Final Technical Report

Report Number: DOE-OSU-EE0007005

DOE Award Number: DE-EE0007005

Recipient Name: The Ohio State University

Project Title: Engineering Clostridia for *n*-Butanol Production from Lignocellulosic Biomass and CO_2

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Acknowledgment: This material is based upon work supported by the Department of Energy's Office of Energy Efficiency and Renewable Energy under the Bioenergy Technologies Office, Award Number DE-EE0007005.

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Executive Summary

The objective of this project was to engineer novel *Clostridium* strains to produce *n*-butanol from low-cost lignocellulosic biomass and gases (CO_2 and H_2). Butanol is an important industrial solvent and potentially a superior fuel that can fit the existing fuel infrastructure and replace gasoline in auto engines without modification. This project focused on metabolic engineering of cellulolytic *Clostridium cellulovorans* for directly converting cellulose to *n*-butanol and carboxydotrophic *Clostridium carboxidivorans* to convert CO_2 and H_2 to butanol and ethanol. These clostridial strains were engineered using synthetic biology, gene overexpression and gene knockout strategies to produce *n*-butanol as the main product from lignocellulose and gaseous substrates. With these metabolically engineered strains, high-titer and high-yield *n*-butanol production from lignocellulosic biomass were achieved with minimal CO_2 released to the environment in a sustainable and co-cultured consolidated bioprocess (CBP) integrated with *in situ* butanol separation by gas stripping. A well-to-pump life cycle analysis showed that the integrated biobutanol production process could reduce CO_2 and greenhouse gas (GHG) emissions by ~75% compared to traditional chemical process. Moreover, the production of cellulosic butanol from CBP would not require enzymatic hydrolysis, an expensive step in current biorefinery using lignocellulosic biomass. The successful development of CBP using engineered clostridia for *n*-butanol production from lignocellulosic biomass and CO_2 can meet the BETO 2022 cost target of $3/gallon gasoline equivalent (gge) for advanced biofuels. With the higher energy content, butanol will be cheaper than ethanol and similar to gasoline at $2.50/gal in terms of energy cost ($ per MJ). With the reduced production cost and product value, biobutanol from lignocellulose and CO_2 can one day become a major liquid transportation biofuel, replacing bioethanol and gasoline.

Strain development via metabolic engineering and fermentation process optimization, assisted by omic analysis of the mutation and process effects, helped us to achieve our goal of producing *n*-butanol and ethanol (biofuel) from lignocellulosic biomass at a cost of $3/gge (gallon gasoline equivalent) or less. The co-fermentation technology using both cellulose and CO_2/H_2 for biofuel production can greatly increase product yield from the biomass feedstock while also reduce GHG emissions, and is novel and advantageous compared to existing ethanol and acetone-butanol-ethanol fermentation technologies. The same metabolic engineering and process approaches can also be applied to other microorganisms to produce bio-based chemicals from renewable resources. Overall, the CBP technology using the novel engineered clostridia is unique and innovative, and complements well with the Biochemical Conversion technologies in the BETO Multi-Year Program Plan (MYPP), which largely focuses on the hydrolysis of cellulosic biomass and utilization of the hydrolysates for ethanol production.

Introduction

Biobutanol is an attractive with superior fuel properties (high energy density, lower volatility, etc.) and fits the existing fuel infrastructure with lower water miscibility, flammability, and corrosiveness than ethanol, and can directly replace gasoline in car engines without modification. *n*-Butanol can also be dehydrated to 1-butene and catalyzed into longer chain oligomers for aviation fuel applications. In this project, we aimed at the development of CBP using engineered clostridia for *n*-butanol production from lignocellulosic biomass and CO_2, which potentially can meet the BETO 2022 cost target of $3/gal gasoline equivalent for advanced biofuels.

The use of lignocellulosic feedstocks for ethanol fermentation has been problematical given the complexity of pre-treatment and cellulose hydrolysis and the associated capital and operational costs. In addition, harsh pre-treatments generate inhibitors that affect fermentation performance. One alternative is to engineer microbes to utilize cellulose directly in a process generally referred to as CBP. This approach has been pursued for ethanol via the heterologous expression of cellulases in *S. cerevisiae* and engineered strains of *E. coli*, *Klebsiella oxytoca* and *Zymomonas mobilis* but the results have generally been disappointing. Meanwhile, efforts on the cloning cellulases in solventogenic *Clostridium acetobutylicum* have not generated a strain capable of producing butanol directly from cellulose either.

Novel low cost feedstocks are important because in the conventional acetone-butanol-ethanol (ABE) fermentation process, starch feedstocks contribute to about 70% of the overall production cost. Also, approx. 34% of the carbon from the feedstock is converted to CO_2 and the fermentation also produces H_2. These gases are typically just vented to atmosphere. Today, butanol production costs are competitive for the chemical sector but cannot compete in the biofuel market. Cellulose, CO_2 and syngas are readily available and low cost but difficult to ferment. There are microbes that can efficiently utilize cellulose or gases to produce ethanol and organic acids, but not *n*-butanol. In this project, metabolic engineering was applied to both cellulolytic and acetogenic clostridia to produce *n*-butanol directly from cellulose and CO_2. A novel and integrated process for *n*-butanol production from cellulose and CO_2 using the engineered microbes was envisioned and evaluated in this project for potential commercial development.

This project focused on the engineering of *C. cellulovorans*, a cellulosome-producing acidogen, for converting cellulose and hemicellulose to *n*-butanol. Cellulolytic clostridia with cellulosome are highly efficient in degrading and hydrolyzing crystalline cellulose and hemicellulose, and thus offer exceptional bioprocessing applications. In particular, *C. cellulovorans*, which produces butyrate as the major fermentation product, with some acetate, lactate and formate as byproducts (see **Figure 1**), is an idea host for biobutanol production from

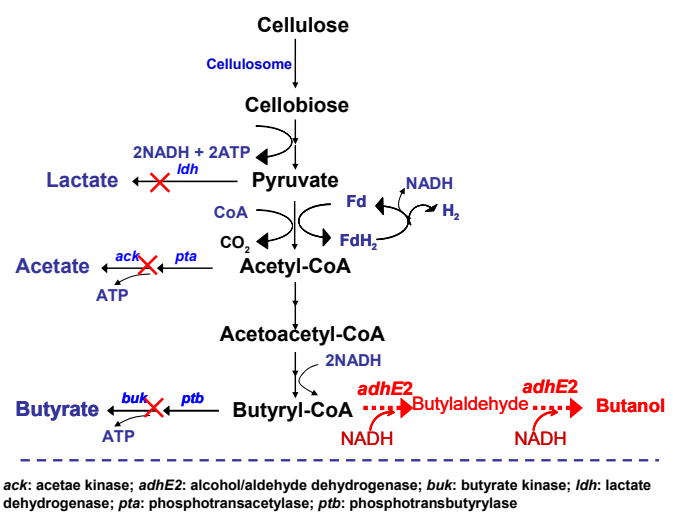


Figure 1. A novel butanol fermentation pathway in *C. cellulovorans* with *adhE*2 or *aad* cloned from *C. acetobutylicum* and acetate, lactate and butyrate pathways knocked out to produce only butanol from cellulose.

cellulose. Only one heterologous gene, *aad* or *adhE*2, is required to make butanol from cellulose because native metabolic pathway from cellulose to butyryl-CoA already exists in *C. cellulovorans.* This not only would simplify the cloning process, but also could give higher butanol productivity. In addition, the acidogenic pathways in *C. cellulovorans* could be knocked down to further increase butanol yield from cellulose. With a simple butanol biosynthesis pathway, the engineered *C. cellulovorans* would be able to produce more butanol as the sole or main fermentation product, with a theoretical maximum butanol yield of 0.42 g/g cellulose. Butanol as the only or main fermentation product also would simplify the downstream purification process and lower the butanol production cost significantly compared to conventional ABE fermentation. In addition, acetogenic *Clostridium* that could convert CO_2 and H_2, byproducts from clostridial fermentations, to acetate (and butyrate) with high yield, titer and productivity, would also be engineered to produce *n*-butanol. By converting the CO_2 and H_2 produced in cellulose fermentation can increase the overall butanol yield by 50%, reaching a theoretical max value of 0.63 g/g cellulose.

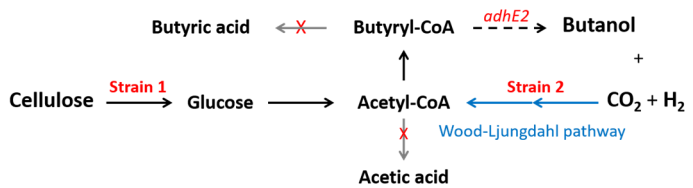


Figure 2. Production of *n*-butanol from cellulose by engineered cellulolytic *C. cellulovorans* (Strain 1) and CO_2-fixing acetogenic bacteria (Strain 2) overexpressing *adhE*2. The genes in acetate and butyrate biosynthesis pathways can be knocked out to produce butanol as the main product from cellulose.

Figure 2 illustrates the concept of a co-cultured fermentation process with the acetogen producing *n*-butanol from CO_2 and H_2 generated by the cellulolytic clostridia, which has the potential to achieve an overall butanol production of >0.45 g/g cellulose, with minimal CO_2 released to the environment, at a reactor productivity of 0.25–1.0 g/L·h suitable for commercial scale up. The engineered clostridia mutant strains would be used in a high-cell density (HCD) fermentation process integrated with online butanol recovery for economical production of *n*-butanol from selected lignocellulosic biomass.

Introducing and overexpressing genes in the CoA-dependent n-butanol biosynthesis pathway into cellulolytic and acetogenic Clostridia enabled them to produce *n*-butanol directly from cellulose and CO_2/H_2. This novel approach could significantly improve butanol yield and reduce greenhouse gas **(**GHG) emissions. Yield improvements from lower cost feedstocks would transform process economics. We aimed to produce *n*-butanol from cellulosic biomass and CO_2/H_2 at a projected cost of <$2.25/gal, which is significantly lower than the current market price of $6.75/gal for synthetic butanol derived from oil in a market of 1.4 B gal/yr.

In this report, we summarize important accomplishments and main activities with most significant findings and data obtained in this three-year (FY 2016-2018) project carried out by three partners at the Ohio State University (OSU), Green Biologics (GB), and University of Alabama at Birmingham (UAB).

Accomplishments

Goals and Objectives

The goal of this project was to develop engineered clostridial strains and fermentation process that can directly utilize cellulose and fix CO_2 for *n*-butanol production from lignocellulosic biomass at a targeted cost of $2.25/gal or less than $3/gge (gallon gasoline equivalent). Through metabolic engineering and process improvements, butanol titer, yield, and production rate would be increased significantly in fermentation to lower the production cost from cellulosic biomass. This project had three partners - Ohio State University (OSU), Green Biologics (GB), and University of Alabama at Birmingham (UAB) and the following four objectives or main tasks:

Task A. Engineering clostridia for *n*-butanol production from cellulose and CO_2/H_2 (OSU)
Task B. Fermentation kinetics studies and process optimization (GB & OSU)
Task C. Omic analysis of mutants in fermentation (UAB)
Task D. Process design & cost analysis (GB & OSU)

All tasks described in the original proposal were completed, meeting our milestones and targets as summarized below.

