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

March 9, 2017
Biochemical Conversion

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Goal Statement

- The goal of this project is to develop engineered clostridia strains and fermentation process that can directly utilize cellulose and fix CO_2 for *n*-butanol production from lignocellulosic biomass.

- The engineered strains can be used in fermentation to produce *n*-butanol from lignocellulosic biomass at a targeted cost of $2.25/gal or less than $3/gge (gallon gasoline equivalent).

Quad Chart Overview

Timeline

- Start date: October 1, 2015
- End date: September 30, 2017
- Completion: 55%

Barriers

- Ct-H: Efficient catalytic upgrading of sugars/aromatics, gaseous and bio-oil intermediates to fuels and chemicals
- Im-E: Cost of production
- Target: Develop cost-effective biological synthesis technologies

Budget

	Total Costs FY 12 –FY 14	FY 15 Costs	FY 16 Costs	Total Planned Funding (FY 17-Project End Date)
DOE Funded	-	-	$625,688	$606,460
Project Cost Share (Comp.)*	-	-	$149,547	$162,738

Partners

- Ohio State University (61.6%); Green Biologics (19.6%); University of Alabama (18.8)

1. Project Overview

- This project has three partners - Ohio State University (OSU), Green Biologics (GB), and University of Alabama (UA) - with a long collaboration history working on biobutanol production.
- The proposal was submitted to DOE-EERE Biotechnology Incubator program in 2014.
- The project has four specific 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 (UA)
  - Task D. Process design & cost analysis (GB & OSU)

2 – Approach (Management)

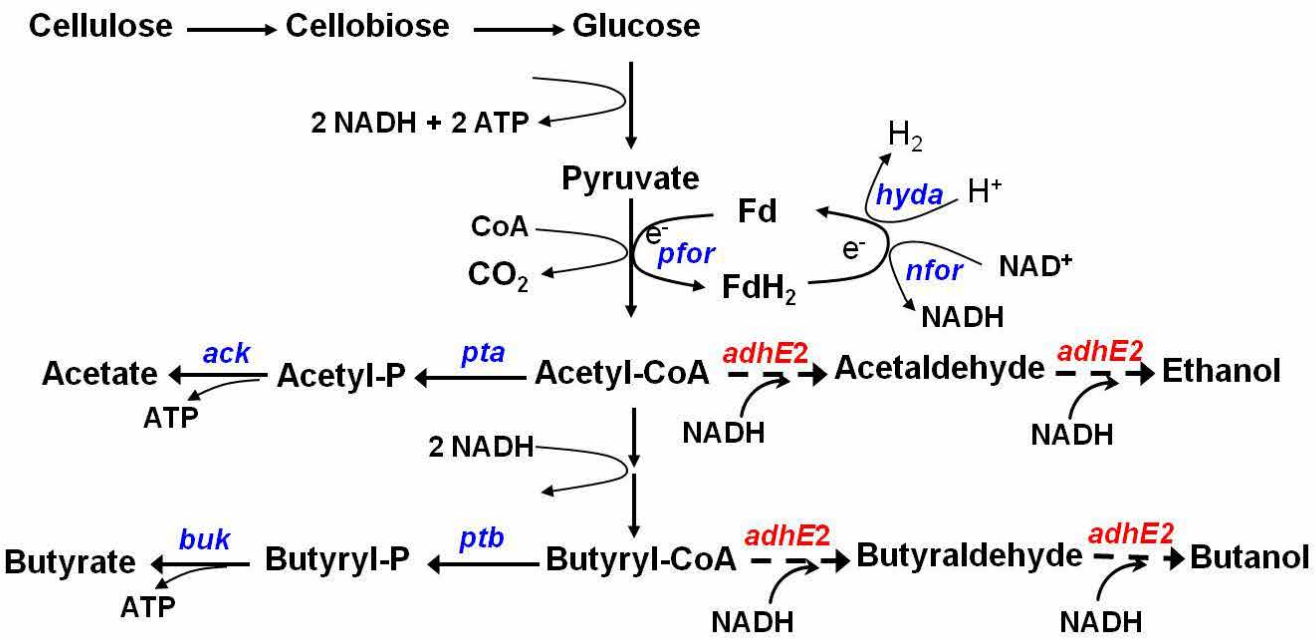
- Project is managed by OSU sponsored research program office (Amy Dudley).
- Project is directed by the PI, Prof. S.T. Yang of Ohio State University (OSU), with 2 subaward Co-PIs, Dr. Tim Davies of Green Biologics (Chief Technology Officer, GB) and Prof. Margret Liu of University of Alabama (UA).
- PI and Co-PI's each leads a major task:

Task A	Task B	Task C	Task D
OSU	OSU / GB	UA	GB
Yang	Davies / Yang	Liu	Davies

- We meet regularly to discuss project progress and exchange data.
- Project progress is monitored with quarterly milestones in each major task.
- GB has an ABE fermentation plant in Minnesota and will seek to commercialize the project outputs.

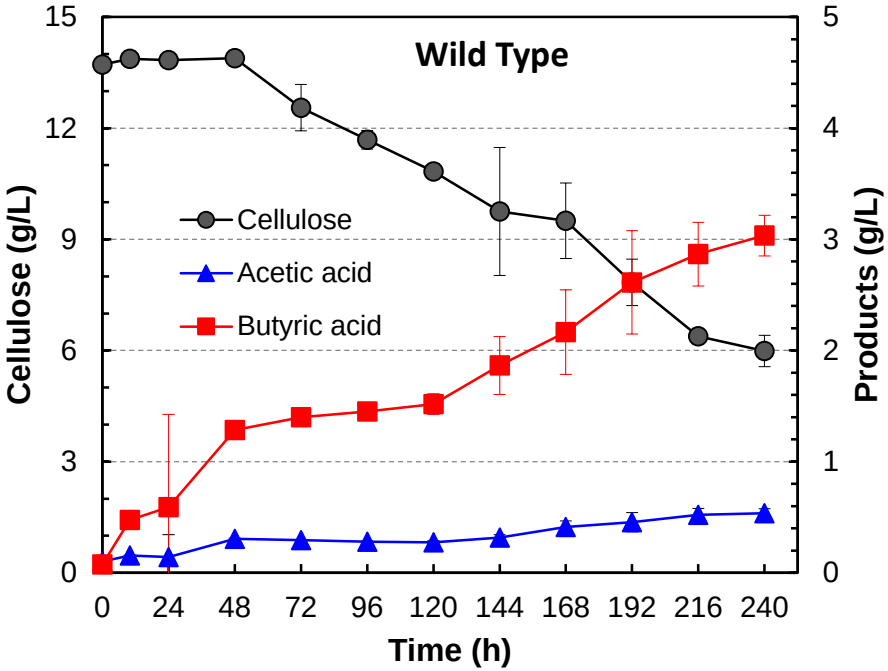
2 – Approach (Technical)

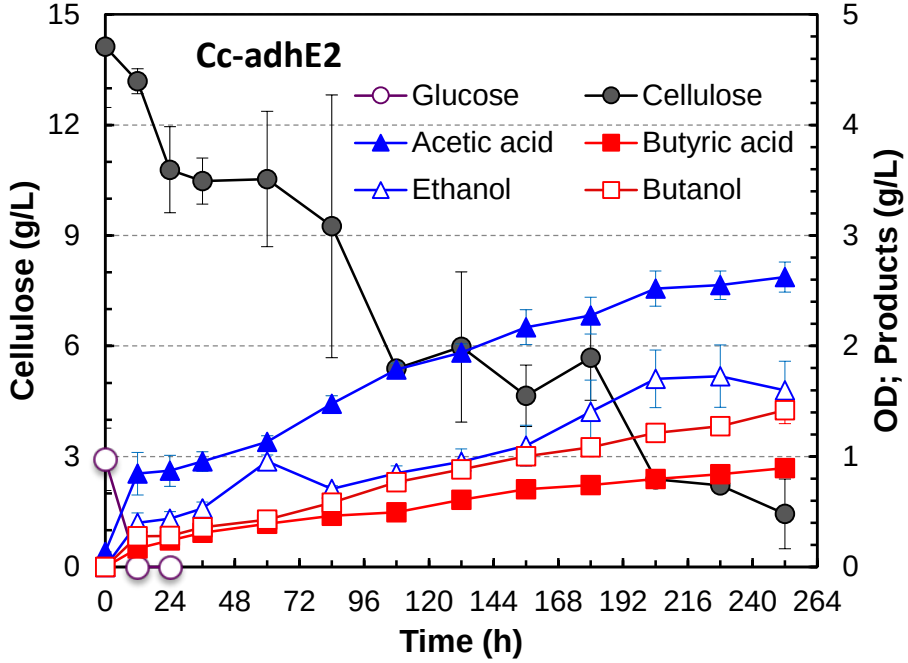
- Consolidated bioprocessing can save ~50% cost in using lignocellulosic biomass for biofuels production, but no organism naturally can produce *n*-butanol directly from cellulose.
- Engineering cellulolytic acidogen *Clostridium cellulovorans* to produce *n*-butanol and ethanol directly from cellulose by introducing the heterologous bi-functional aldehyde/alcohol dehydrogenase gene, *adhE2*



