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## Development of a high temperature microbial fermentation process for butanol

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### Abstract

Transforming renewable biomass into cost-competitive high-performance biofuels and bioproducts is key to the U.S. future energy and chemical needs. Butanol production by microbial fermentation for chemical conversion to polyolefins, elastomers, drop-in jet or diesel fuel, and other chemicals is a promising solution. A high temperature fermentation process could decrease energy costs, capital cost, give higher butanol production, and allow for continuous fermentation. In this paper, we describe our approach to genetically transform *Geobacillus caldoxylosiliticus*, using a pUCG18 plasmid, for potential insertion of a butanol production pathway. Transformation methods tested were electroporation of electrocompetent cells, ternary conjugation with *E. coli* donor and helper strains, and protoplast fusion. These methods have not been successful using the current plasmid. Growth controls show cells survive the various methods tested, suggesting the possibility of transformation inhibition from a DNA restriction modification system in *G. caldoxylosiliticus*, as reported in the literature.

### Introduction

*Geobacillus* bacteria are rod-shaped, aerobic or facultative anaerobic, endospore-forming microbes. Depending on the strain, the temperature range for growth can extend as low as 35 °C or as high as 80 °C, but typically isolates grow between 45 and 70 °C (Nazina et al. 2001). These characteristics make *Geobacillus* bacteria attractive as platforms for biofuel production (Cripps 2009, Taylor 2008). These bacteria have been isolated from sources on all seven continents as well as the Pacific Ocean and the Mediterranean Sea. The most frequently reported natural sources for *Geobacillus* isolates have been hot springs. Most *Geobacillus* isolates are equipped with a number of sensors, transporters and hydrolytic enzymes specifically adapted to extract energy and metabolic building blocks from plant biomass (Zeigler 2014). Efficient transformation methods for various strains of *Geobacillus* have previously been established through conjugative transfer (Suzuki 2012), electroporation (Cripps 2009), and protoplast fusion (Zeigler 2001). An advantage of using a thermophilic organism for genetic engineering is the possibility of utilizing temperature as a way to modulate and balance both the engineered pathway and the host's metabolism (Keller 2015). Using renewable biomass like switchgrass and corn stover and converting it into cost-competitive high-performance biofuels and bioproducts is important for the US long-term energy needs. The production of butanol through microbial fermentation and conversion to polyolefins, elastomers, drop-in jet or diesel fuel, and other chemicals is a promising solution for alternative energy.

*Clostridia* are currently the most studied and industrially used butanol fermenters (Lee et al. 2008), but they have limitations. *Clostridia* have a low tolerance for butanol, they grow slowly and at ambient temperatures, they require batch production, and they give low product yields, creating high product recovery cost since the product is so dilute (Vane 2008, Weber et al. 2010). One of the most effective ways of effectively harvesting butanol from fermentation is gas stripping, however gas stripping at lower temperatures requires higher gas flow rates, which causes the cost of recovery to increase. When conducted at high temperatures, the efficiency of gas stripping is directly related to vapor pressure of

the gas. It has been shown that raising the temperature from 25 to 55°C can increase butanol recovery from 46% to 86% at the same gas flow rate, thus lowering the cost of product recovery (Ollivier et al. 2011).

Developing and demonstrating a high temperature simultaneous saccharification and butanol fermentation system will provide many benefits. Thermophilic conditions will lead to faster hydrolysis rates during the saccharification step of biomass, which can lead to maximized butanol production. It can decrease the energy costs of reactor cooling and also decrease the capital cost of reactor design. At high temperatures there could be a decrease in reactor contamination, which would help lower the cost of maintaining a sterile environment. High temperatures will also allow more efficient gas stripping of butanol, which would facilitate the use of continuous fermentation. Continuous fermentation would improve the economics of the process by drastically reducing sterilization and reinoculation times, reducing separation costs, and reducing butanol toxicity to the microorganisms (Kumar 2011, Ezeji 2007).