Task A: Metabolic engineering of *C. cellulovorans* and acetogens for butanol production from cellulose and CO_2 and H_2

During the first year, we developed new cloning vehicles (plasmids) with better compatibility with *C. cellulovorans* that greatly increased transformation efficiency to facilitate metabolic engineering study and strain development. After that, we successfully constructed 20 engineered strains overexpressing various heterologous genes and evaluated their fermentation kinetics in serum bottles (details see **Activities**). The best *C. cellulovorans* strain met all of our milestones.

Milestone	Description (Targeted Quarter to Meet)	Status
M1.1	*C. cellulovorans* producing butanol from cellulose at a yield >0.1 g/g (Q1); >0.15 g/g (Q2); >0.2 g/g (Q3)	√
M1.2	A strain producing butanol and ethanol from CO_2 and H_2 at >0.1 g/L (Q4); >0.4 g/L (Q6)	√
M1.3	Strains producing little or no acids, with butanol and ethanol at >0.3 g/g (Q5); >0.35 g/g (Q6)	√
M1.4	A high butanol tolerant strain capable of producing butanol at >2.5 g/L (Q4); >5 g/L (Q6)	√
Go/No-Go #1	Select strains producing *n*-butanol at titer of >2.5 g/L, yield of 0.2 g/g cellulose (Q4)	√

We first evaluated 4 acetogens for their ability to use CO_2/H_2 in serum bottles. For metabolic engineering of acetogens for butanol and ethanol production from CO_2 and H_2, We found that all acetogens also have very robust restriction modification (RM) systems, hindering effective transformation of recombinant plasmids into host cells. We analyzed RM systems based on available genomic sequences to identify key restriction sequences and methylation method to protect plasmids. Then, we developed new plasmids with better compatibility with *C. aceticum* that greatly increased transformation efficiency to facilitate metabolic engineering study and strain

development. However, we later found that *C. aceticum* was complex to engineer for alcohol production from syngas due to redox imbalance issue. On the other hand, *C. carboxidivorans* appeared to be a better candidate for alcohol production. Finally, we constructed recombinant plasmids for expressing *adhE2* and additional dehydrogenase genes for ethanol and butanol production, and obtained several engineered mutant strains of *C. carboxidivorans*, which met our milestone (M1.2).

Task B: Fermentation kinetics studies and process optimization

Engineered *C. cellulovorans* strains developed in Task A were evaluated for butanol production from cellulose and glucose in serum bottles and stirred-tank bioreactors (1-5 liters) with process parameters (pH, substrate concentration, etc.) optimized. Different pretreatment methods for enhancing cellulose degradability were evaluated for crystalline cellulose as well as several lignocellulosic biomass (corn fiber, soybean hull, sugarcane bagasse, cotton stalk, etc.). In general, acid pretreatment improved fermentability of biomass, although *C. cellulovorans* was able to use untreated cellulose and hemicellulose present in the biomass for butanol production.

Gas fermentation kinetics was evaluated in serum bottles and different types of bioreactors (stirred-tank, bubble column, packed bed). Medium optimization was attempted to increase cell density, activity, and productivity. In general, a rich medium with cell immobilization could increase the cell density to the level sufficient to meet the productivity target.

Milestone	Description (Targeted Quarter to Meet)	Status
M2.1	Fermentation kinetics profiles showing butanol production >2.5 g/L, yield >0.2 g/g (Q4)	√
M2.2	Optimized medium to support cell growth at density >OD 10 (Q4)	√
M2.3	Reactor for high cell density fermentation, >OD 20 (Q5), productivity >0.1 g/L·h (Q7)	√
M2.4	Mixed fermentation process with cellulosic and gaseous substrates producing butanol and ethanol at >5 g/L	√
M2.5	Fermentation process and reactor design producing *n*-butanol and ethanol at 10 g/L, 0.3 g/g, and 0.2 g/L·h in cellulose-gaseous fermentation	√

The total alcohol (butanol and ethanol) production from cellulose reached about 9 g/L, slightly lower than our final target of 10 g/L (M2.5) (see **Activities**). We found that butanol production ceased after reaching ~5 g/L due to sporulation, which limited cells for continued fermentation. Attempts to adapt cells for a higher butanol tolerance was not successful, again due to sporulation. It is thus necessary to knock down genes initiating sporulation to allow continued fermentation for butanol biosynthesis, which should be a high priority research in the future. Nevertheless, *In situ* product recovery by adsorption or gas stripping can be used to alleviate butanol toxicity, which would allow the process to achieve a final butanol titer of >10 g/L as demonstrated with different strains.

Task C: Omics analysis of mutant strains under various fermentation conditions

We completed comparative proteomics analysis for *C. cellulovorans-adh*E2 in batch fermentations with glucose and cellulose as carbon source, respectively. Compared to the wild type, several proteins (enzymes) in glycolysis and metabolic pathways leading to butanol biosynthesis were up-

regulated or down-regulated, which would be the targets for metabolic engineering. We also completed comparative metabolomics analysis for *C. cellulovorans-adh*E2 in batch fermentations with glucose and cellulose as carbon source, respectively. Finally, with these omic data and available fermentation kinetics data, a dynamic metabolic flux control model was established and used to identify metabolic flux bottlenecks (rate-limiting steps) and possible gene targets for further metabolic engineering to improve butanol production from cellulose.

Milestone	Description (Targeted Quarter to Meet)	Status
M3.1	Proteomics profiling of mutants generated and suitable cell engineering strategy identified (Q4)	√
M3.2	Core metabolites responsible for carbon, energy and redox balance identified to assist process development and scale-up (Q7)	√

Task D: Process design and cost analysis

New process concepts and designs for butanol production from lignocellulosic feedstocks were evaluated based on available fermentation data from Task B, starting with a baseline case detailing separate unit operations and then build out designs with increasing complexity based on process consolidation. The integration of unit operations was made possible through advances in fermentation technology and from the introduction of engineered microbes utilizing cellulose and gases. Mixed-culture fermentation process using cellulolytic, solventogenic, and carboxidotrophic clostridia was evaluated for its feasibility and potential advantages compared to monoculture fermentations. Integrated fermentation process with *in situ* product recovery was also evaluated. Finally, life cycle and cost analyses were performed, demonstrating the advantages of the proposed integrated process for biobutanol production at a projected cost of $2.25/gal or $3/gallon gasoline equivalent with more than 75% reduction in GHG emissions compared to conventional chemical synthesis process (see **Activities**).

Milestone	Description	Status
M4.1	Pre-treatment process selected. The process and conceptual plant design with outline butanol production costs achieved	√
M4.2	Process and conceptual plant design of advanced fermentation defined	√
M4.3	Process and conceptual plant design for CBP and co-culture completed	√
M4.4	A conceptual plant for butanol production from lignocellulosic biomass at $2.25/gal with 90% reduction in GHG emissions	√

Project Activities

Task A: Engineering Clostridia for *n*-butanol production from cellulose and CO_2/H_2

Planned Activities

Subtask A.1: Expression of butanol biosynthesis pathway in *C. cellulovorans*
The current mutant strain of *C. cellulovorans* expressing *adhE2* also produces a large amount of ethanol, which greatly reduces butanol production. One strategy to minimize ethanol production and maximize butanol production is to replace the bifunctional acetaldehyde/alcohol dehydrogenase (*adhE2*), which has a low selectivity for butanol, with aldehyde dehydrogenase

(*ald*) and butanol dehydrogenase (*bdh*), which has a higher selectivity for butanol, in *C. cellulovorans*. Another strategy is to overexpress thiolase (*thl*) of *C. tyrobutyricum* in *C. cellulovorans* to increase the metabolic flux from acetyl-CoA to butyryl-CoA. In addition, it is necessary to increase intracellular NADH availability for butanol biosynthesis and redox balance by, such as, overexpressing formate dehydrogenase and supplying artificial electron carriers (e.g., methyl viologen).

Subtask A.2: Expression of butanol biosynthesis pathway in acetogenic clostridia
Acetogenic clostridia such as *C. aceticum* capable of converting CO_2 and H_2 to acetate with a high yield, titer, and productivity will be engineered to produce ethanol and butanol by overexpressing *adhE2*, *aor*, and genes (*thl*, *hbd*, *crt*, and *bcd-etfA-etfB* or *ter*) in the pathway from acetyl-CoA to butyryl-CoA.

Subtask A.3: Knockout genes in competing pathways to enhance butanol yield
In order to have a high butanol yield, it may be necessary to inactivate the competing acetate, butyrate and ethanol pathways in *Clostridium* by knocking out corresponding genes.

Subtask A.4: Adaptive evolutionary engineering to enhance butanol tolerance
High butanol tolerance of butanol-producing bacteria is a prerequisite for reaching a high butanol titer in fermentation. Butanol tolerance and production can be greatly increased by adaptation or evolutionary engineering under gradually increased butanol stress. Through mutagenesis, adaptation, and screening, strains that can tolerate 10 g/L butanol may be isolated.

Completed Activities and Major Findings

Engineering C. cellulovorans for n-butanol production from cellulose
Totally, we constructed 20 recombinant plasmids for overexpressing various genes in *C. cellulovorans* (**Table 1**). The effects of overexpressing these genes on butanol biosynthesis were evaluated in serum bottles with glucose and cellulose, respectively, as the substrate, and the fermentation kinetics results are summarized in **Table 2** and **Table 3**. We also performed metabolic flux analysis to further evaluate the gene expression effects on butanol production and identify possible metabolic bottlenecks for future metabolic engineering studies.

Table 1. *C. cellulovorans* mutants overexpressing various genes for butanol production.

#	Gene	Purpose	Results or Findings
1	*adhE2*	*adh*E2 overexpression for butanol and ethanol production from butyryl-CoA and acetyl-CoA, respectively	Mutant produced butanol and ethanol with acetate and butyrate as two byproducts.
2	*adhE2-bdhB*	*adhE2* and *bdhB* overexpression for butanol production with increased selectivity over ethanol	Mutant produced a similar amount of butanol but more butyrate and less ethanol, resulting in higher C4/C2 and butanol/ethanol ratios.
3	*adhE1-bdhB*	*adhE1* and *bdhB* overexpression for butanol production with increased selectivity over ethanol	Mutant produced similar amounts of butanol and ethanol but more butyrate, resulting in a higher C4/C2 ratio.
4	aor^{CC}	aor^{CC} (from *C. carboxidivorans* P7) overexpression for butanol and ethanol production from aldehyde, respectively	No butanol and little ethanol were produced because of the lack of butanol dehydrogenase gene in the genome.
5	*adhE2-*aor^{CL}	*adhE2* and aor^{CL} (from *C. ljungdahlii* DSM 13528) for alcohol production with reduction of acetate and butyrate	Mutant produced little alcohol, probably because the AOR from *C. ljungdahlii* worked in the reverse direction.