Metabolic Engineering of *C. cellulovorans*

- *C. cellulovarns* produces various cellulases, both secreted and cellulosome
- Wild type produced only butyrate and acetate; Mutant overexpressing *adhE2* also produced butanol and ethanol





$C_6H_{12}O_6 \rightarrow 0.8\ CH_3CH_2CH_2COOH + 0.4\ CH_3COOH + 2\ CO_2 + 2.4\ H_2$

adhE2 → $CH_3CH_2CH_2CH_2OH$

adhE2 → C_2H_5OH

2 – Approach (Technical)

- Approx. 34% of the carbon from the biomass feedstock is converted to CO_2 and the fermentation also produces H_2.
- Carboxydotrophic (CO_2-fixing) acetogens can convert CO, CO_2 and H_2 to acetate via Wood-Ljungdahl pathway

  Glucose → 3 Acetate

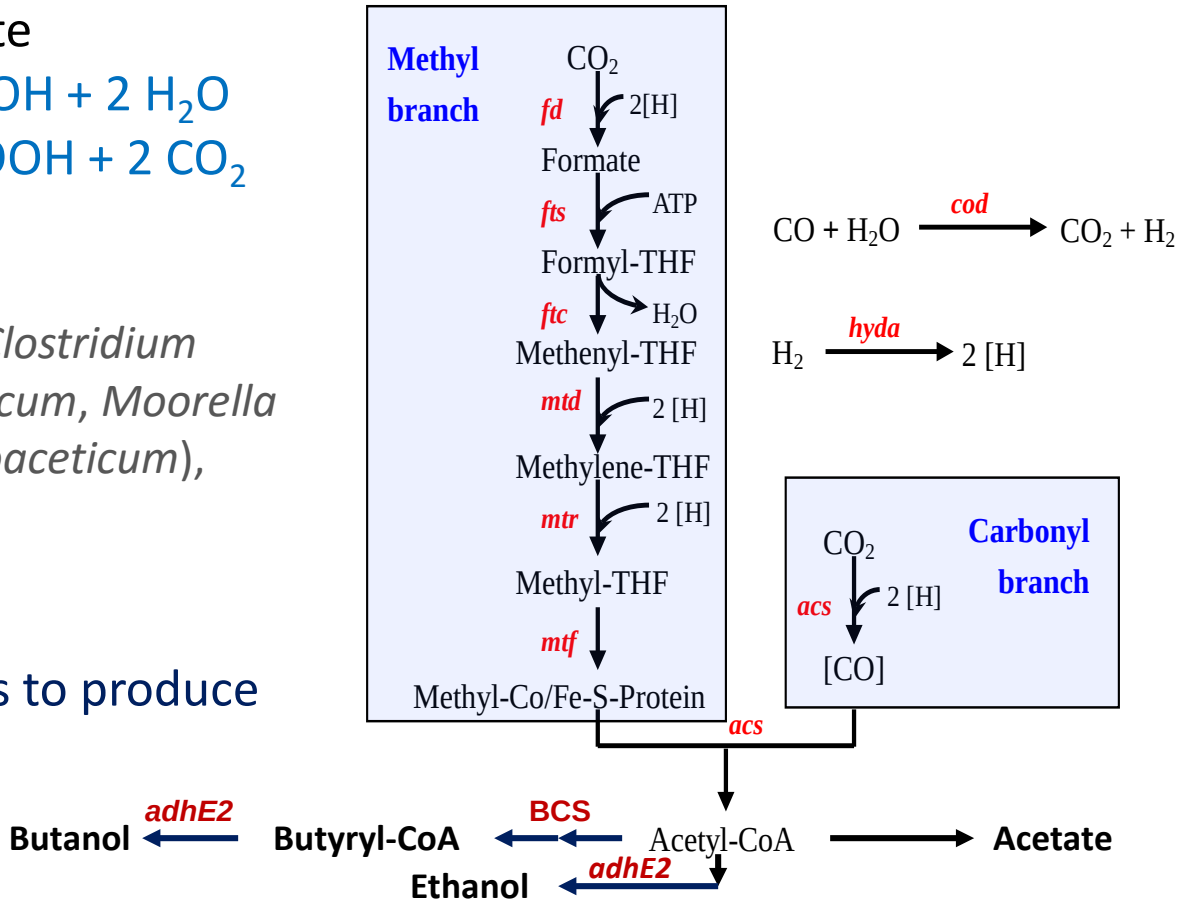
  $2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$

  $4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2$

Homoacetogens:

Acetobacterium woodii, Clostridium aceticum, C. formicoaceticum, Moorella thermoacetica (*C. thermoaceticum*), *Acetogenium kivui*

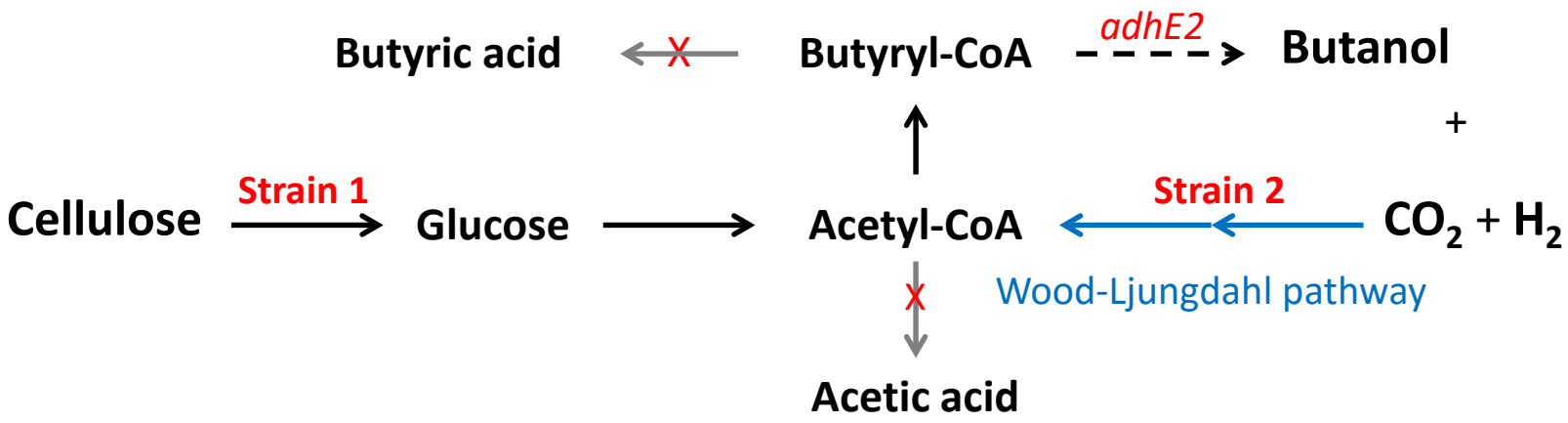
Engineering acetogens to produce ethanol and butanol



2 – Approach (Technical)

Overall Process Design

- Cellulolytic clostridia converts cellulose to butanol, with CO_2 and H_2 as by-products (Theoretical yield: 0.42 g/g cellulose)
- Carboxydotrophic clostridia further converts CO_2 and H_2 to butanol



- With the co-culture, total butanol yield from cellulose could be increased by 50% (Theoretical yield: 0.63 g/g cellulose) if all CO_2 were converted to butanol .
- GHG emissions could be reduced by additional 50%.