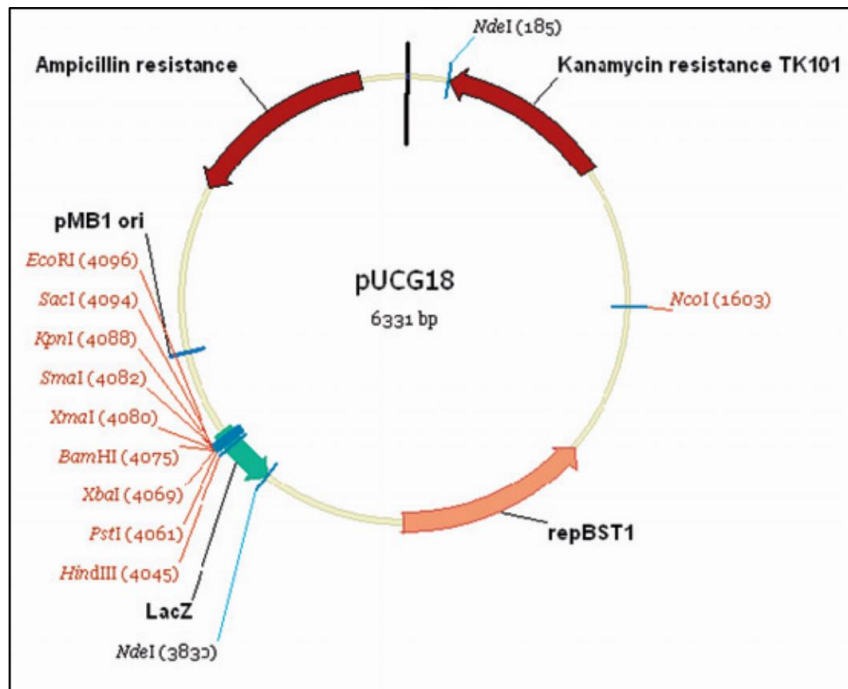
## Methods

### Strains

*Geobacillus caldoxylosilyticus* ATCC 700356 (ATCC, Manassas, VA, USA), *Geobacillus caldoxyloliticus* NCIMB 14283 (NCIMB, Bucksburn, Aberdeen, UK), *Bacillus licheniformans* GBL 2635, *Ureibacillus thermophaericus* GBL 2643, and *Ureibacillus thermophaericus* GBL 2666 (Green Biologics Ltd, Milton, Abingdon, United Kingdom) potential butanol-tolerant/butanol-producing bacterial strains were either purchased or provided to the Idaho National Lab. *Escherichia coli* strains C2925 and NEB 10B were purchased from New England Biolabs (Ipswich, Massachusetts, USA) and used for general preparation of plasmid DNA using the Qiagen miniprep kits. DNA prepared from the C2925 strain is unmethylated DNA, with respect to *dam* and *dcm* methylation genes from *E. coli*. *E. coli* strain HB101/pRK2013 was purchased from ATCC and used as a helper strain for plasmid transfer during ternary conjugation experiments.

*Geobacillus* strains were screened for their ability to convert both glucose and xylose into butanol. The strains were grown aerobically on 2YT (16 g tryptone, 10 g yeast extract, 5 g sodium chloride, 1 L H<sub>2</sub>O ) then transferred to an optical density at 600 nm (OD<sub>600</sub>) of 0.5 for growth anaerobically on 2YT plus 0.25% glucose and 0.5% xylose. Cell growth rates were determined by OD<sub>600</sub> and quantification of butanol was performed by gas chromatography and mass spectrometry using a standard curve.

To establish a protocol for insertion of a butanol pathway for increased butanol production in *G. caldoxylosilyticus*, various transformation methods were tested including electroporation of electrocompetent cells, ternary conjugation, and protoplast transformation. The *Geobacillus* shuttle vector pUCG18 (*Bacillus* Genetic Stock Center, Columbus, OH, USA) has a thermostable selectable marker gene conferring kanamycin resistance in *E. coli* at 37 °C and in *Geobacillus* up to 68 °C (Figure 1). Plasmid DNA was prepared for transformation from *E. coli* strains that are wild type as well as mutated with respect to *dam* and *dcm* methylation genes (Taylor 2008).