6	*adhE2-aor*^{CC}	*adhE2* and *aor*^{CC} for butanol and ethanol production.	Mutant produced more butanol and butyrate but less ethanol, resulting in higher C4/C2 and butanol/ethanol ratios.
7	*adh*E2-*thl*^{CA}	*adh*E2 and *thl*^{CA} (from *C. acetobutylicum* ATCC 824) for increased flux toward C4 over C2 metabolites	Mutant gave a higher C4/C2 ratio but less butanol.
8	*adh*E2-*thl*^{V5A}	*adh*E2 and *thl*^{V5A} (from *C. acetobutylicum* BMK19) for increased flux toward C4 over C2 metabolites	Mutant gave a higher C4/C2 ratio but less butanol.
9	*adh*E2-*thl*^{CT}	*adh*E2 and *thl*^{CT} from *C. tyrobutyricum* ATCC 25755 for butanol production with increased flux toward C4 over C2 metabolites	Mutant produced much more butyrate with a significantly higher C4/C2 ratio, but butanol production was low.
10	*adh*E2-*thl*	*adh*E2 and *thl* from *C. cellulovorans* for butanol production with increased flux toward C4 over C2 metabolites	No significant effect - mutant produced comparable amounts of alcohols and acids compared to overexpressing *adhE2* only.
11	*adh*E2-*hbd*	*adh*E2 and *hbd* from *C. tyrobutyricum* for butanol production with increased flux toward C4 over C2 metabolites	Mutant gave a higher C4/C2 ratio but less butanol.
12	*adh*E2-*thl*^{CA}-*hbd*	*adhE2*, *thl*^{CA}, and *hbd* overexpression for butanol production with increased flux toward C4 over C2 metabolites	Mutant produced more butyrate but less butanol and ethanol with a much higher C4/C2 and butanol/ethanol ratios. Butanol production increased to >5 g/L and butyrate production decreased when MV was added in the fermentation.
13	*adh*E2-*fdh*	*adhE2* and *fdh* overexpression for increased NADH availability to favor butanol biosynthesis over acids	Mutant produced little alcohol, probably because the FDH worked in the reverse direction.
14	*adh*E2-*fnr*	*adhE2* and *fnr* overexpression for increased NAD(P)H availability to favor butanol biosynthesis over acids	Mutant produced comparable amount of butanol but less ethanol, acetate, and butyrate.
15	*adh*E2-*thl*^{CA}-*fnr*	*adhE2*, *thl*^{CA}, and *fnr* overexpression for butanol production with increased flux toward C4 over C2 metabolites	Mutant gave a higher C4/C2 ratio with much more butyrate but slightly less butanol.
16	*adh*E2-*ctfAB*	*adhE2* and *ctfAB* overexpression for acetate and butyrate re-assimilation to increase alcohol production	Mutant produced more ethanol and butanol and less acetate and butyrate, resulting in a higher alcohol/acid ratio.
17	*adhE2-thl*^{CA}-*ctfAB*	*adhE2, thl*^{CA}, and *ctfAB* overexpression for butanol production with increased C4/C2 and alcohol/acid ratios	Mutant produced more butyrate but less alcohols, resulting in a low alcohol/acid ratio.
18	*adhE2-pk*	*pk* overexpression for glycolysis acceleration	No significant effect - mutant produced a comparable amount of butanol; the specific growth rate was slightly lower.
19	*adh*E2-*pgk*	*pgk* overexpression for glycolysis acceleration	No significant effect - mutant produced a comparable amount of butanol; no significant change in the specific growth rate.
20	*adh*E2-*pfk*	*pfk* overexpression for glycolysis acceleration	No significant effect - mutant produced less butanol; no significant change in the specific growth rate.

Table 2. Fermentation kinetics of glucose in serum bottles by various strains of *C. cellulovorans*.

Strain	Ethanol (g/L)	Butanol (g/L)	Acetate (g/L)	Butyrate (g/L)	Total alcohol (g/L)	Butanol yield (g/g)	Alcohol/ acid ratio	Butanol/ ethanol ratio	C4/C2 ratio
*adh*E2	2.14	1.76	3.09	0.96	3.90	0.09	0.96	0.82	0.52
*adh*E1-*bdh*B	0.21	1.47	2.41	4.88	1.68	0.08	0.23	**7.00**	**2.42**
*adh*E2-*bdh*B	1.47	1.66	1.99	4.61	3.31	0.09	0.47	1.10	1.81
*adh*E2-*aor*^{CC}	0.61	**2.44**	3.99	3.66	3.05	**0.14**	0.40	**4.00**	1.37
*adh*E2-*aor*^{CL}	0.16	0.00	1.96	6.30	0.16	0.00	0.02	0.00	2.96
*adh*E2-*thl*^{CA}	0.62	0.94	2.11	4.23	1.56	0.06	0.25	1.53	**1.90**
*adh*E2-*thl*^{V5A}	1.24	1.59	2.94	1.77	2.84	0.11	0.60	1.28	0.80
*adh*E2-*thl*^{CT}	0.39	0.27	1.73	4.51	0.63	0.02	0.66	0.68	**2.25**
*adh*E2-*thl*	2.42	1.58	2.91	0.77	4.00	0.09	1.09	0.65	0.44
*adh*E2-*hbd*	0.75	1.17	2.61	3.08	1.92	0.07	0.34	1.56	1.26
*adh*E2-*thl*^{CA}-*hbd*	0.23	0.82	1.94	5.28	1.05	0.05	0.15	3.57	**2.82**
*adh*E2-*thl*^{CA}-*hbd* (MV)	0.83	**5.50**	1.78	3.02	6.33	**0.27**	**1.32**	**6.63**	**2.84**
*adh*E2-*fdh*	0.19	0.20	1.98	6.43	0.39	0.01	0.05	1.00	3.06
*adh*E2-*fnr*	0.35	**2.36**	1.99	4.61	2.70	**0.15**	0.41	**6.81**	**2.99**
*adh*E2-*thl*^{CA}-*fnr*	0.51	1.33	2.33	3.76	1.84	0.10	0.30	2.61	1.79
*adh*E2-*ctfAB*	2.28	2.10	2.71	0.86	4.37	0.11	**1.23**	0.92	0.59
*adh*E2-*thl*^{CA}-*ctfAB*	1.83	1.25	1.90	3.80	3.09	0.08	0.54	0.68	1.23
*adh*E2-*pk*	1.15	1.84	2.29	0.60	2.99	0.13	1.03	1.60	0.71
*adh*E2-*pgk*	1.30	1.48	3.06	0.93	2.78	0.12	0.70	1.14	0.55
*adh*E2-*pfk*	1.61	1.12	2.53	0.72	2.74	0.09	0.84	0.70	0.45

Table 3. Fermentation kinetics of cellulose in serum bottles by various strains of *C. cellulovorans*.

Strain	Ethanol (g/L)	Butanol (g/L)	Acetate (g/L)	Butyrate (g/L)	Total alcohol (g/L)	Butanol yield (g/g)	Alcohol/ acid ratio	Butanol/ ethanol ratio	C4/C2 ratio
*adh*E2	2.01	**2.00**	2.00	1.57	4.01	0.11	1.12	0.99	0.89
*adh*E2-*bdh*B	0.26	0.32	2.32	6.32	0.58	0.02	0.07	1.22	2.57
*adh*E1-*bdh*B	0.20	1.11	2.26	4.75	1.30	0.06	0.19	**5.65**	2.38
*adh*E2-*aor*^{CC}	0.58	**2.29**	1.92	3.97	2.87	0.12	0.49	3.95	2.50
*adh*E2-*aor*^{CL}	0.17	0	2.14	6.61	0.17	0	0.02	0	2.86
*adh*E2-*thl*^{CA}	1.83	2.13	2.50	3.29	3.96	0.12	5.79	0.68	1.25
*adh*E2-*thl*^{CA} (MV)	**4.74**	**4.31**	0.68	0.89	**9.05**	**0.23**	**5.75**	0.91	0.96
*adh*E2-*hbd*	1.94	1.92	2.32	1.92	3.86	0.10	4.24	0.91	0.90
*adh*E2-*thl*^{CA}-*hbd*	0.09	0.10	1.47	4.50	0.19	0.01	0.03	1.11	2.95
*adh*E2-*thl*^{CA}-*hbd* (MV)	0.33	**4.02**	0.62	1.55	4.36	**0.26**	2.01	**12.18**	**5.87**
*adh*E2-*fnr*	0.96	3.06	2.37	2.24	4.01	0.16	0.87	3.20	1.59
*adh*E2-*fnr* (MV)	1.93	**5.28**	0.63	0.73	7.22	**0.33**	**5.30**	2.74	2.35
*adh*E2-*fdh*	0.41	1.01	2.63	5.42	1.42	0.06	0.18	2.45	2.12

Metabolic flux analysis. As shown in **Figure 3**, system-metabolic engineering strategies were applied for enhancing *n*-butanol biosynthesis in *C. cellulovorans*, including 1) optimizing butanol biosynthesis pathway by screening and selecting the most efficient bifunctional acetaldehyde/alcohol dehydrogenase (*adh*E2) for converting butyryl-CoA to *n*-butanol; 2) increasing carbon flux from acetyl-CoA to butyryl-CoA by overexpressing thiolase (*thl*^{CA}) and NADPH-dependent 3-hydroxybutyryl dehydrogenase (*hbd*^{CT}); 3) improving the intracellular

NADH availability by overexpressing ferredoxin dehydrogenase (*fnr*); and 4) weakening competitive acids biosynthesis by overexpressing CoA transferase (*ctf*AB) and aldehyde oxidoreductase (*aor*), which facilitate the re-assimilation of butyrate to butyryl-CoA and butyraldehyde, respectively. In this quart, we further compared these mutants using available fermentation kinetics data for metabolic flux analysis. The measured specific uptake rate of glucose or cellulose and production rates of the different products were used for estimating the intracellular fluxes. Flux distributions for various mutants are compared and shown in **Figure 4**.