2 – Approach (Technical)

Go/No Go Milestone

- Target fermentation process performance parameters: *n*-butanol titer >10 g/L, yield >0.35 g/g cellulose, productivity >0.1 g/L ·h, which are necessary for the process to be economically competitive
- Project Go/No Go decision point (Month 12 or 9/30/2016): obtaining engineered clostridia strains capable of converting cellulose and CO_2 to *n*-butanol at >2.5 g/L and yield of 0.2 g/g cellulose for further evaluation in a consolidated bioprocess (CBP), providing a good base for further metabolic engineering improvement and use for process optimization
- The performance is evaluated in batch fermentation with free cells in serum bottles. Samples are analyzed with HPLC and GC for sugars and fermentation products. Cellulose is analyzed after hydrolysis following the NREL protocol.
- Selected bacterial strains are further studied in 1-5 L bioreactors.

2 – Approach (Technical)

Economic and Technical Metrics

- Compared to conventional ABE fermentation, the new process with higher butanol yield from low-cost biomass feedstock could reduce biobutanol cost by ~50% to less than $2.25/gal or $3.0/gge.

	Conventional ABE fermentation	Novel biobutanol fermentation
Products	Acetone, Butanol, Ethanol (3:6:1)	Mainly butanol (>80%)
Substrate cost ($/kg)	Corn: $170/ton 70% starch $0.24/kg corn starch	Corn stover: $70/ton 60% cellulose + hemicellulose $0.12/kg cellulose
Process	Semi-continuous process with 6–8 fermentors (CSTR) in series with a total retention time of more than 60 hours Recovery by distillation; energy intensive	Sequential batch process with high cell density and online gas stripping for butanol recovery to reduce energy input
Butanol concentration Productivity Butanol yield	1.2 % (w/v) 0.3 g/L·h ~0.25 g/g sugar	~1 % in broth; 15% after gas stripping 0.10–0.25 g/L·h 0.30–0.45 g/g cellulose
Product cost	$4.50/gal	$2.25/gal

Cost estimation by comparing with commercial ABE plant with corn as feedstock and assuming in situ butanol separation by gas stripping with fermentation off gas to alleviate butanol toxicity

2 – Approach (Technical)

Methods

- Strain development
  - Overexpressing genes in butanol biosynthesis pathway
  - Knockout genes in acid biosynthesis pathway
  - Redox engineering to increase NADH availability
  - Adaptive evolutionary engineering to increase butanol tolerance
- Process development
  - Medium optimization
  - Novel bioreactor design
  - High cell density fermentation
  - *In situ* product separation
- Omics analysis
  - Proteomics analysis
  - Metabolomics analysis

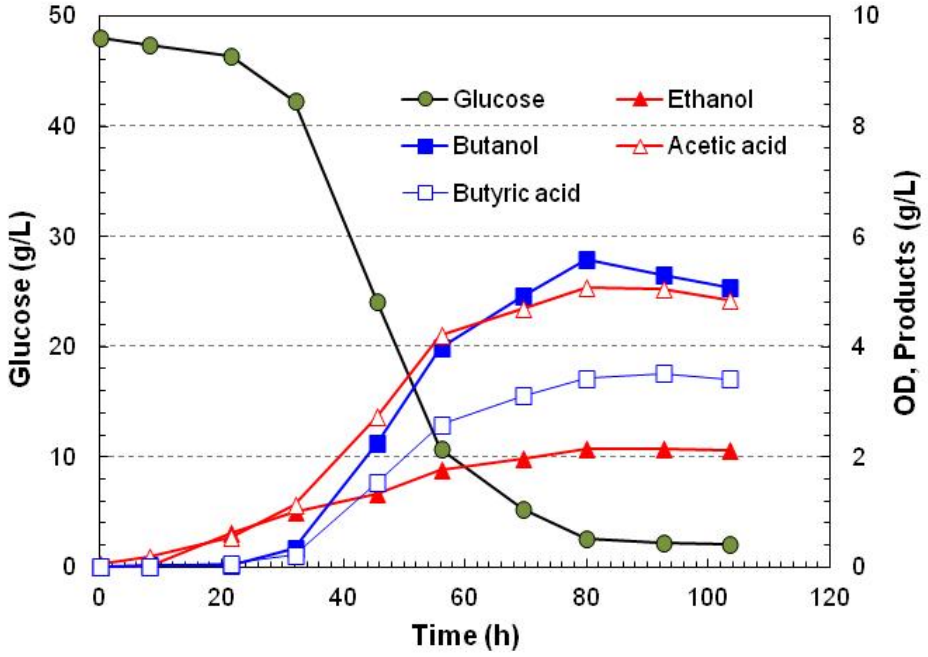
3 – Technical Accomplishments/Progress/Results

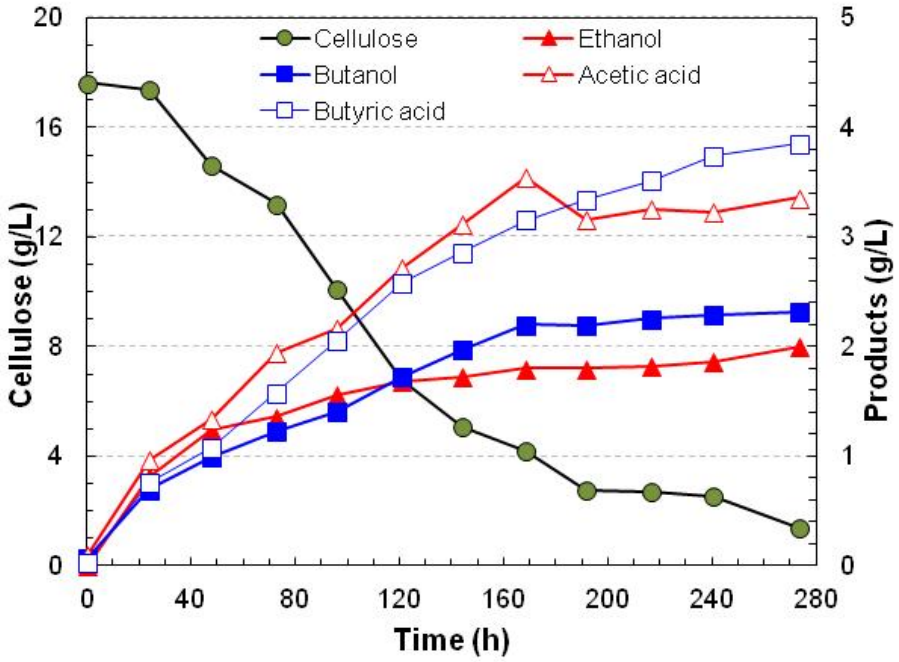
- Metabolic engineering of *C. cellulovorans* for butanol production from cellulose
  - Developed new cloning vehicles (plasmids) with better compatibility with the host cells that greatly increased transformation efficiency to facilitate metabolic engineering study and strain development
  - Constructed 7 engineered strains overexpressing various heterologous genes and evaluated their fermentation kinetics in serum bottles
  - The best studied strain overexpressing *adhE2* meets all of our quarterly milestones to date

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)	Achieved
M1.3	Strains producing little or no acids, with butanol and ethanol at >0.3 g/g (Q5); >0.35 g/g (Q6)	Achieved
M1.4	A high butanol tolerant strain capable of producing butanol at >2.5 g/L (Q4); >5 g/L (Q6)	Achieved Q4
Go/No-Go #1	Select strains producing *n*-butanol at titer of >2.5 g/L, yield of 0.2 g/g cellulose (Q4)	Meet

