay master mix included 50 mM potassium phosphate pH 7.0, 2.5 mM $MgCl_2$, 0.1 mM ThDP, 0.1 mM FAD, 2.5 U ADH and 0.2 mM NADH with the keto acid substrate. Pyruvate and KIV at various concentrations were used for characterization of *C. thermocellum* IlvB activity. 5 mM KIV was used for characterization of other thermophilic decarboxylases. Mastermixes were made based on concentrations after the addition of 10 or 20% enzyme.

Assays were activated with either 50 or 100 µl of enzyme in a 500 µl total reaction. Cuvettes and assay buffers were preheated to 37 °C using a water bath that circulated water of the desired temperature outside the cuvettes. Reactions were activated by addition of the enzyme with mixing by inversion. Change in absorbance of NADH at 340 nm was measured by an Agilent Spectrophotometer. Activity was calculated using the extinction coefficient of NADH, 6022 M^{-1} cm^{-1}.

2.3 Characterization of *C. thermocellum* IlvB for decarboxylase activity

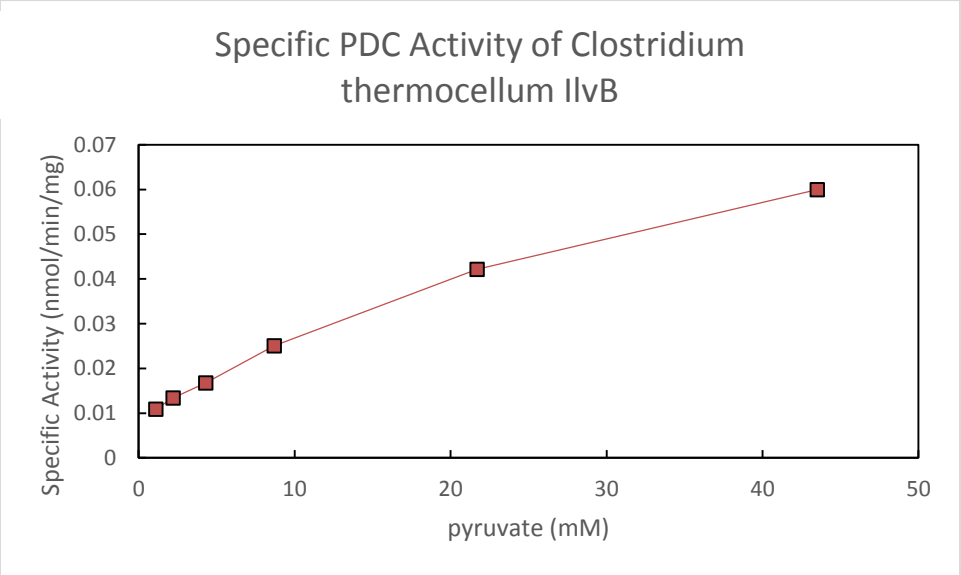
Since native isobutanol production by *C. thermocellum* was demonstrated and it is a desired host for CBP, its annotated genome was investigated for potential KIVD enzymes. A Basic Local Alignment Search Tool (BLAST) search with *L. lactis* Kivd as the query resulted in only two major "hits" based on query coverage (82% or greater) and E value (e-30 or better). Both putative enzymes were annotated as acetolactate synthase, aka. AHAS, which is also the first enzymes in the branched chain amino acid (BCAA) biosynthesis and the node to produce isobutanol from pyruvate. The annotation of these two enzymes as AHAS catalytic (or large) subunits was supported by their conserved operon organization as well as their homology to this

highly conserved protein family. The enzyme described as IlvB (encoded by *Cthe_2516* in strain ATCC27405) was named as such due to the gene's location upstream of the small, regulatory AHAS subunit (named IlvN) and the fact that the small subunit's start codon begins within the IlvB gene. This gene structure of *ilvB* upstream of *ilvN* is highly conserved among bacteria [67]. We describe the other BLAST "hit" as IlvI, based on the naming system of *E. coli* AHAS isozymes. Although it does not have a regulatory subunit gene downstream, its homology to the class of AHASs and its vicinity to the BCAA gene, *ilvD*, implies that it may also function as an AHAS. The similarity of these two enzymes–Kivd and AHAS–as identified by BLAST was not surprising due to the similarity of their catalytic activity. Both enzymes are thiamin diphosphate ThDP-dependent decarboxylases that catalyze the CO_2 removal from a 2-keto acid substrate, although also AHAS has synthase, or C-C bond formation, activity following decarboxylation of the substrate, pyruvate [68].