**Figure 1.** pUCG18 plasmid map (Taylor 2008).

### *Electroporation of Electrocompetent Cells*

*G. caldoxylosilyticus* cells were revived by streaking directly from the frozen stock onto a Tryptose Blood Agar Base (TBAB) plates (10 g tryptose, 3 g beef, 5 g NaCl, 15 g agar, 1 L H<sub>2</sub>O) and incubating overnight at 60°C. Four or five colonies were picked and grown overnight in 30 mL of 2YT at 60°C in a shaking incubator set to 220 rpm. The cells were subcultured into a baffled flask with 200 mL of 2YT, at an OD<sub>600</sub> of 0.2 until an OD<sub>600</sub> of 1.4 was reached. Cultures were cooled on ice for 10 min and centrifuged at 3,400 x g for 20 min at 4°C. The cells were resuspended in ice-cold electroporation media (91 g mannitol, 91 g sorbitol, and 10 % glycerol (v/v)) and washed 3 times before being resuspended in electroporation media at 2% of the original volume. Cells were transferred to ice-cold microcentrifuge tubes in 50 µL aliquots and mixed with 1-5 µL of pUCG18 plasmid (methylated and unmethylated). Cells were cooled for 5 min on ice then transferred to an ice-cold 0.1 cm BioRad electroporation cuvette. An exponential decay program for the BioRad GenePulser II was used with the capacitance range of 10-25 µF, voltage range 1500-2500 V, and the pulse controller set to 600 Ω. After the pulse was delivered, cells were immediately mixed with 0.5 mL of 2YT and allowed to recover for 4 hours at 60°C in a shaking incubator set to 220 rpm. The cultures were centrifuged at 1,800 x g for 2 min and cell pellets were resuspended in 100 µL of 2YT then plated on TBAB plates supplemented with kanamycin at 4 µg/mL. Plates were incubated at 60°C for 24-48 hours.

### *Ternary Conjugation with E. coli*

Cells were revived by streaking directly from the frozen stock onto the appropriate agar. *E. coli* strains C2925 (*dam*<sup>-</sup>/*dcm*<sup>-</sup>) and HB101/pRK2013 were grown on LB agar plates (10 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar (for plates), 1 L H<sub>2</sub>O) supplemented with kanamycin at 40 µg /µL and incubated overnight at 37°C. *G. caldoxylosilyticus* was grown on TBAB and incubated overnight at 60°C. Four or five colonies were picked from each strain and grown overnight; *E. coli* at 37°C in 30 mL of LB supplemented with kanamycin at 40 µg /mL and *G. caldoxylosilyticus* in 30 mL of 2YT at 60°C in a shaking incubators at

220 rpm. *E. coli* strains were subcultured into 20 mL of LB with antibiotic at a starting OD<sub>600</sub> of 0.1 and *G. caldoxylosilyticus* was subcultured into 100 mL of 2YT at a starting OD<sub>600</sub> of 0.1. Cells were incubated until an OD<sub>600</sub> between 0.3 and 0.6. A 10 mL aliquot of the *E. coli* C2925 (with pUCG18) donor strain and the HB101/pRK2013 helper strain were centrifuged together at 3,400 x g for 20 min. The supernatant was discarded and cells were washed and resuspended in LB media a total of 3 times. Similarly, 80 mL of *G. caldoxylosilyticus* was pelleted and washed 3 times. After the third centrifugation, cells were resuspended in 1 mL of LB and all three strains were combined in an 8:1:1 ratio into the same centrifuge tube and centrifuged at 3,400 x g for 20 min. The pellet was placed in the middle of an LB plate and incubated at 37°C for 6-16 hours, after which the pellet was resuspended in 2 mL of LB and plated on LB supplemented with kanamycin at 4 µg/mL and grown at 60°C for 24-48 hours.