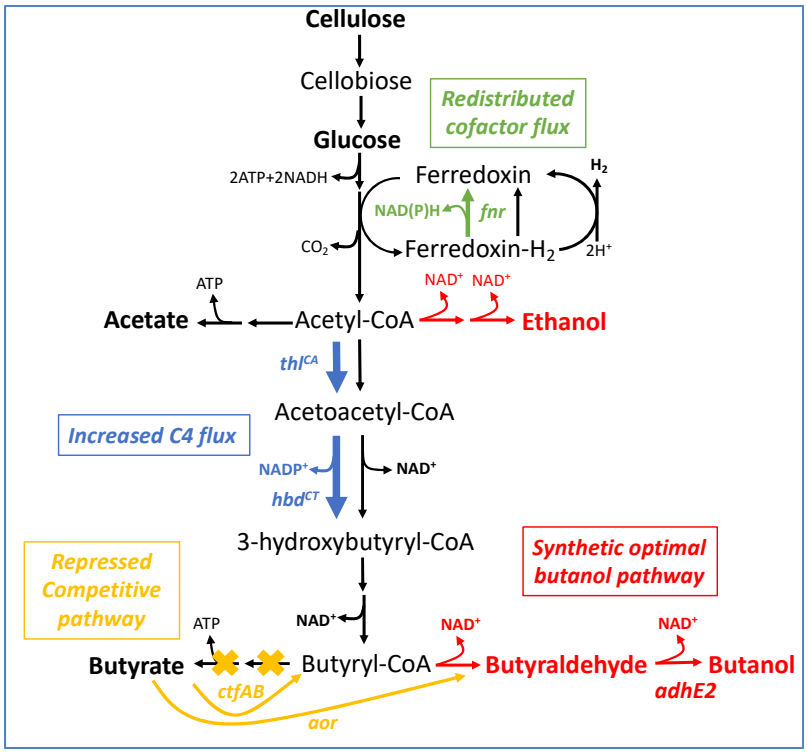


Figure 3. Metabolic pathways for *n*-butanol biosynthesis in *C. cellulovorans*. Key genes studied for their effect on the metabolic flux: *adh*E2 (acetaldehyde/alcohol dehydrogenase), *thl* (thiolase), *hbd* (3-hydroxybutyryl dehydrogenase), *fnr* (ferredoxin dehydrogenase), *ctf*AB (CoA transferase) and *aor* (aldehyde oxidoreductase).

According to the flux distribution shown in **Figure 4**, the fluxes varied at four main key nodes: acetyl-CoA/butyryl-CoA, ethanol/butanol, acetate/butyrate and ferredoxin/NADH nodes. Compared with that of wild-type strain (Fig. 4A), the mutant overexpressing *adh*E2 distributed more carbon flux for alcohol production, although the fraction to ethanol was in the majority, leading to lower fraction of carbon flux from acetyl-CoA to butyryl-CoA (41 vs. 72). As expected, overexpressing thl^{CA} and *hbd* with *adh*E2 affected the fraction from C2 to C4 (Fig. 4B), which contributed to butyrate and butanol formation. However, most of increased C4 flux was distributed to butyrate formation (15 vs. 61 compared with overexpression of *adh*E2). Meanwhile, the flux from ferredoxin for NADH generation also decreased to 136, indicating that a lack of NADH for the reduction of butyryl-CoA to butanol. In contrast, as shown in Fig. 4C, acids production flux was inhibited while alcohols production flux increased with the addition of 100 μM methyl viologen (MV). Unlike the carbon flux distribution in *adh*E2 mutant, in which ethanol production flux was more prominent than in butanol, the mutant over-expressing *adh*E2, thl^{CA}, and *hbd* further increased butanol production flux to 56. On the other hand, as shown in Fig. 4D, the mutant co-expressing *fnr* with *adh*E2 also showed a higher C4 flux and butanol production flux, probably because overexpressing *fnr* can increase NADH availability and thus favor butanol production. However, Significant amounts of acids were still produced by this mutant. Down-regulation of butyrate biosynthesis by knocking out *ptb* (phosphotransbutyrylase) and/or *buk* (butyrate kinase) is thus desirable for further enhancing butanol production in *C. cellulovorans*.

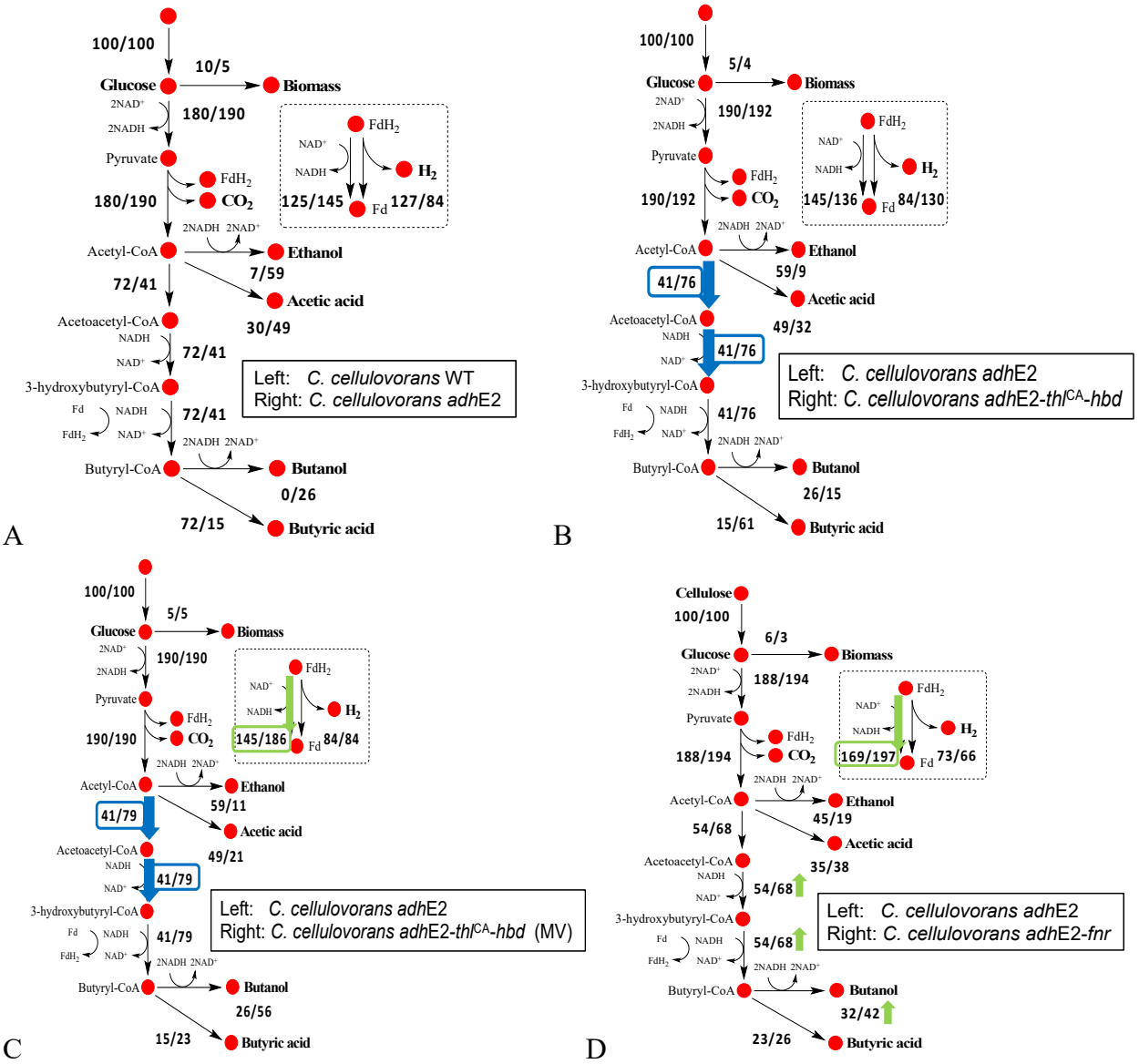


Figure 4. Flux distributions of wild-type (WT) and mutant *C. cellulovorans* strains in batch fermentations with glucose or cellulose as substrate. Gene expression effects on the fluxes are illustrated by the values shown next to each metabolic step and bold arrows indicating increased flux.

CRISPR-Cas9 genome-editing system for C. cellulovorans

During this project, 8 CRISPR-Cas9n systems (pClosN1.0) and CRISPRi systems (pdClos1.0) had been studied for gene deletion and down-regulation, respectively, in *C. cellulovorans* (**Table 4**). However, none of them resulted in any positive mutant after numerous attempts in electro-transformation and screening. The negative results from these attempts led to the conclusion that a new vector based on the host's own CRSIPR system and Cas9 nickase should be developed for genome editing in *C. cellulovorans*. The failed attempts could also be attributed to the fact that acetate and hydrogen biosyntheses are essential for cell survival. Nevertheless, we have designed new plasmids and our preliminary data showed the redesigned plasmids worked in *C.*

cellulovorans and were able to knock out targeted genes. Further work is needed to confirm the knockout of targeted genes and their effects on the mutants.

Table 4. Plasmids for CRISPR-Cas9 genome editing in *C. cellulovorans*.

#	Plasmid	Purpose	Results or Findings
1	pdClos1.0-*hyd*	CRISPRi with inactivated Cas9 nickase and homologous arms of hydrogenase (*hyd*) for down-regulation of hydrogenase	Mutant produced less acetate and more butyrate at a significantly higher butyrate yield and butyrate/acetate ratio
2	pdClos1.0-*hyd*1	CRISPRi with inactivated Cas9 nickase and homologous arms of hydrogenase (*hyd*) for down-regulation of hydrogenase	Mutant produced less acetate and more butyrate at a significantly higher butyrate yield and butyrate/acetate ratio
3	pdClos1.0-*ack*	CRISPRi with inactivated Cas9 nickase and homologous arms of acetate kinase (*ack*) for *ack* down-regulation	Mutant showed no significant difference in acetate or butyrate production, suggesting no effect on the fermentation
4	pdClos1.0-*pta*	CRISPRi with inactivated Cas9 nickase and homologous arms of phosphotransactylase (*pta*) for *pta* down-regulation	Mutant showed no significant difference in acetate or butyrate production, suggesting no effect on the fermentation
5	pClosN1.0-*ack*	CRISPR with Cas9 nickase and homologous arms of *ack* for *ack* deletion	No mutant obtained from transformants screening, indicating that acetate biosynthesis is essential for cell survival and cannot be knockout
6	pClosN1.0-*pta*	CRISPR with Cas9 nickase and homologous arms of *pta* for *pta* deletion	No mutant obtained from transformants screening, indicating that acetate biosynthesis is essential for cell survival and cannot be knockout
7	pClosN1.0-*ptb*	CRISPR with Cas9 nickase and homologous arms of *ptb* for *ptb* deletion	No mutant obtained from transformants screening.
8	pClosN1.0-*ptb*-*adh*E2	CRISPR with Cas9 nickase and homologous arms of *ptb* for *ptb* deletion and *adh*E2 for its knockin	No mutant obtained from transformants screening.

Engineering acetogenic clostridia for butanol production from CO_2/H_2

Totally, 9 recombinant plasmids based on pMTL82151 were constructed for overexpressing various genes (*aor*, *adh*E2, *fnr*, *thl*, and *hbd*) in *C. carboxidivorans* (**Table 5**). The results including the effects of overexpressing these genes on butanol biosynthesis are summarized in **Table 5**. Unfortunately, we have not been successful in obtaining mutants with plasmids for overexpressing *thl* or *hbd* to improve C4/C2 ratio and butanol production. Nevertheless, we have laid the ground work for metabolic engineering of *C. carboxidivorans* for enhanced butanol production from gaseous substrates (CO, CO_2 and H_2).

Table 5. Recombinant plasmids used for overexpressing various genes for butanol biosynthesis in *C. carboxidivorans*.