C. cellulovorans – adhE2

Batch fermentation in serum bottles – Cellulose vs.Glucose

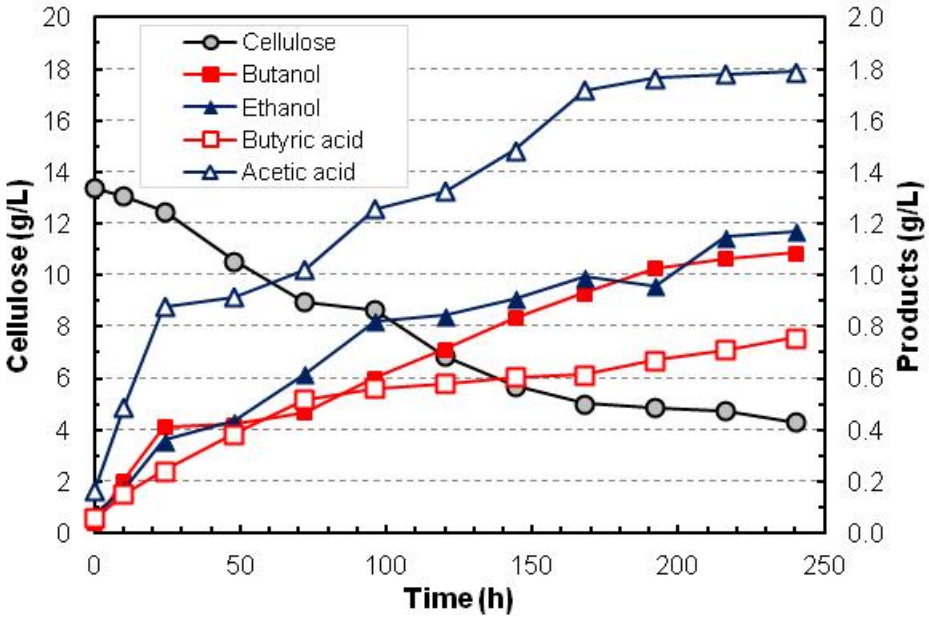


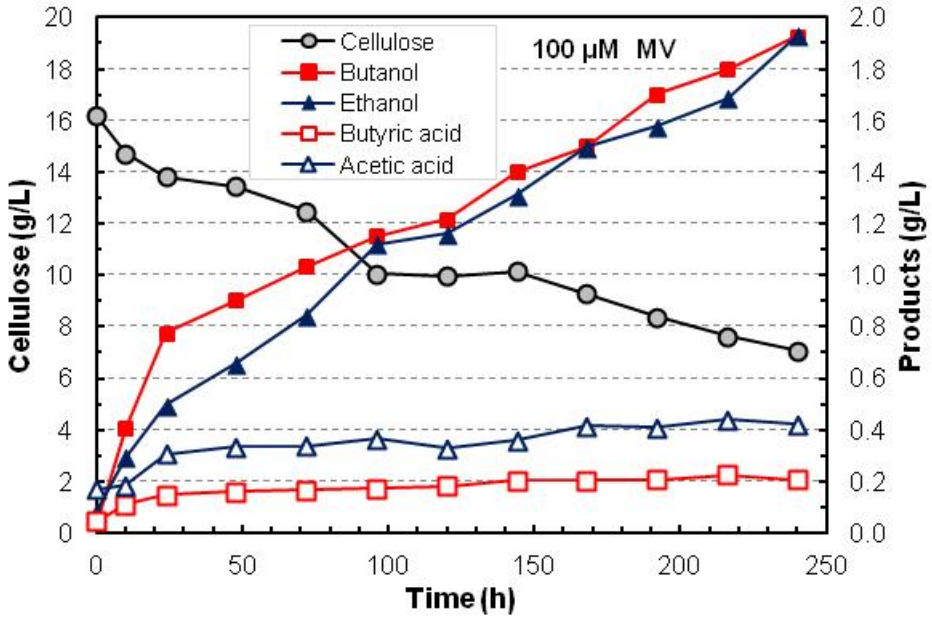


Substrate	Butanol Yield (g/g)	Ethanol Yield (g/g)	Butanol Productivity (g/L h)
Glucose	0.122	0.046	0.070
Cellulose	0.164	0.141	0.013

C. cellulovorans – adhE2

Batch fermentation in serum bottles – Effects of MV



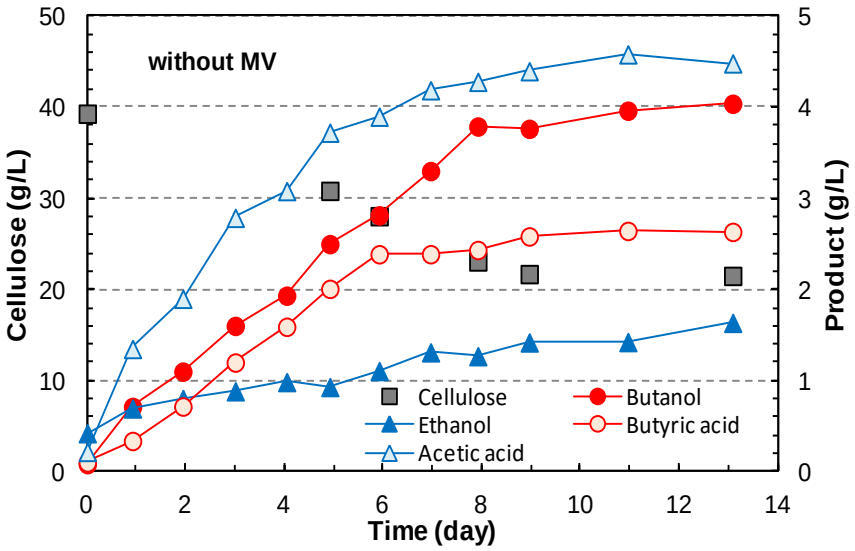


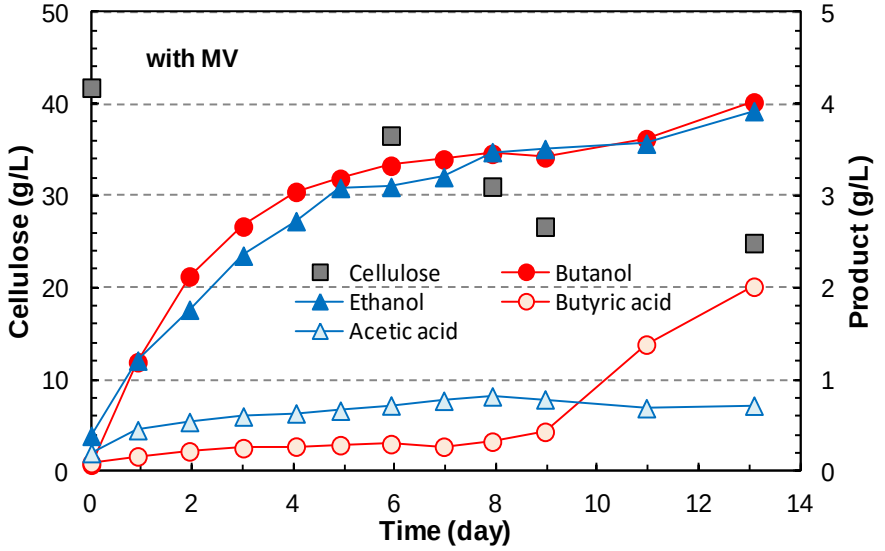
Methyl viologen (MV) as an artificial electron carrier was added to shift metabolic flux to increase NADH availability for alcohol production

MV	Butanol Yield (g/g)	Ethanol Yield (g/g)	Butanol Productivity (g/L h)
Without MV	0.098	0.105	0.005
With MV	0.196	0.187	0.008

Butanol production from cellulose

- High cell density fermentation in serum bottles
- Effects of MV on batch fermentation, pH 6.5-7.0
- More alcohols and less acids were produced with MV