C. thermocellum IlvB was his-tagged and purified from recombinant *G. thermoglucosidasius* and purified using Ni-NTA beads. Keto acid decarboxylase activity was measured using a kinetic coupled assay with a commercial alcohol dehydrogenase, measuring decreasing absorbance of NADH. Activity was measured on both the native AHAS substrate, pyruvate, and the desired substrate, KIV, over a range of substrate concentrations from about 1 mM to more than 40mM.

The activities of *C. thermocellum* IlvB on 1mM pyruvate and KIV were very similar, but were more than a thousand times less active than the specific activity of *L. lactic* Kivd at 81 U/mg [69]. Activity on pyruvate increased with additional substrate following Michaelis-Menten-like kinetics curve (Figure 5a). On the other hand, activity on KIV remained stagnant as substrate concentration increased to 20 mM, and decreased at higher concentrations (Figure 5b).

a.



b.

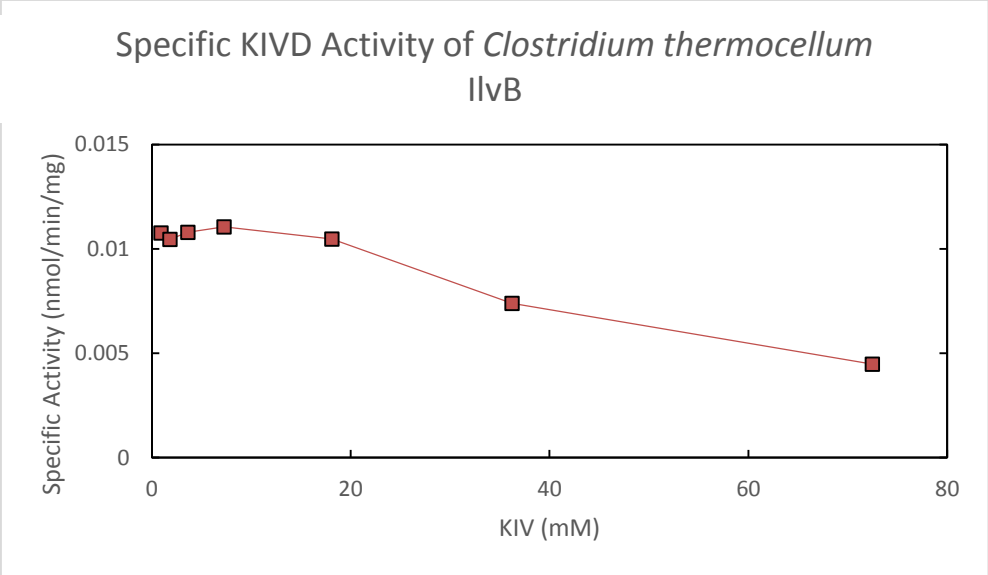


Figure 5. Specific decarboxylase activity of C. thermocellum *IlvB*

Specific decarboxylase activity on (a) pyruvate and (b) KIV measured by a coupled assay with NADH-consuming alcohol dehydrogenase.

C. thermocellum IlvB demonstrated extremely low decarboxylase activity and had poor expression in three strains of *E. coli* (results not shown). Consequently, enzymes from other thermophiles were investigated for comparable KIVD activity to the *L. lactis* enzyme, as well as improved *E. coli* expression.