Plasmid	Purpose	Results or Findings
*adh*E2	*adh*E2 overexpression for butanol and ethanol production from butyryl-CoA and acetyl-CoA, respectively	Mutant produced more alcohol, resulting in higher alcohol/acid ratio.

aor^{CC}	*aor*^{CC} from *C. carboxidivorans* P7 overexpression for butanol production	Decreased the alcohol/acid ratio with syngas as the carbon source but increased the ratio with glucose as the carbon source.
aor^{CL}	*aor*^{CC} from *C. ljungdahlii* DSM 13528 overexpression for butanol production	On-going efforts to transform the plasmid into *C. carboxidivorans*.
aor^{CC}-*fnr*	*aor*^{CC} and *fnr* overexpression for increased NAD(P)H availability to favor butanol biosynthesis over acids	Decreased the alcohol/acid ratio with syngas as the carbon source but increased the ratio with glucose as the carbon source.
*adh*E2-*fnr*	*adhE2* and *fnr* overexpression for increased NAD(P)H availability to favor butanol biosynthesis over acids	Mutant produced more alcohol, resulting in higher alcohol/acid ratio.
*adh*E2-*thl*^{CA}	*adh*E2 and *thl*^{V5A} (from *C. acetobutylicum* BMK19) for increased flux toward C4 over C2 metabolites	On-going efforts to transform the plasmid into *C. carboxidivorans*.
*adh*E2-*thl*^{V5A}	*adh*E2 and *thl*^{CT} from *C. tyrobutyricum* ATCC 25755 for butanol production with increased flux toward C4 over C2 metabolites	On-going efforts to transform the plasmid into *C. carboxidivorans*.
*adh*E2-*thl*^{CT}	*adhE2* and *pfk* overexpression for glycolysis acceleration	On-going efforts to transform the plasmid into *C. carboxidivorans*.
*adh*E2-*hbd*	*adh*E2 and *hbd* from *C. tyrobutyricum* for butanol production with increased flux toward C4 over C2 metabolites	On-going efforts to transform the plasmid into *C. carboxidivorans*.

Summary: We had successfully expressed many genes in *C. cellulovorans* and screened/identified mutants that improved butanol production from cellulose compared to the initial strain overexpressing *adhE2* only (Subtask A.1). The new mutants met our targeted butanol titer (>5 g/L) and yield (>0.35 g/g cellulose). Engineering *C. aceticum* to overexpress butanol biosynthesis pathway genes (Subtask A.2) did not produce any transformant with significant alcohol production. However, we had engineered *C. carboxidivorans*, which was capable of producing *n*-butanol and ethanol from $CO/CO_2/H_2$. Overexpressing *adh*E2, *aor*, and *fnr* in this acetogen showed significantly increased alcohol production from syngas ($CO/CO_2/H_2$) and glucose compared to the wild-type strain. For industrial application, we'll need to further engineer *C. carboxidivorans* to increase C4/C2 ratio and butanol production. Genome editing with CRISPR-Cas9 for gene knockout (*ack*, *pta*, and *ptb*) and knockdown (*hyd*) had been applied, aiming to reduce acids production and increase alcohols production (Subtask A.3). However, the CRISPR system for genome editing (which was not in the original proposal) developed in this project did not produced any positive mutant. To increase butanol productivity, we had tried to adapt *C. cellulovorans* aiming to increase its butanol tolerance to higher than ~10 g/L butanol (Subtask A.4). However, sporulation in *C. cellulovorans* appeared at 3-5 g/L butanol and limited butanol production. This limitation can be circumvented by using co-cultured fermentation with solventogenic clostridia (e.g., *C. beijerinckii*, *C. acetobutylicum*, and engineered *C. tyrobutyricum*) that can tolerate and produce butanol at >10 g/L. Alternatively, sporulation and stress related genes such as *spo*0A and histidine kinase would be considered as targets for metabolic engineering.

Task B: Fermentation kinetics studies and process optimization

Planned Activities

Subtask B.1: Fermentation kinetics studies of mutants generated in Task A
Clostridium mutant strains generated in Task A will be studied in serum bottles and bench-top fermenters to evaluate their ability to produce butanol and the effects of gene expression and knockout on cell growth and metabolism. This task is performed mainly by OSU.

Subtask B.2: Medium optimization
Factorial experimental design will be used to find the optimum medium composition (minerals and salts, pH, etc.) for the growth of *C. cellulovorans* on cellulose and acetogens on CO_2/H_2 in fermentation. Industrial carbon (corn stover, waste paper, etc.) and nitrogen sources (corn steep liquor) will be tested. This task is performed mainly by GB.

Subtask B.3: Fermentation process optimization
Current high-performance ABE fermentation processes that are economically viable at commercial scale will provide the guiding principles to the utilization of cellulosic and gaseous feedstocks. The fermentation process with metabolically engineered mutants in bioreactors will be studied at a laboratory scale to determine its feasibility and to optimize the process variables. In the case of cellulosic feedstocks, processes will be required to maximize both cellulose hydrolysis and solvent production. New fermentation and associated equipment designs for fermentation with solid substrates will be employed and optimized. *In-situ* and *ex-situ* methods for solvent removal will also be assessed. For the fermentation with gaseous substrates, the main challenge is to ensure that the gasses are available for uptake by cells. A number of process parameters (such as bubble size, reactor pressure, gas sparging rate, hold-up rate and inlet and outlet gas pressures) affecting gas solubility and gas-liquid mass transfer will be optimized by varying process conditions and also reactor design. Gas stripping for solvent removal may be conducted with the supply of nutrient gasses. The optimization of nutrient supply with solvent removal will be critical for optimal process performance. A number of reactor and solvent removal strategies will be tested. This task is performed mainly by GB.

Subtask B.4: Mixed fermentation process with cellulosic and gaseous substrates
Co-cultured fermentation with both cellulolytic *C. cellulovorans* and carboxydotrophic acetogen will be studied for converting cellulose and CO_2/H_2 to biofuel.

Subtask B.5: Integrated fermentation design validation
Optimized fermentation processes for each of the feedstocks or the combined mixed fermentation will be studied and validated at the lab scale. This data will be used to generate process economics and design models for future pilot plant study.

Completed Activities and Major Findings

All engineered mutant strains developed in this project have been evaluated for their fermentation kinetics with glucose or cellulose as the carbon source. **Figure 5** shows the fermentation kinetics for *C. cellulovorans-adh*E2 grown on cellulose (Fig. 5A) and *C. cellulovorans-adh*E2-*thl*^{CA}-*hbd* grown on glucose in the presence of methyl viologen (Fig. 5B). More than 5.3 g/L of *n*-butanol was produced from glucose by *C. cellulovorans-adh*E2-*thl*^{CA}-*hbd* with a high butanol yield of >0.33 g/g. High butanol titer and yield from cellulose were also obtained with *C. cellulovorans-adh*E2-*thl*^{CA}-*hbd, C. cellulovorans adh*E2-*thl*^{CA} and *C. cellulovorans adh*E2-*fnr*^{CA} (see Tble 3).

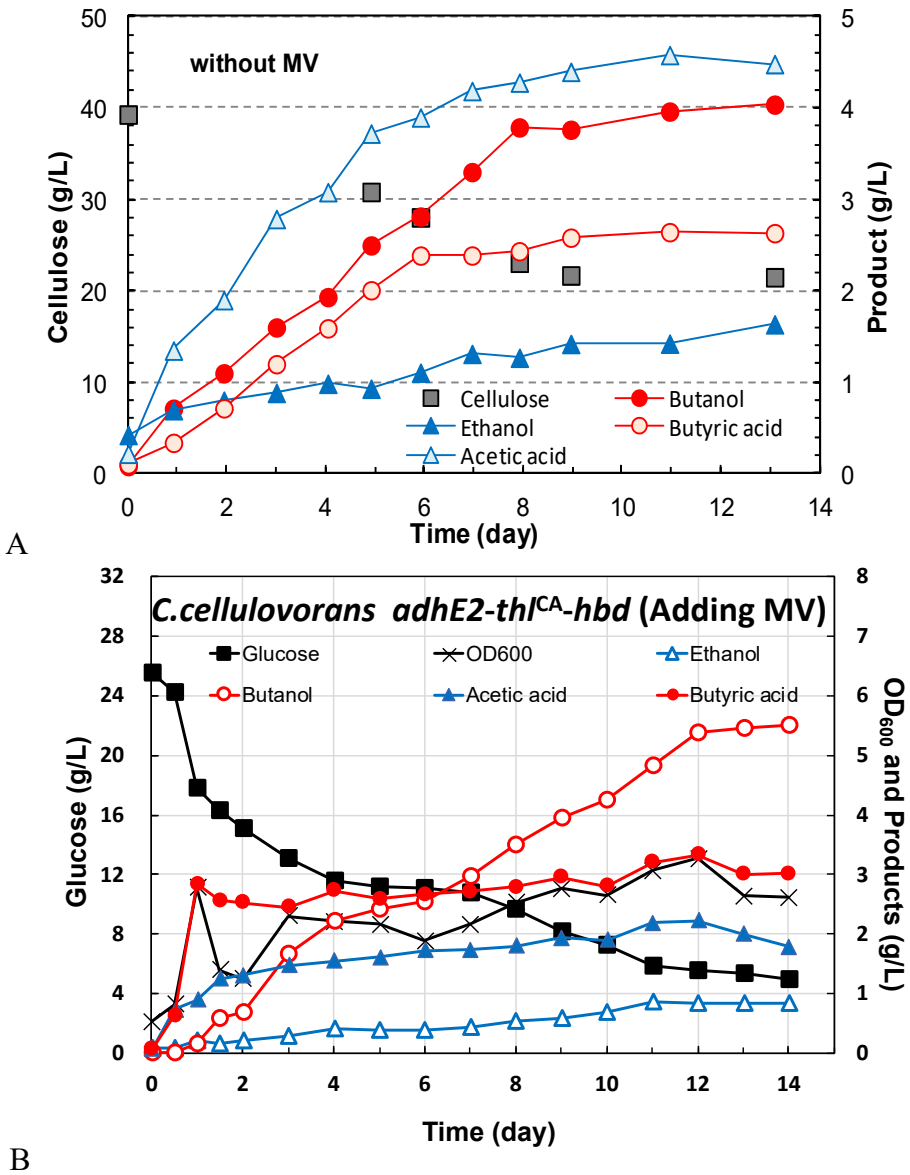


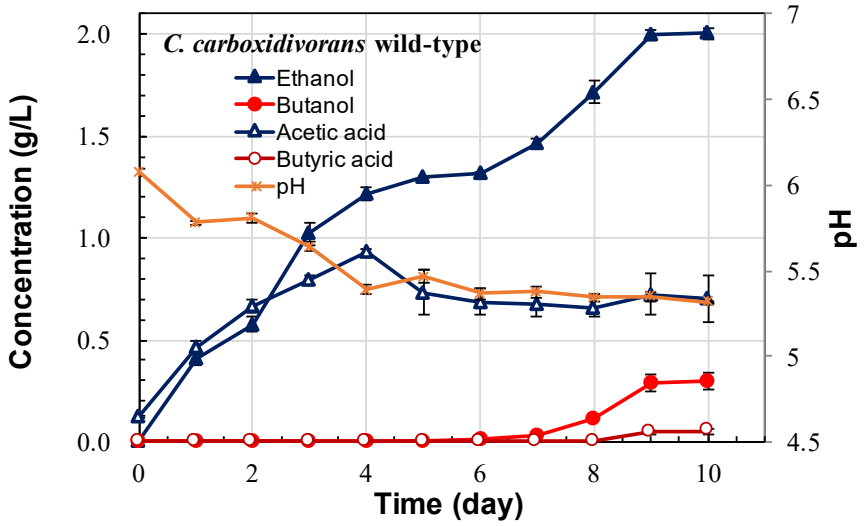
Figure 5. Fermentation kinetics for *C. cellulovorans-adh*E2 grown on cellulose (A) and *C. cellulovorans-adh*E2-*thl*^{CA}-*hbd* grown on glucose in the presence of methyl viologen (B).