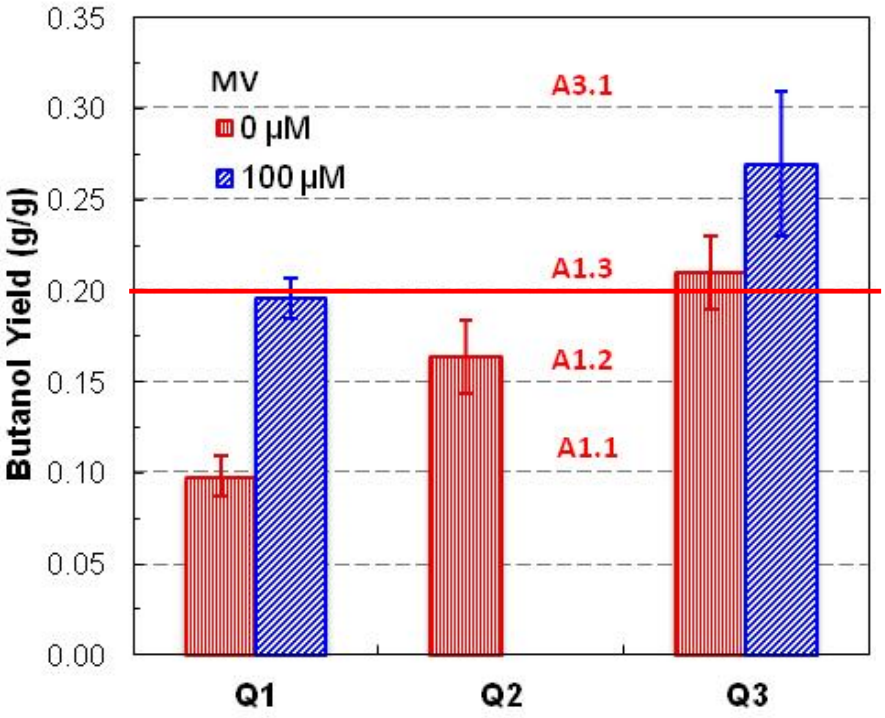


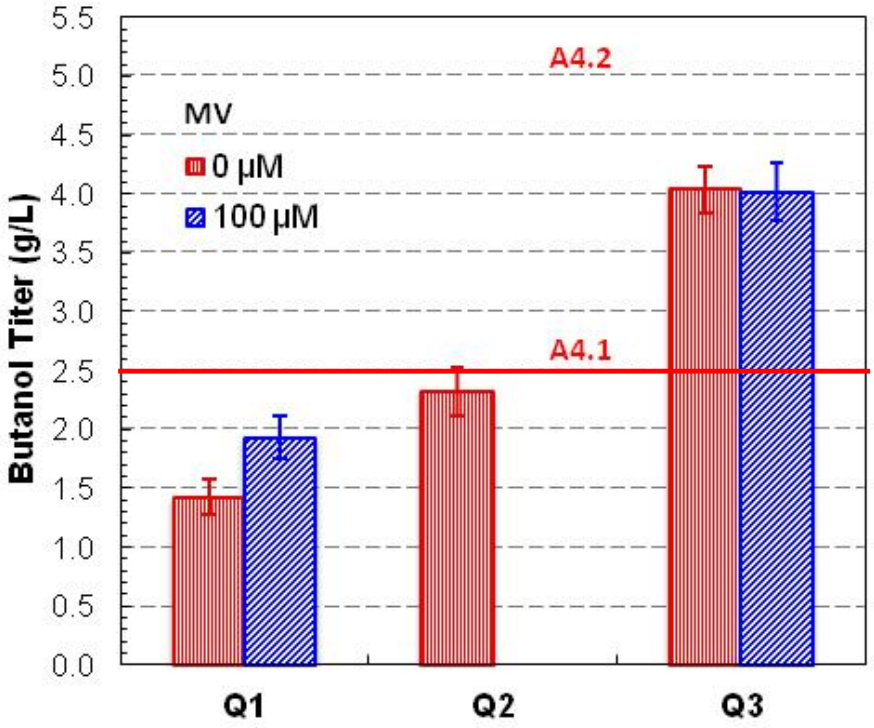


Yield (g/g)	Butanol	Ethanol	Butyrate	Acetate	Alcohols/Acids
Without MV	0.21	0.06	0.13	0.22	0.77
With MV	0.22	0.20	0.11	0.03	3.23

Butanol production from cellulose

Batch fermentation with high cell density

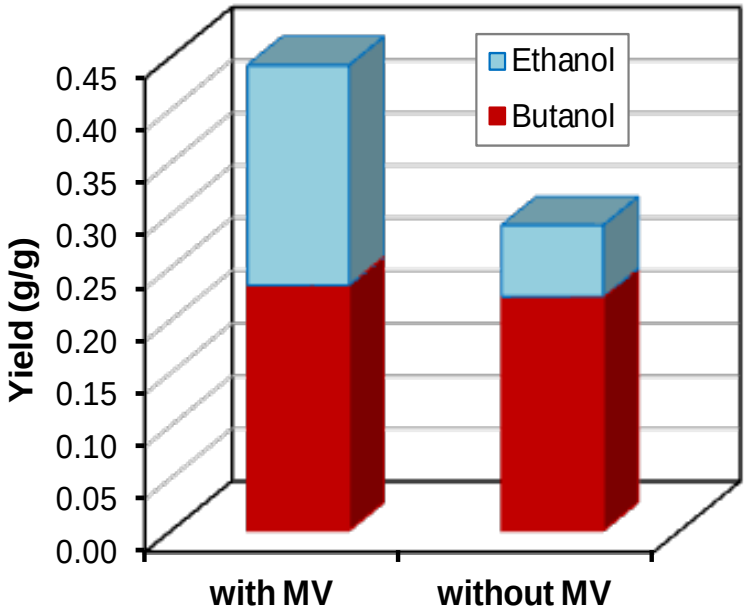


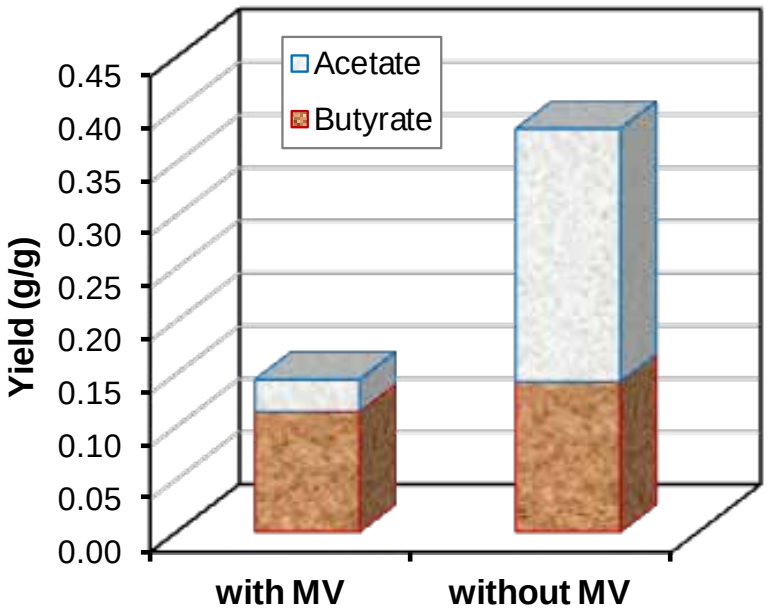


C. cellulovorans mutant overexpressing *adhE2* meets the Go criteria:
Butanol yield >0.2 g/g cellulose, Butanol titer >2.5 g/L

Butanol production from cellulose

Batch fermentation with high cell density



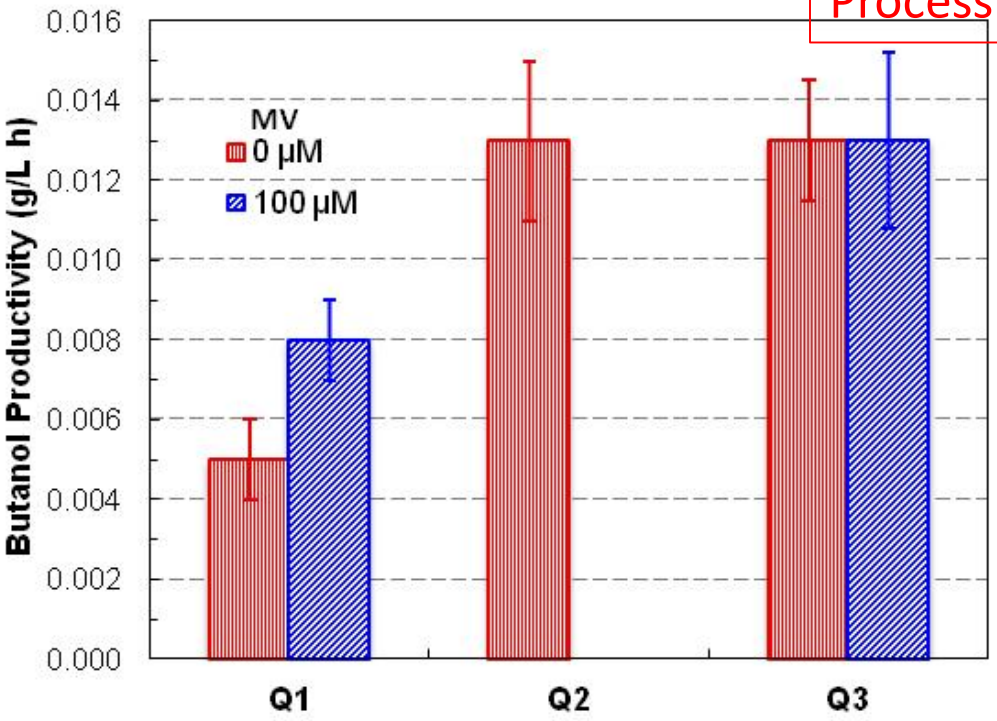


The total alcohol (butanol and ethanol) yield from cellulose consumed in the fermentation was 0.27 g/g without adding MV and 0.42 g/g with MV, meeting our **milestone M1.3** (butanol and ethanol as the main products at >0.3 g/g (Q5) and >0.35 g/g (Q6).