2.4 Characterization of other thermophilic decarboxylases for KIVD activity

A second BLAST search was run against the model Kivd with known thermophile genomes as the search set. Five homologs were cloned into an expression vector backbone, overexpressed in *E. coli* and purified to measure decarboxylase activity (see Table 2). Four of the genes were successfully overexpressed and purified, excluding *C. bescii ilvBN* (Figure 6). Three enzymes had better specific activity than *C. thermocellum* IlvI, by 2-7 fold, but improvements were only modest compared to the activity of *L. lactis* Kivd (Table 3).

Table 2. Thermophilic KIVD homologs

Plasmid	Organism	Gene(s)/ORF(s)
pRW09	*Anoxybacillus flavithermus*	ilvB
pRW10	*Caldicellulosiruptor bescii*	ilvBN
pRW11	*Caldicellulosiruptor saccharolyticus*	837
pRW12	*Caldicellulosiruptor saccharolyticus*	1142
pRW13	*Thermus thermophilus*	ilvBH

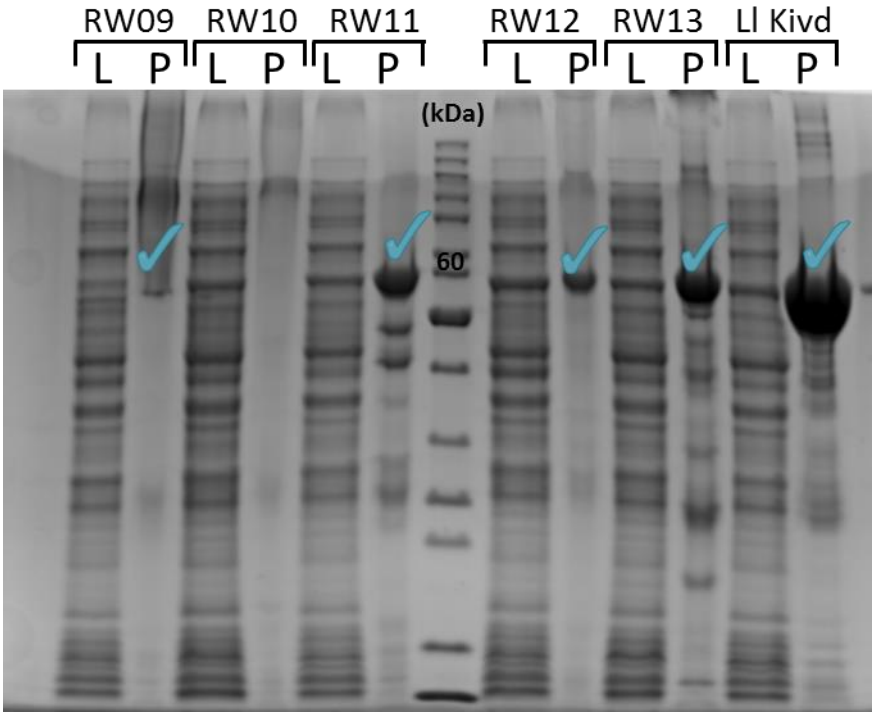


Figure 6. SDS PAGE gel of purified thermophilic KIVD homologs

SDS PAGE gel of his-tag purified protein from strains RW09-13 and KIVD. Check marks denote successfully purified protein product. L: lysate; P: purified protein.

Table 3. Specific Activity of purified thermophilic KIVD homologs

Organism	Enzyme	Specific Activity (nmol/min/mg)
A. flavithermus	IlvB	0.081
C. saccharolyticus	837	0.021
C. saccharolyticus	1142	0.030
T. thermophilus	IlvBH	0.009
C. thermocellum	IlvI	0.011

2.5 Summary

AHAS enzymes from thermophilic hosts were cloned, purified and assayed for KIVD activity to examine the feasibility for use in thermophilic isobutanol production. Enzymes from *C. thermocellum*, *A. flavithermus*, *C. saccharolyticums* and *T. thermophilus* were successfully

cloned and purified, but their activity was more than a thousand times less active than *L. lactis* Kivd, which has some activity in thermophilic conditions (55-60 °C). With such a substantial gap in base activity, protein engineer of these enzymes was not pursued. Instead, the alternative approach of improving Kivd's thermostability is recommended for improving the temperature stability of the isobutanol pathway.

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