#### *Protoplast Fusion (Zeigler 2001)*

*G. caldoxylosilyticus* was streaked on an mLB agar plate (10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar (for plates), 1.25 mL 10% (w/v) NaOH per liter) and grown at 60°C overnight. Four or five colonies from the fresh plate were grown in 30 mL of mLB at 60°C in a shaking incubator set to 220 rpm overnight. *G. caldoxylosilyticus* was subcultured into 20 mL of mLB to give a starting OD<sub>600</sub> of 0.3-0.5. The culture was grown at 60°C in a shaking incubator set to 220 rpm until late exponential phase, an OD<sub>600</sub> around 0.8-1.0. The culture was centrifuged at 1800-1900 x g for 5 min at room temperature. The pellet was resuspended in 4 mL of protoplasting media (mLB supplemented with 10% (w/v) lactose and 10 mM MgCl<sub>2</sub>). Lysozyme was added to 10 µg/mL by adding 40 µL of a 1 mg/mL lysozyme suspension in protoplasting media. Cells were incubated in a shaking water bath at 50°C and 130 rpm for 10 min. Protoplasts were diluted by adding 5 mL protoplasting media, and then gently pelleted by centrifuging at 700-800 x g for 7 min at room temperature. The pellet was suspended in 2 mL of protoplasting media and 0.1 mL of this protoplast suspension was mixed with 5-20 µL of pUCG18 plasmid DNA (methylated or unmethylated) and 0.9 mL of freshly prepared 40% (w/v) PEG 600 or PEG 10,000, prepared in protoplasting media. The culture was incubated in a shaking water bath at 50°C and 130 rpm for 2 min. The transformation mix was diluted with 2.5 mL of protoplasting media, and then pelleted by centrifugation at 700-800 x g for 7 min at room temperature. The pellet was resuspended in 0.1 mL of protoplasting media. The culture was incubated in shaking water bath at 50°C and 130 rpm for 1 h. A serial dilution of the culture was performed up to 1 x 10<sup>-5</sup>. Dilutions of 1 x 10<sup>-3</sup>, 1 x 10<sup>-4</sup>, and 1 x 10<sup>-5</sup> were plated onto regeneration plates (protoplasting media supplemented with 20 mM CaCl<sub>2</sub> and 1% agar) supplemented with kanamycin at 4 µg/µL and grown at 50°C for 12 hours, then at 60°C for 24-48 hours until colony formation. Once there was formation, colonies were immediately transferred to TBAB plates supplemented with kanamycin at 4 µg/mL and grown at 60°C for 24-48 hours to help reduce the loss of cell viability.

## **Results**

### *Electroporation of Electrocompetent Cells*

Settings were adjusted to give a 5-6 millisecond pulse decay time which allows for optimal efficiency, as reported in the literature. Growth of the controls showed that the cells are able to withstand the shock

and still be viable after recovery. Freshly prepared electrocompetent cells and electrocompetent cells from a frozen stock have been tested in the electroporation protocol to transform the plasmid.

#### *Ternary Conjugation with E. coli*

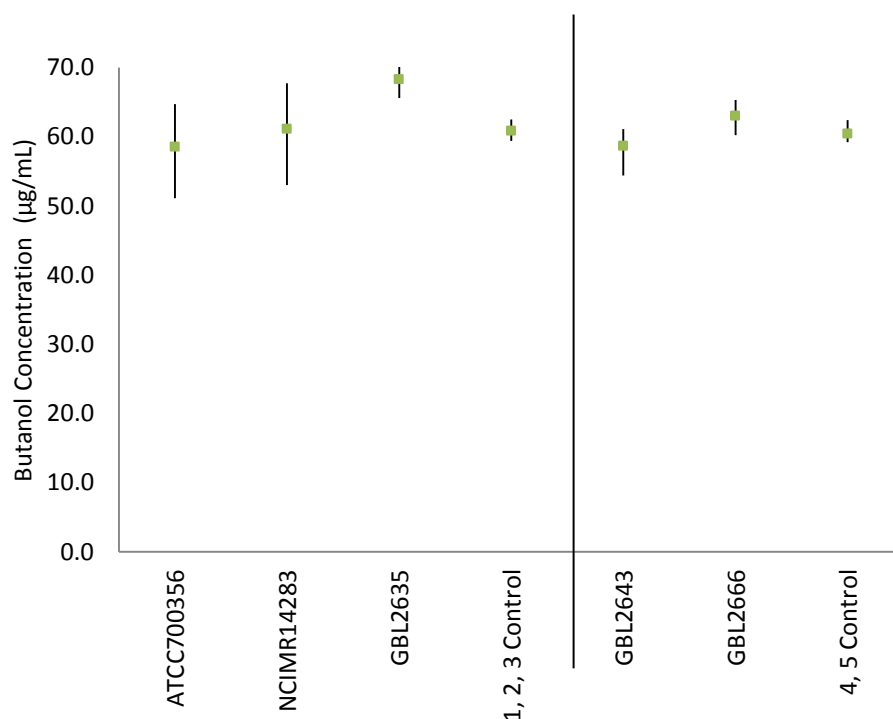
In an attempt to optimize growth of the *Geobacillus* bacteria, cells were plated without diluting. A ratio of *Geobacillus*: *E. coli* donor: *E. coli* helper cells of 8:1:1 was used to avoid overgrowth of the *E. coli* strains, as previously reported in the literature.