Butanol production from lignocellulosic biomass

We studied butanol production from pretreated corn cob in bioreactor. Additional lignocellulosic materials including soybean hulls, sugarcane bagasse, and corn stover were also studied in bioreactors with pH control, focusing on the effects of acid and base pretreatments of cellulosic biomass on enhancing cellulose hydrolysis and fermentation efficiency. In general, *C. cellulovorans* showed a good ability to utilize cellulose, xylan, and plant biomass for acids (acetate and butyrate) and alcohols (ethanol and butanol) production. The effect of pH on alcohol production by *C. cellulovorans adh*E2 was also evaluated in bioreactor, which showed the optimal pH of ~6.5.

Butanol production from CO_2, CO and H_2

Batch fermentations with engineered *C. carboxidivorans* under autotrophic and mixotrophic conditions in serum bottles and bioreactors were studied, focusing on the optimization of medium composition and pH. **Figure 6** shows fermentation kinetics for *C. carboxidivorans* wild type and *C. carboxidivorans-adh*E2 grown on CO_2, CO and H_2. Overexpressing *adh*E2 in *C. carboxidivorans* allowed the cells to reassimilate acetate for ethanol production, resulting in a higher ethanol production compared to the wild type. However, both butanol and butyrate were produced at a relatively low level, indicating that the flux from C2 (acetyl-CoA) to C4 (butyryl-CoA) was limiting their biosynthesis. Further metabolic engineering to increase C2 to C4 flux by overexpressing *thl* and *hbd* is thus recommended.



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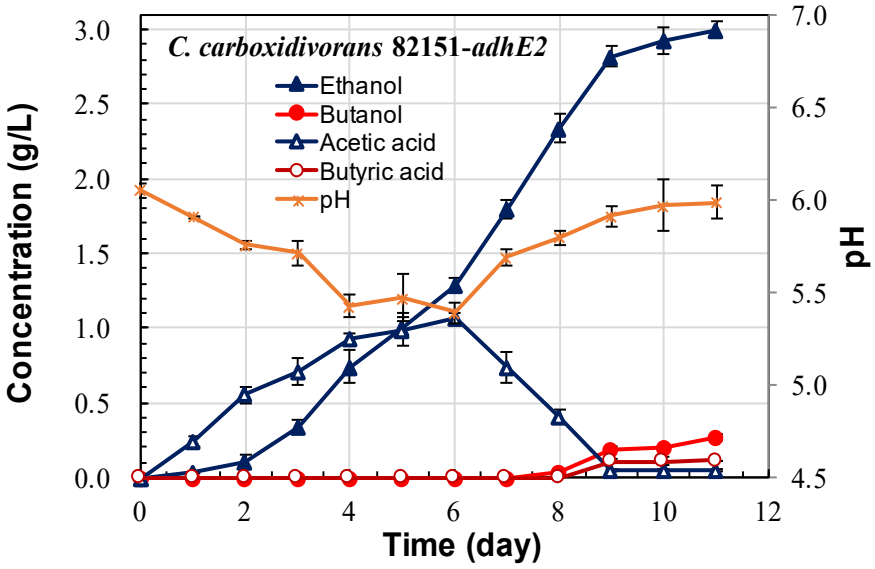

B

Figure 6. Fermentation kinetics for *C. carboxidivorans* wild type (A) and *C. carboxidivorans-adh*E2 (B) grown on CO_2, CO and H_2.

Effects of medium composition. The effects of medium on cell growth and alcohol production by *C. carboxidivorans* wild-type strain were studied in serum bottles. The modified Wilkins-Chalgren medium (with additional 20 mg/L molybdate), CGM medium, and previously used basic medium were compared for their growth and fermentation kinetics in serum bottles and the results are summarized in **Table 6**. As expected, the medium composition affected both cell growth and alcohol production. The modified Wilkins-Chalgren medium gave significantly higher cell growth and ethanol production compared to the other two media.

Table 6. Acids and alcohols production from glucose by *C. carboxidivorans* in different mediums in serum bottles with pH 6.0.

Medium	OD_{600}	Ethanol (g/L)	Butanol (g/L)	Butyrate (g/L)	Acetate (g/L)	Ethanol yield (g/g)	Alcohol/acid ratio (g/g)
Basic	0.63	0.37	0.00	0.00	0.18	0.07	2.07
CGM	0.75	0.40	0.00	0.00	0.27	0.08	1.48
Wilki-Chalgren	3.51	0.50	0.00	0.00	0.18	0.07	2.75

Effects of pH. Enzymes related to alcohol production are sensitive to the pH and the medium pH significantly affects both cell growth and fermentation kinetics. In this quarter, the effects of pH on cell growth and acids and alcohols production from glucose by *C. carboxidivorans* were studied in serum bottles at pH 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. The results are summarized in **Table 7**. In general, the fermentation was faster, more glucose was consumed, and more acids and ethanol were produced with increasing the pH from 5.0 to 6.5. Good cell growth and the highest ethanol yield were obtained at pH 6.5, although the highest OD_{600} was obtained at pH 7.0.

Table 7. Effects of pH on acids and alcohols production from glucose by *C. carboxidivorans* in serum bottles with pH controlled at various values between 5.0 and 7.0.

pH	OD_{600}	Ethanol (g/L)	Butanol (g/L)	Butyrate (g/L)	Acetate (g/L)	Ethanol yield (g/g)	Alcohol/acid ratio (g/g)
5.0	2.05	0.53	0.00	0.00	0.15	0.083	3.10
5.5	2.62	0.62	0.00	0.03	0.13	0.046	3.98
6.0	3.09	1.01	0.00	0.02	0.20	0.064	4.45
6.5	3.64	2.88	0.00	0.03	0.28	0.254	9.48
7.0	4.32	0.89	0.00	0.02	0.19	0.059	4.20

Mixed culture fermentation

A coculture of solventogenic *C. acetobutylicum* and *C. carboxidivorans* was studied with glucose as the substrate to demonstrate the interaction between these two strains and its effect on alcohol production. As shown in **Figure 7**, more alcohols and acetate were produced from glucose by the coculture compared to the fermentation with *C. acetobutylicum*. The results clearly demonstrated that more products, including butanol, can be produced from glucose in the cocultured fermentation due to the utilization of CO_2 and H_2 by the carboxydotrophic strain. A fermentation process with cellulolytic, solventogenic, and carboxydotrophic clostridia, as illustrated in **Figure 8**, thus can be developed for enhanced butanol production with a greatly increased butanol yield

of >0.4 g/g cellulose. Further optimization of fermentation conditions for such a mixed culture process is necessary for further process development.

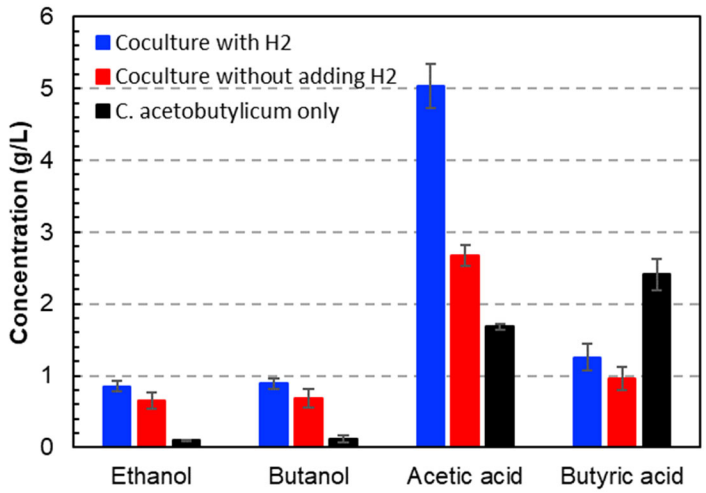

Figure 7. Comparison of products produced from glucose in a cocultured fermentation of *C. acetobutylicum* and *C. carboxidivorans* with monoculture of *C. acetobutylicum*. More acetate and alcohols were produced by the coculture, especially when external hydrogen was added, confirming that CO_2 (and H_2) released in glucose fermentation was reassimilated by *C. carboxidivorans*.

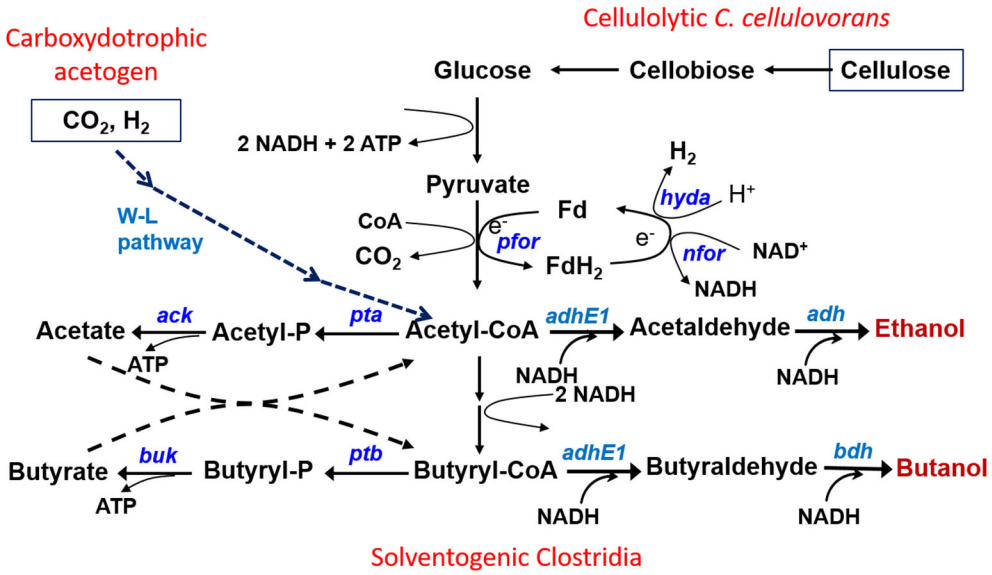

Figure 8. Mixed-culture fermentation with cellulolytic, carboxydotrophic, and solventogenic clostridia for butanol and ethanol production from cellulose and CO_2/H_2.