Butanol production from cellulose

- Compared to glucose, butanol productivity from cellulose is low due to low cell density/activity and slow cellulose degradation

Glucose: 0.049 g/L h, or 3.8-fold
Process goal: 0.10-0.25 g/L h



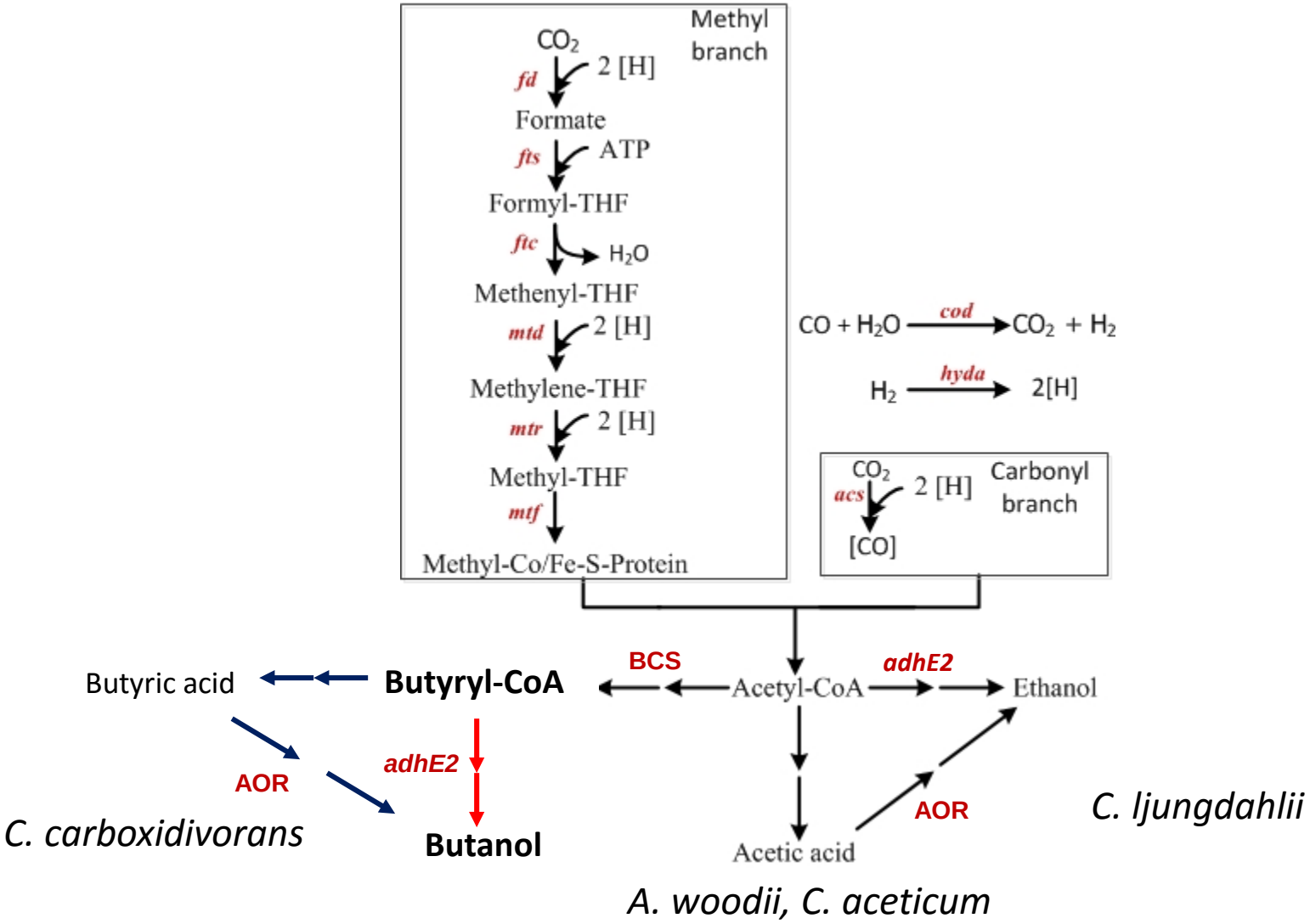
- Productivity can (will) be improved (>10-fold) in bioreactor with process optimization and by better pretreatment of cellulose to increase its accessibility to cells for degradation

3 – Technical Accomplishments/Progress/Results

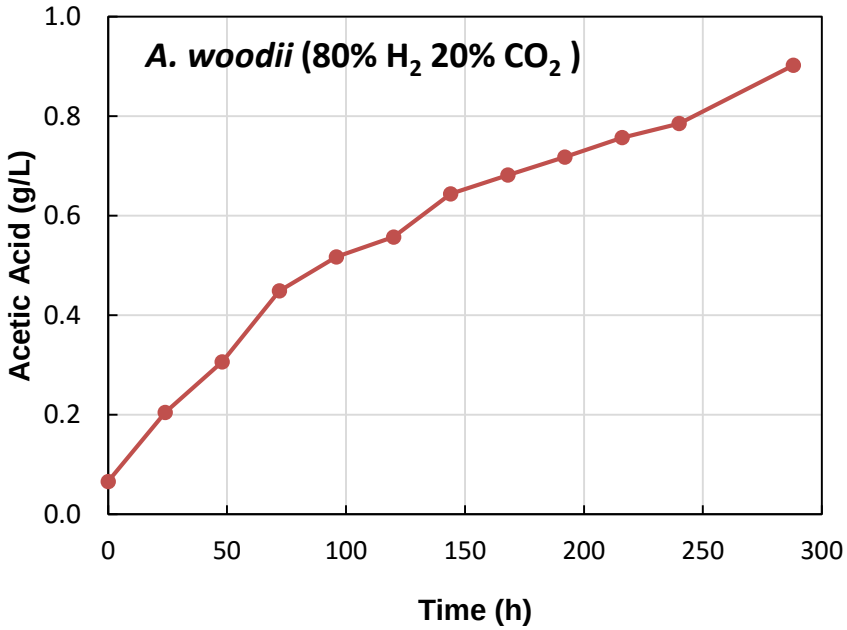
- Metabolic engineering of acetogens for butanol and ethanol production from CO_2 and H_2
  - All acetogens have very robust restriction modification (RM) systems, hindering effective transformation of recombinant plasmids into host cells.
  - Analyzed RM systems based on available genomic sequences to identify key restriction sequences and methylation method to protect plasmids
  - Developed new plasmids with better compatibility with *C. aceticum* that greatly increased transformation efficiency to facilitate metabolic engineering study and strain development
  - Constructed recombinant plasmids for expressing *adhE2* and BCS operon genes for ethanol and butanol production (transformation and mutant screening are ongoing)
  - Evaluated 4 acetogens for their ability to use CO_2/H_2 in serum bottles
  - The best strain meets our quarterly milestones

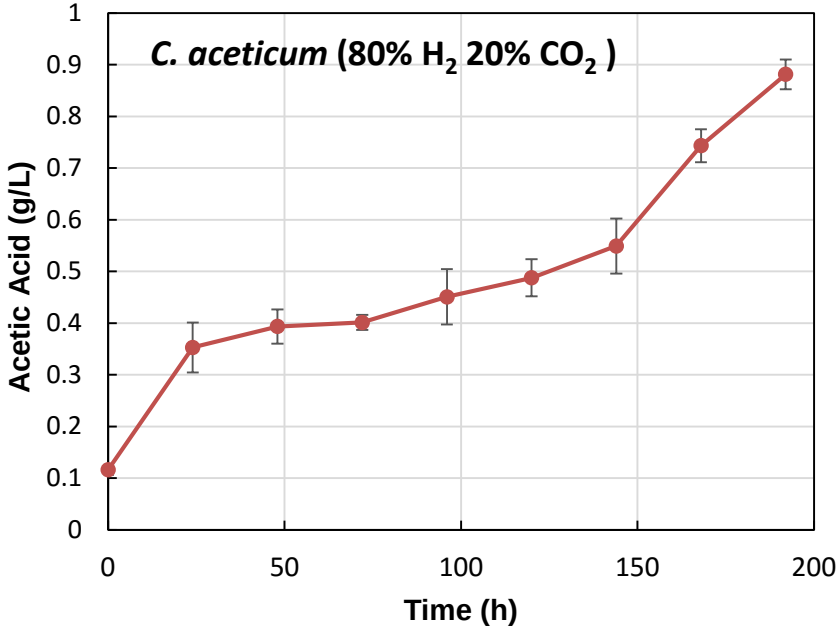
Milestone	Description (Targeted Quarter to Meet)	Status
M1.2	A strain producing butanol and ethanol from CO_2 and H_2 at >0.1 g/L (Q4); >0.4 g/L (Q6)	Achieved