#### *Protoplast Fusion*

*G. caldoxylosilyticus* was previously shown to be inhibited at 4 µg/mL on LB plates, but on the regeneration plates there was no inhibition. The lack of complete inhibition allowed cells without the plasmid, thus without the kanamycin selectable marker, to grow. When colonies were transferred to TBAB plates supplemented with kanamycin at 4 µg/µL, cells would die due to lack of the plasmid. Different concentrations of kanamycin were screened for inhibition and *G. caldoxylosilyticus* grew up to 55 µg/µL when added to the TBAB plates.

#### *Butanol Production*

None of the provided strains produced any significant levels of butanol above the background of the no growth control (media only), as shown in Figure 2.



**Figure 2.** The concentration of butanol produced from *Geobacillus caldoxylosilyticus* (ATCC700356), *Geobacillus caldoxyloliticus* TK4 (NCIMB14283), *Bacillus licheniformans* (GBL2635), *Ureibacillus thermophaericus* (GBL2643), *Ureibacillus thermophaericus* (GBL2666) grown on 2YT media with xylose compared to a no growth control (media only control).

*Geobacillus caldoxylosilyticus* was the strain selected for further development based on the genetic analysis for butanol homologs and its ability to grow well on xylose. The experimental conditions for electroporation are shown in Table 1. The experimental conditions for ternary conjugation are shown in Table 2.

**Table 1. Electroporation** conditions tested for electrocompetent cells.

Electroporation run conditions <sup>a</sup>	Pulse Time (ms)	Growth
E1		
E1, 1µl, 2.5 µl, 5µl of P1, RTRM, R1	14.8-15.1	No
E1, 1µl, 2.5 µl, 5µl of P2, RTRM, R1	14.8-15.1	No
E1, 1µl, 2.5 µl, 5µl of P2, ICRM, R1	14.8-15.1	No
E1, 1µl, 2.5 µl, 5µl of P1, ICRM, R2	14.8-15.1	No
E1, 1µl, 2.5 µl, 5µl of P2, ICRM, R2	14.8-15.1	No
E2		
E2, 1µl, 2.5 µl, 5µl of P1, RTRM, R1	14.8-15.1	No
E2, 1µl, 2.5 µl, 5µl of P2, RTRM, R1	14.8-15.1	No
E2, 1µl, 2.5 µl, 5µl of P1, ICRM, R1	14.8-15.1	No
E2, 1µl, 2.5 µl, 5µl of P2, ICRM, R1	14.8-15.1	No
E2, 1µl, 2.5 µl, 5µl of P1, ICRM, R2	14.8-15.1	No
E2, 1µl, 2.5 µl, 5µl of P2, ICRM, R2	14.8-15.1	No
E3		
E3, 1µl, 2.5 µl, 5µl of P1, ICRM, R3	15.0-15.2	No
E3, 1µl, 2.5 µl, 5µl of P2, ICRM, R3	15.0-15.2	No
E4		
E4, 1µl, 2.5 µl, 5µl of P1, RTRM, R3	5.9-6.1	No
E4, 1µl, 2.5 µl, 5µl of P2, RTRM, R3	5.9-6.1	No
E4, 1µl, 2.5 µl, 5µl of P1, ICRM, R3	5.9-6.1	No
E4, 1µl, 2.5 µl, 5µl of P2, ICRM, R3	5.9-6.1	No
E4, 1µl, 2.5 µl, 5µl of P1, 55RM, R3	5.9-6.1	No
E4, 1µl, 2.5 µl, 5µl of P2, 55RM, R3	5.9-6.1	No