Summary: Both cellulose and CO_2/H_2 are energetically unfavorable and difficult to use by cells. In both cellulose and gas fermentations, poor substrate (cellulose and CO_2/H_2) uptake, long process time, low productivity, and low alcohol titer were major challenges for achieving the milestone of producing 10 g/L butanol and ethanol from cellulose at yield of 0.3 g/g cellulose and productivity of 0.2 g/L·h in a co-cultured fermentation (Milestone 2.5). Co-culturing *C. cellulovorans* with *C carboxidivorans* and other solventogenic strains such as *C. beijerinckii* could help to achieve our

process goal, but would require further optimization of the fermentation conditions. Supplementing the gas with CO showed a benefit in improving the energetics and fermentation performance. It was also necessary to increase butanol tolerance and cell growth on cellulose and CO_2/H_2. Increasing the cell density (>OD 20) in fermentation could help to achieve the targeted productivity of 0.2 g/L·h. An OD 20 in fermentation could be achieved by first culturing cells in rich media with fermentable sugars such as glucose and then switching to cellulose and/or gas substrates. Proper pretreatments of plant biomass significantly increased substrate availability and fermentability. For commercial application, it would be necessary to further optimize process parameters affecting cellulose availability and mass transfer for gas substrate in bioreactors. In addition, external addition of cellulases (at a reduced dosage) could also accelerate the cellulose fermentation. Finally, mixotrophic cultures could enhance CO_2 uptake and metabolism by cells, but would require further process optimization. The mixed culture fermentation would also require further kinetic studies and optimization before such a process could be properly designed for the anticipated benefits.

Task C: Omic analysis of mutants in fermentation

Planned Activities

Subtask C.1: Proteomics assisted metabolic engineering
The objective is to evaluate and finalize the target of metabolic engineering of *C. cellulovorans* and *C. aceticum* (or C. *carboxidivorans*) for Task A using comparative proteomics. The expression of the key enzymes in central carbohydrate metabolism and end products will be analyzed and compared to narrow down the carbon and redox regulation genes and guide the metabolic engineering.

Subtask C.2: Metabolomics assisted process development and scale-up
The objective is to metabolically engineer the butanol fermentation process by newly engineered Clostridia using metabolomics. This study will support butanol fermentation process optimization and scale-up. The interaction of cellular metabolism and fermentation process parameters will be analyzed and used to support the development of bioreactor operation parameters and fed-batch process.

Completed Activities and Major Findings

UAB researchers successfully built the first-generation mathematical model to integrate the proteomics (**Figure 9**) and metabolomics (**Figure 10**) data, targeting to predict the key regulators for metabolic engineering. The pairwise analysis of intracellular metabolites showed different metabolic shift patterns, amino acids metabolism and lipids metabolism in wild type vs. mutant and cellulose vs. glucose. The primary static flux analysis indicated that cellulose favored butyrate production and the metabolic flux distribution from C2 to C4. An enzyme catalyzed reactions-based dynamic model was also developed to simulate intracellular metabolite fluctuation and predict limiting steps at the system level. Flux control analysis showed that butanol biosynthesis from glucose and cellulose were limited by the flux from C2 to C4 and the reactions catalyzed by acetyl-CoA acetyltransferase or thiolase (Thl) and β-hydroxbutyryl-CoA dehydrogenase (Hbd) had major effects on butanol production (**Figure 11**). Therefore, overexpressing *thl* and *hbd* can greatly enhance butanol production by *C. cellulovorans* as demonstrated in the metabolic engineering study.

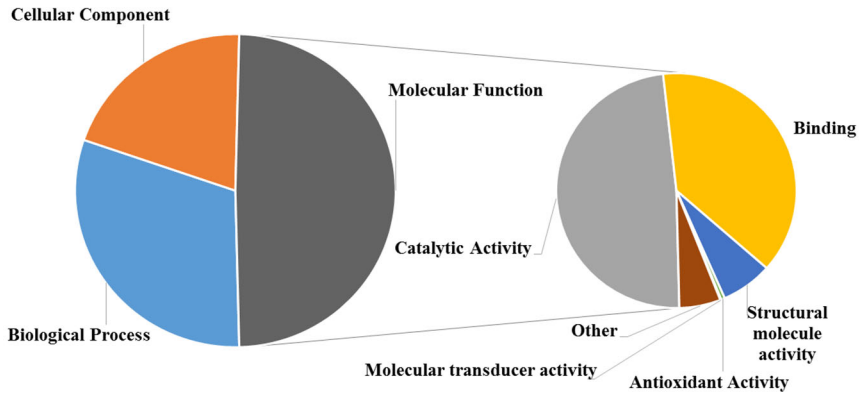

Figure 9. Classification of proteins in *C. cellulovorans* found in the proteomics analysis. 624 proteins are grouped into cellular component, biological process, and molecular function based on gene ontology.

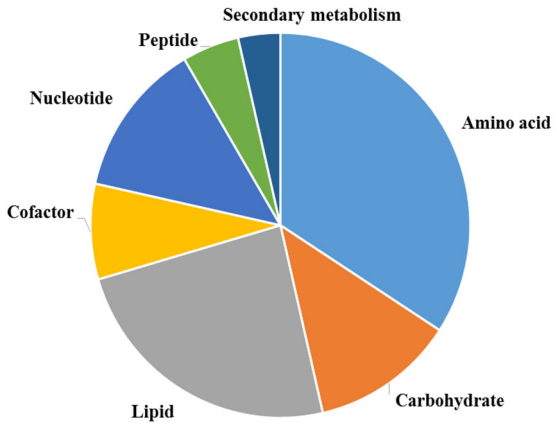

Figure 10. Classification of metabolites in *C. cellulovorans* found in the metabolomics analysis. A total of 474 intracellular metabolites, including some unstable metabolites such as cofactors and peptides, were extracted and identified from *C. cellulovorans.* They are grouped as amino acid, carbohydrate, lipid, cofactor, nucleotide, peptide, and secondary metabolite.

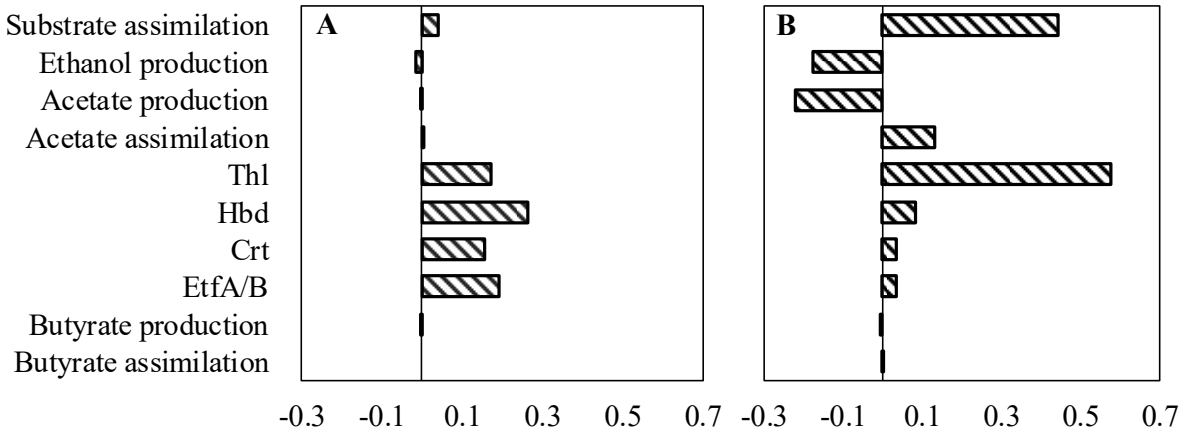

Figure 11. The metabolic flux control coefficients in butanol fermentation by *C. cellulovorans-adhE2* with glucose (A) and cellulose (B) as the substrate. A higher control coefficient indicates higher impact. Thl: acetyl-CoA acetyltransferase; Hbd: β-hydroxbutyryl-CoA dehydrogenase; Crt: 3-hydroxyacyl-CoA dehydrogenase; EtfA/B: electron transfer flavoprotein A/B subunit.

Summary: We developed, for the first time, a multi-Omics experimental data-based model to rationally guide metabolic engineering of clostridia. Details on the dynamic model integrating the metabolomics, proteomics, and fermentation kinetics data are given in Appendix E.

Task D: Process design & cost analysis

Planned Activities

Subtask D.1: Baseline case
GB will develop a model based on a series of unit operations for feedstock pre-treatment, enzymatic hydrolysis, batch fermentation and solvent recovery via distillation. The preferred pre-treatment process (criteria include cost, performance and sugar quality) will be selected, and the process and conceptual plant with outline butanol production costs will be designed.

Subtask D.2: Advanced fermentation
The second process concept will capitalize on GB's advanced fermentation process (BEST^{TM}) for renewable n-butanol. This technology integrates best in class microbiology with high productivity fermentation and innovative solvent recovery. The BEST process has been proven at commercial demo-scale using corn starch. We will design a process to utilize a cellulosic feedstock with outline butanol production costs.

Subtask D.3: Process consolidation
We will develop two process concepts; one for consolidated bioprocessing using engineered *C. cellulovorans*. In this example, the microbe will utilize cellulose directly and ferment to butanol in a single stage replacing the need to add enzymes. The second concept will be built on the first concept by introducing engineered *C. carboxidivorans* alongside *C. cellulovorans* in order to utilize fermentation gases (CO_2 and H_2). Co-culture technology should provide a step change in butanol yield. In both cases, we will incorporate GBL's advanced design for *in situ* solvent removal (part of the BEST system).

Subtask D.4: Process comparison & LCA
We will determine the energy balance and associated greenhouse gas (GHG) emissions. The four design briefs will be compared with GBI's advanced fermentation process converting corn starch to butanol. We will evaluate technical performance alongside production costs (including capital costs) and associated GHG emissions.

Completed Activities and Major Findings

GB engineers performed techno-economic analyses (TEA) of different process designs for butanol production from corn (baseline case) and a lignocellulosic biomass feedstock using conventional process involving separate unit operation steps of pretreatment, hydrolysis, fermentation and separation, and consolidated bioprocess (**Figure 12**). The analysis showed a significant potential for commercial production of butanol directly from cellulosic biomass if the technology is successfully developed and scaled up. The economics for the integrated process with a coculture for CO_2 utilization showed a potential to further reduce butanol production cost by more than 10% due to a 30% increase in butanol production yield from consumed cellulosic feedstock. Based on GB's analysis, we also developed a process model for butanol production from lignocellulosic biomass in a consolidated bioprocess and used it for cost analysis for a plant size of 50,000 metric tons of butanol per year (**Figure 13**).