Metabolic Pathway for Butanol and Ethanol Production from CO_2 and H_2 in Acetogens



Acetate production from CO_2 and H_2

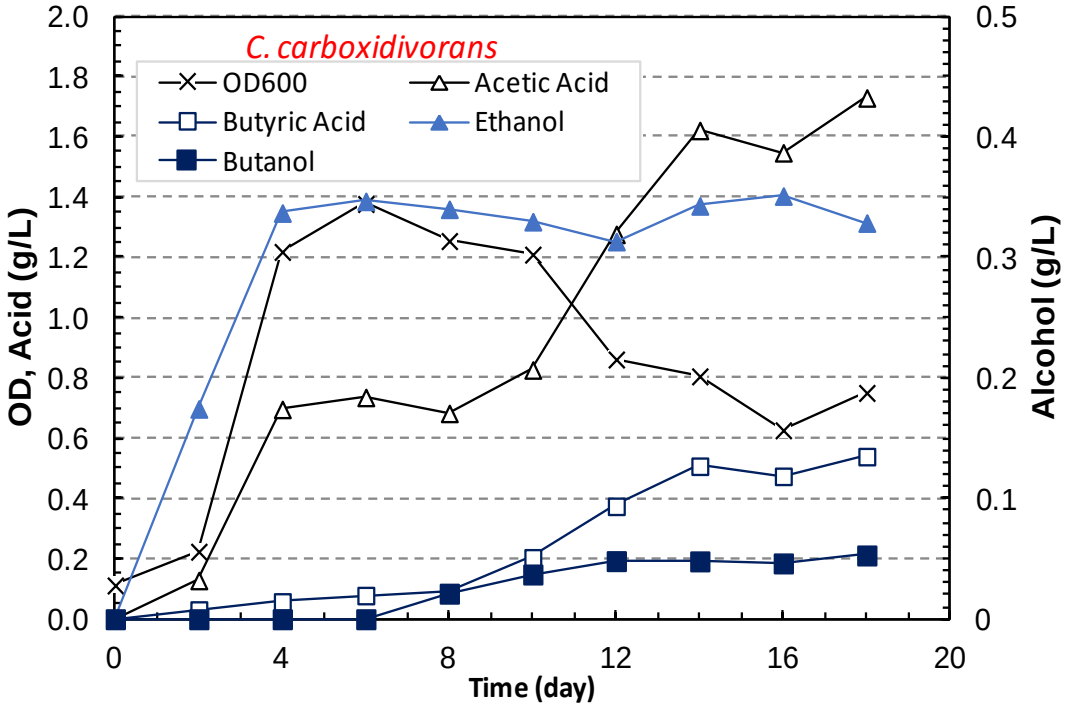




Strain	*A. woodii*	*C. formicoacetium*		*C. aceticum*	
Gas component	20% CO_2 80% H_2	20% CO_2 80% H_2	20% CO_2 80% N_2	20% CO_2 80% H_2	20% CO_2 20% H_2 60% N_2
Acetic Acid Yield (g/g CO_2)	0.84	0.27	0.22	0.77	0.51
Productivity (g/L·h)	0.00290	0.000953	0.000751	0.00399	0.00332

High acetate yield but low productivity from CO_2/H_2 due to low cell density and gas solubility

Alcohols production from CO_2 and H_2



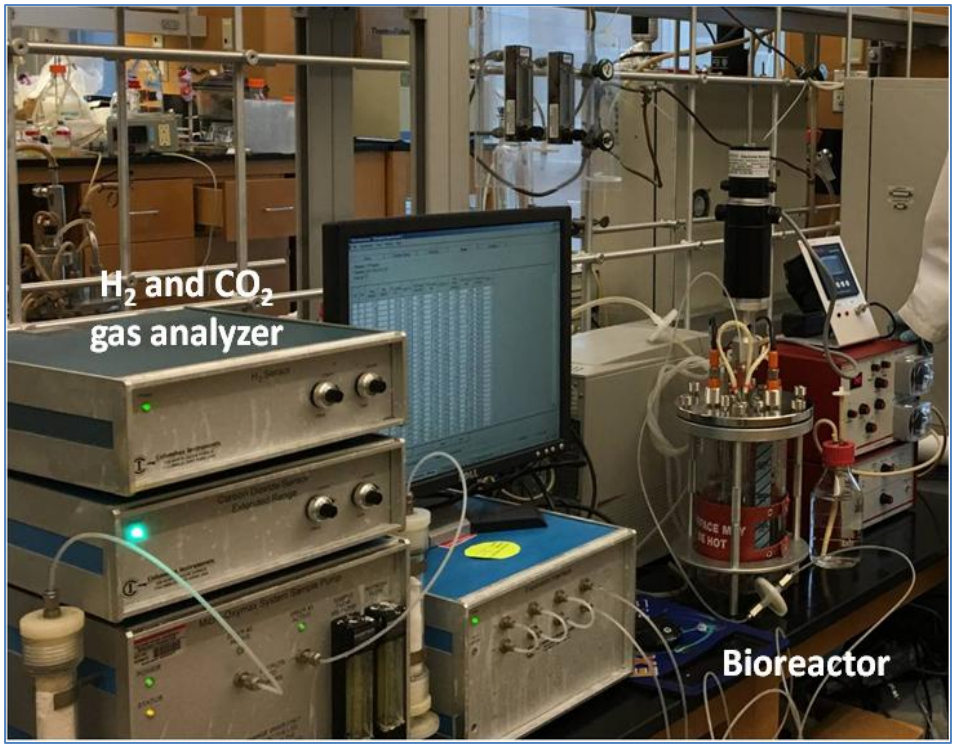
C. carboxidivorans produced not only acetate and butyrate, it also produced significant amounts of ethanol (0.35 g/L) and butanol (0.05 g/L) from CO_2 and H_2, meeting our milestone M1.2 (strain producing butanol and ethanol from CO_2 and H_2 at >0.4 g/L).

3 – Technical Accomplishments/Progress/Results

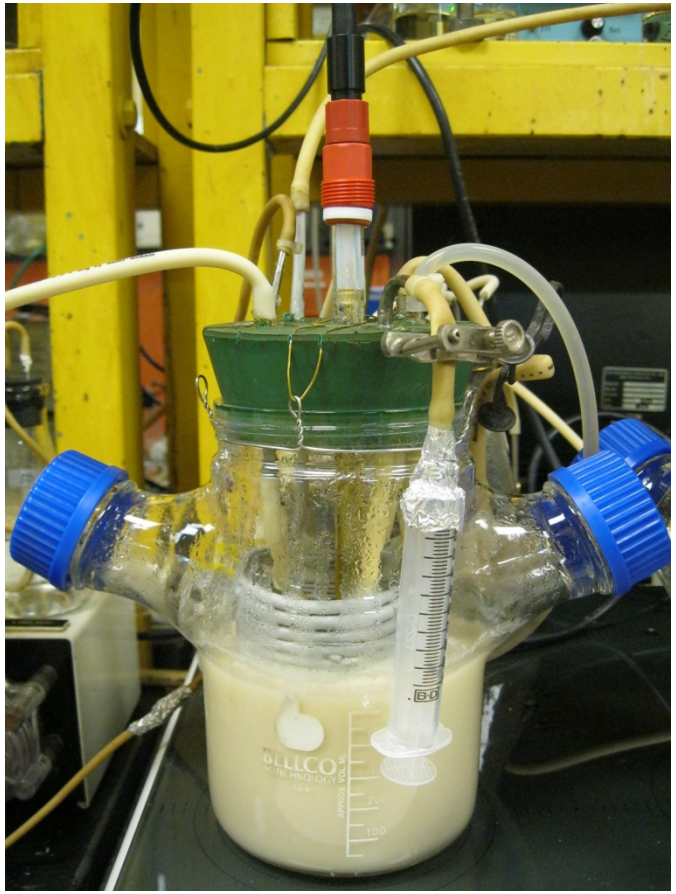
- Fermentation kinetics studies and process optimization
  - Studied fermentation kinetics in serum bottles for various strains developed to date
  - Cellulose fermentation studied in stirred-tank bioreactors (1-5 liters). Process parameters are being optimized.
  - Gas fermentation in different types of bioreactors (stirred-tank, bubble column, packed bed) are in progress.
  - Medium optimization focusing on increasing cell density, activity, and productivity.
  - Different pretreatment methods for enhancing cellulose degradability are being evaluated.
  - In situ product recovery by adsorption and gas stripping to alleviate butanol toxicity will be done when butanol production is >10 g/L.