E1 denotes 2500V, 25uF, 600Ω. E2 denotes 1500V, 25uF, 600Ω. E3 denotes 2000V, 25uF, 600Ω. E4 denotes 2000V, 10uF, 600Ω. P1 denotes pUCG18 met<sup>+</sup>. P2 denotes pUCG18 met<sup>-</sup>. RTRM denoted room temperature recovery media. ICRM denotes ice-cold recovery media. 55RM denotes 55 °C recovery media. R1 denotes 55 °C, 220rpm, 16 hours. R2 denotes 40 °C, 0rpm, 4 hours. R3 denotes 50 °C, 220rpm, 2 hours.

**Table 2. Ternary conjugation** conditions for *E. coli* strains.

Starting OD <sub>600</sub>	Ratio <sup>a</sup>	Incubation (hours)	Growth
0.6	2:1:1	12	No
0.6	8:1:1	12	No
0.3	8:1:1	6	No
0.3	8:1:1	12	No
0.3	8:1:1	16	No

The ratio was *Geobacillus caldoxylosilyticus*:*dam*<sup>-</sup>/*dcm*<sup>-</sup> *E. Coli*:HB101



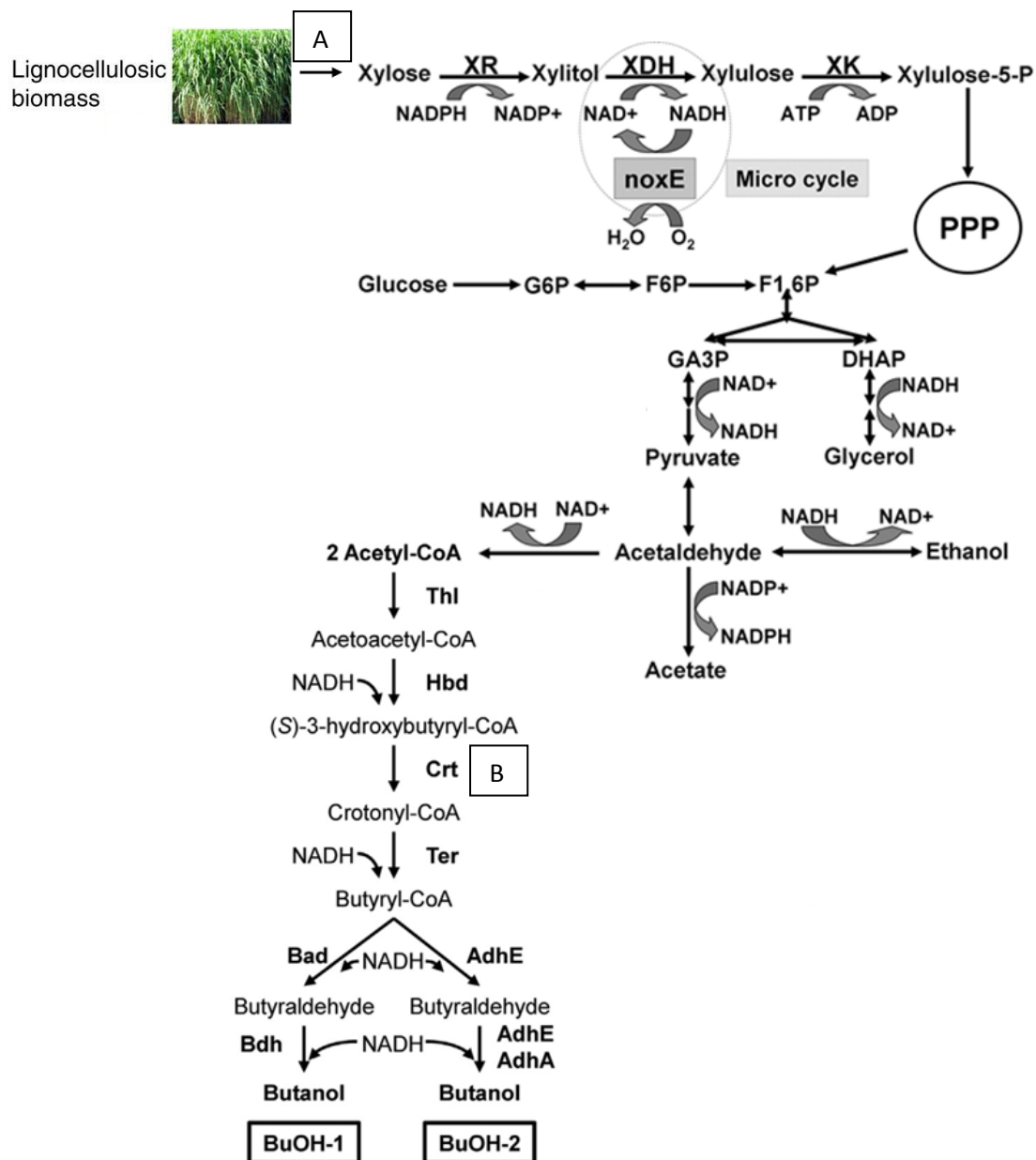
## Discussion

To date there have been no successful transformations of *G. caldoxylosilyticus* using the pUCG18 plasmid and testing the 3 transformation methods discussed.

*G. caldoxylosilyticus* was chosen for testing based on certain characteristics desired for potential butanol production. First, it has the ability to grow anaerobically on xylose and glucose at temperatures between 43 and 75°C, with optimal growth at 65°C. Literature reports that the enzymatic activities, xylose isomerase and xylulokinase were produced at high levels when grown on glucose, suggesting simultaneous consumption of xylose and glucose; a desired characteristic for efficient simultaneous saccharification. *G. caldoxylosilyticus* also has the ability to grow anaerobically on the substrates cellobiose, starch, and xylan. It was reported that alcohol dehydrogenase activity was detected when grown anaerobically, suggesting the possibility low ethanol production. Protein