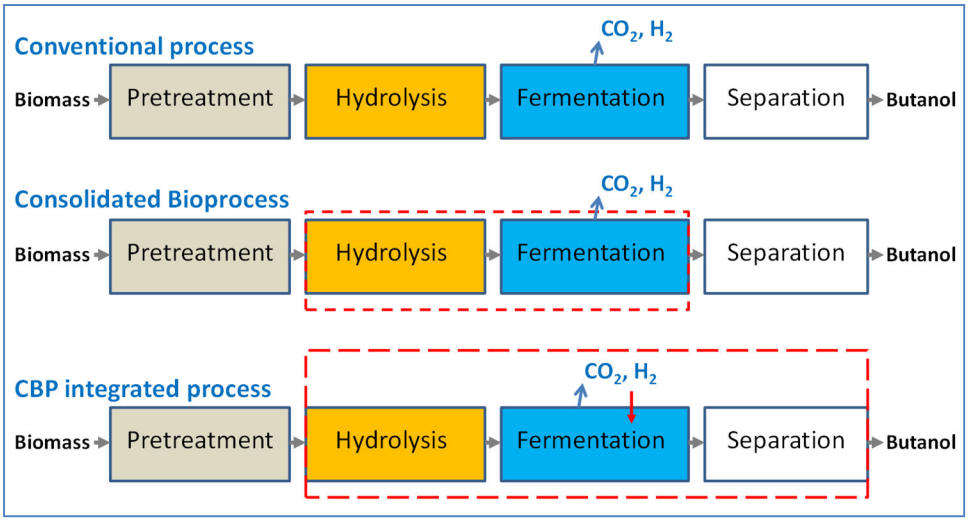

Figure 12. Different process designs for butanol production from biomass feedstock. The conventional process has 4 separate steps: pretreatment, hydrolysis, fermentation, and separation, while the consolidated bioprocess (CBP) uses cellulolytic clostridia to directly convert cellulose to the fermentation products. The integrated CBP also includes a carboxydotrophic clostridia to reutilize CO_2 and H_2 for butanol production. In situ separation of butanol is also integrated in the process to reduce butanol toxicity to the fermentation.

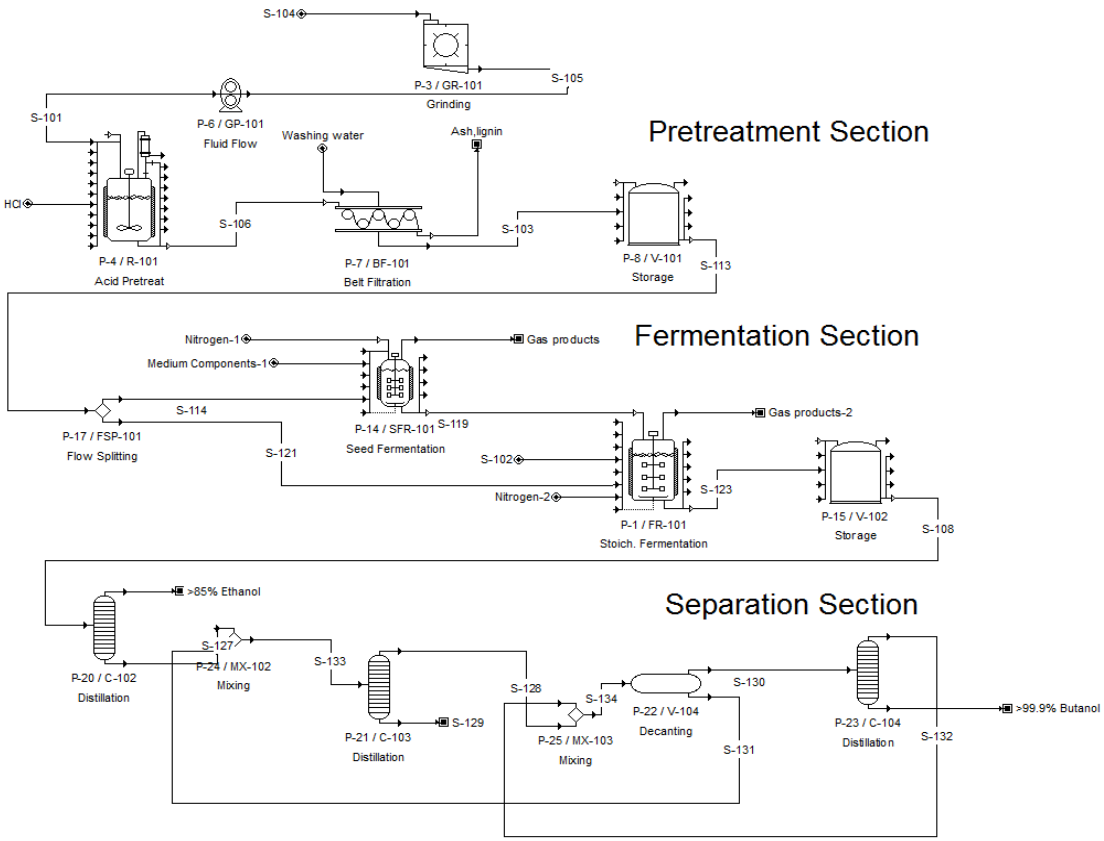

Figure 13. A process flowsheet for butanol production from lignocellulosic biomass in a consolidated bioprocess.

Our cost analysis shows that butanol can be produced from corn cob at $0.71/kg or $2.13/gal, assuming that corn cob (40% cellulose, 30% hemicellulose, and 20% lignin) costs $50 per dry ton and that the fermentation produced butanol at ~5.5 g/L with a yield of 0.4 g/g cellulose/hemicellulose and productivity of 0.05 g/L·h. The cost is sensitive to the fermentation productivity and butanol titer and yield.

Life Cycle Analysis: A well-to-pump life cycle analysis (LCA) was performed using Argonne National Laboratory's GREET Model (greet.es.anl.gov/). **Table 8** compares CO_2 and GHG emissions from butanol production in various processes: 1) butanol production from petroleum feedstock in a traditional chemical plant; 2) butanol production from corn (starch) in ABE fermentation; 3: butanol production from corn stover (lignocellulosic biomass) in ABE fermentation. Compared to the traditional chemical process, biobutanol production by ABE fermentation has a GHG reduction of ~24% from corn (GHG from farming is considered) and 62% from corn stover (excluding GHG from farming since corn stover is a waste from corn farming).

Table 8. Comparison of CO_2 and GHG emissions from different butanol production processes

Emission	Butanol from petroleum feedstock	Butanol from corn in ABE fermentation	Butanol from corn stover in ABE fermentation
CO_2, g	83.42	45.61	33.6
VOC, g	13.46	3.29	7.23
CO, mg	43.63	51.59	19.39
NOx, mg	100	100	35.28
PM10, mg	4.61	14.51	3.51
PM2.5, mg	3.88	6.18	2.67
Sox, mg	0.14	59.48	37.29
CH_4, mg	240	140	79.6
N_2O, mg	2.89	36.87	-6.10E-06
BC, mg	0.58	0.88	0.91
POC, mg	1.22	1.35	0.53
GHG-100, g	91.55	69.86	34.42

The well-to-pump life cycle analysis using Argonne National Laboratory's GREET Model

For the proposed consolidated bioprocess (CBP) for butanol production directly from cellulosic biomass, total GHG emission would be similar to the one for corn stover in ABE fermentation. However, it should be noted that the CBP can significantly reduce resources (including enzyme, chemicals, water, and steam) used in the manufacturing process, which can also reduce GHG emission but the available GREET Model does not account for this. Finally, for the integrated butanol production process with CO_2 reutilization, GHG emission can be further reduced by ~33% to 23.06 since butanol yield would be increased by ~40% and little CO_2 would be released from the fermentation (In ABE fermentation, about 1/3 of the substrate carbon is released as CO_2). Overall, the integrated biobutanol production process has a potential to reduce GHG emissions by ~75% compared to the traditional chemical process.

Products and Technology Transfer Activities

Journal Publication:

1. J Ou, N Xu, P Ernst, C Ma, M Bush, KY Goh, J Zhao, L Zhou, ST Yang, XM Liu, Process engineering of cellulosic n-butanol production from corn-based biomass using *Clostridium cellulovorans*, Process Biochem., 62: 144–150 (2017).
2. T Bao, C Cheng, X Xin, J Wang, M Wang, ST Yang. Deciphering mixotrophic *Clostridium formicoaceticum* metabolism and energy conservation: Genomic analysis and experimental studies, Genomics, published online (2018). doi: 10.1016/j.ygeno.2018.11.020
3. T Bao, J Zhao, J Li, X Liu, and ST Yang, *n*-Butanol and ethanol production by *Clostridium cellulovorans* overexpressing aldehyde/alcohol dehydrogenases from *Clostridium acetobutylicum,* Bioresour. Technol., 285: 121316 (2019).
4. C Cheng, W Li, M Lin, and ST Yang Metabolic engineering of *Clostridium carboxidivorans* for enhanced ethanol and butanol production from syngas and glucose, Bioresour. Technol., 284: 415–423 (2019).
5. T Bao, J Zhao, Q Zhang, and ST Yang Development of a shuttle plasmid without host restriction sites for efficient transformation and heterologous gene expression in *Clostridium cellulovorans*, Appl. Microbiol. Biotechnol., 103: 5391–5400 (2019).
6. C Cheng, T Bao, ST Yang, Engineering *Clostridium* for improved solvents production: Recent progress and perspective, Appl. Microbiol. Biotechnol., 103(14): 5549–5566 (2019).

Conference Presentation:

1. Teng Bao, Jingbo Zhao, and Shang-Tian Yang, System metabolic engineering of *Clostridium cellulovorans* towards consolidated bioprocessing for *n*-butanol production from cellulosic biomass. 2018 AIChE Annual Meeting, Pittsburgh, PA, October 28-November 2, 2018.
2. Tianyi Chen, Chi Cheng, Teng Bao, and Shang-Tian Yang, Improving C4 to C2 ratio for n-butanol production in mixotrophic fermentation by engineered *Clostridium carboxidivorans.* 2018 AIChE Annual Meeting, Pittsburgh, PA, October 28-November 2, 2018.
3. Jing Li, Wenjie Hou, Teng Bao, Shang-Tian Yang, n-Butanol production from cotton stalk using engineered *Clostridium cellulovorans.* 2018 AIChE Annual Meeting, Pittsburgh, PA, October 28-November 2, 2018.
4. Jianfa Ou, Ningning Xu, Chao Ma, Patrick Ernst, and X. Margaret Liu, A computational modeling to integrate multi-Omics in *Clostridium cellulovorans* to guide metabolic engineering, 2017 AIChE Annual Meeting, 11/1/2017.
5. Jianfa Ou, Chao Ma, and X. Margaret Liu. Process engineering of *Clostridium cellulovorans* for butanol production from biomass, 2016 AIChE Annual Meeting, 11/13/2016.
6. Jianfa Ou, Chao Ma, and X. Margaret Liu, Rationally metabolic engineering of *Clostridium cellulovorans* for butanol production, 2016 AIChE Annual Meeting, 11/14/2016

Commercialization:

- GB is our collaborator on this project and a potential commercialization partner
- BioMissions LLC is our new partner for further technology/process development

Patents: None