sequence homology searches revealed glycosyl hydrolase enzyme homologs for *beta*-xylosidase and *alpha*-L-arabinofuranosidase may be present in the genome of *G. caldoxylosilyticus*; these enzymes play a role in the deconstruction of xylan (Figure 3). *G. caldoxylosilyticus* genome sequence also has an annotated *Crt* gene coding for the 3-hydroxybutyryl-CoA, suggesting a butanol pathway or part of a pathway may be present (Figure 3).

The Bacillus Genetic Stock Center catalog was consulted for media recipes and protocols, suggesting TBAB media as the best general-purpose solid media for *Geobacillus*. Each strain formed large, regular colonies on TBAB (Zeigler 2001).

A smaller plasmid pUCG3.8 was synthetically derived by an academic group in the UK and was recently obtained to replace the larger pUCG18. The pUCG3.8 was derived from pUCG18 so it contains all essential genes needed, but the smaller size could help increase the efficiency of the transformation, as reported by Leak, et al. All the transformation methods reported above will be attempted using the smaller pUCG3.8 plasmid (Bartosiak-Jentys 2013). Limited research has been done on *G. caldoxylosilyticus* so there could also be a restriction modification system that is preventing a successful transformation with the pUCG18 plasmid and may even inhibit the pUCG3.8 plasmid.



**Figure 3.** The production pathway of butanol from xylan to the central metabolite acetyl-coA and the enzymes needed to convert acetyl-CoA to butanol. Box A is the process where glycosyl hydrolase enzymes convert xylan to xylose. Box B is the enzyme 3-hydroxybutyryl-CoA, the committed step for butanol production.

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