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)	Achieved
M2.2	Optimized medium to support cell growth at density >OD 10 (Q4)	Achieved
M2.3	Reactor for high cell density fermentation, >OD 20 (Q5), productivity >0.1 g/L·h (Q7)	In progress

Fermentation kinetics studies and process optimization



Bioreactor for gas fermentation with H_2 and CO_2 gas analyzer



Cellulose fermentation

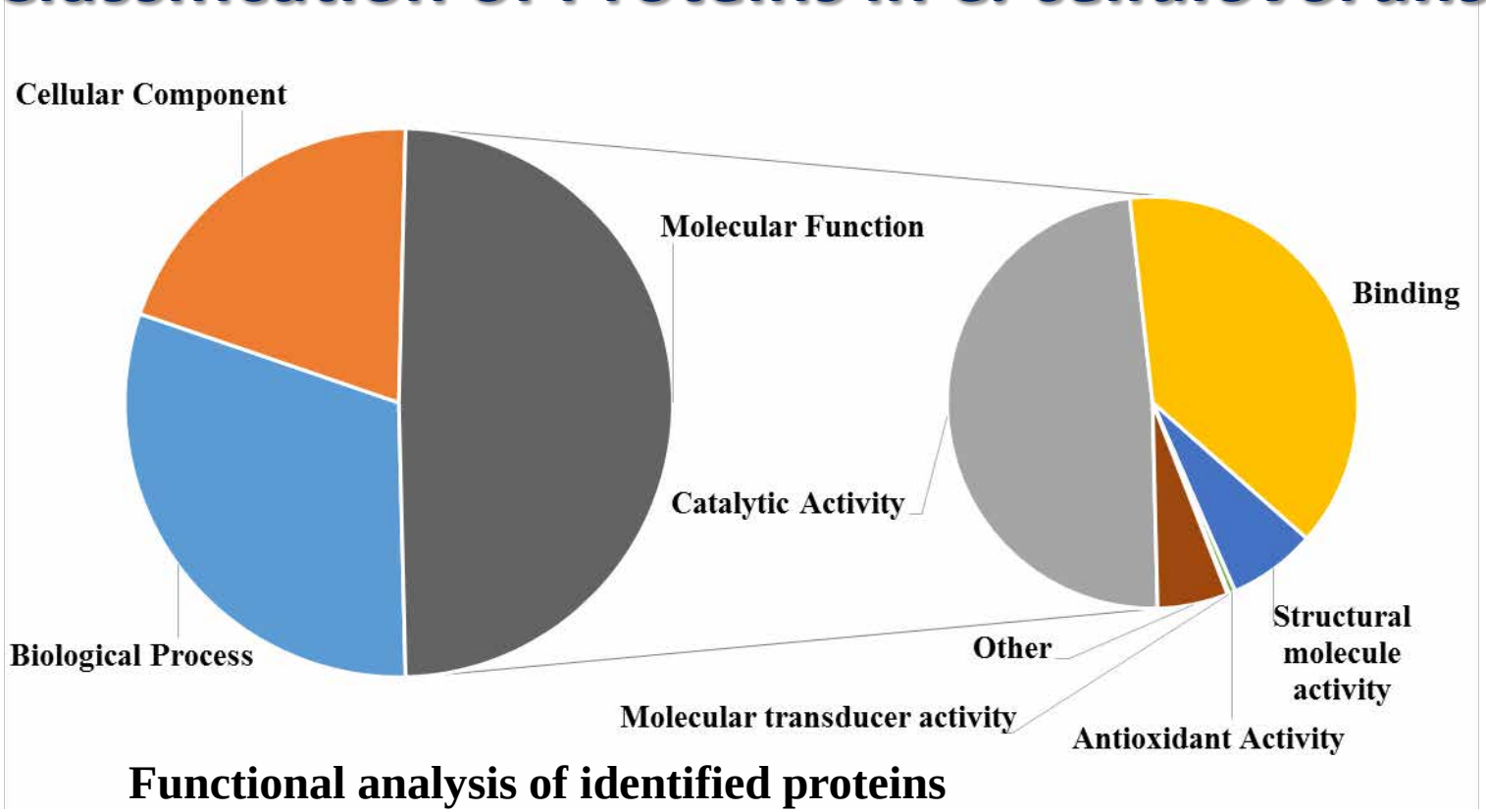
High cell density is achieved with cell recycle and/or immobilization in bioreactor

3 – Technical Accomplishments/Progress/Results

- Omics analysis of mutant strains under various fermentation conditions
  - Completed some comparative proteomics analysis for *C. cellulovorans-adh*E2 in batch fermentations with glucose, cellobiose, 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 could be the targets for metabolic engineering
  - Completed some comparative metabolomics analysis for *C. cellulovorans-adh*E2 in batch fermentations with glucose and cellulose as carbon source, respectively.
  - Possible metabolic flux bottlenecks (rate-limiting steps) are identified for 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)	Achieved
M3.2	Core metabolites responsible for carbon, energy and redox balance identified to assist process development and scale-up (Q7)	In progress

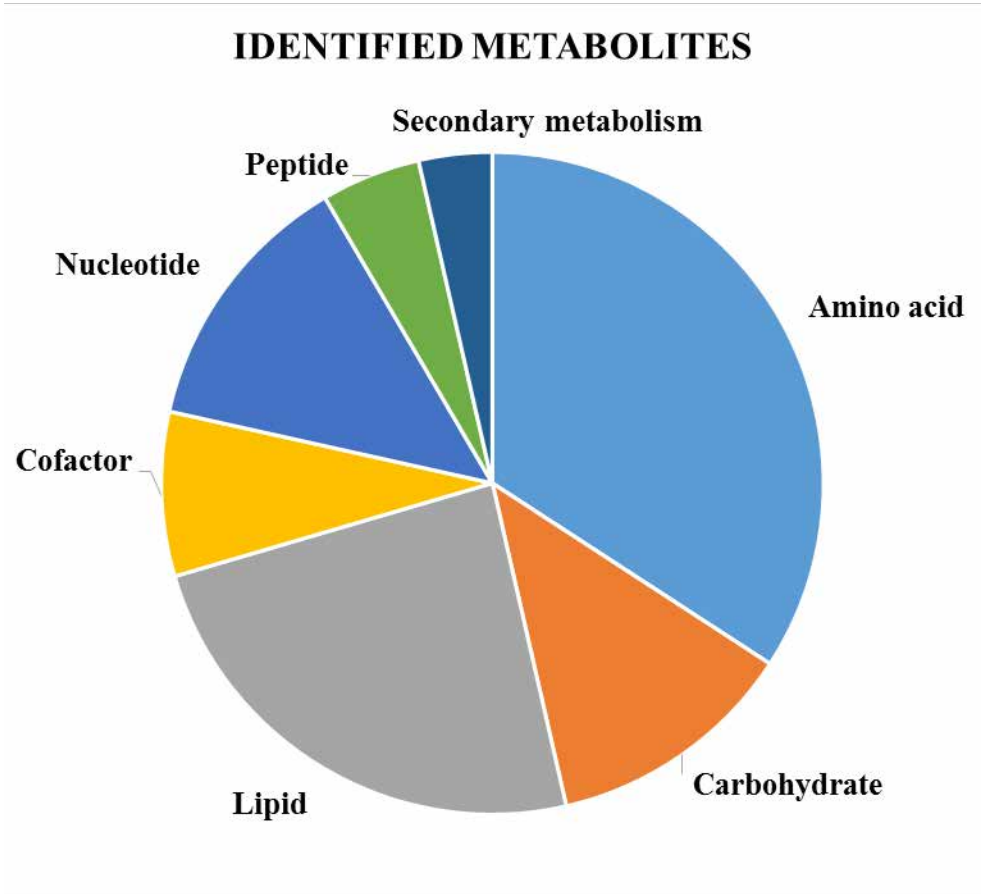
Proteomics Analysis: Classification of Proteins in *C. cellulovorans*



Functional analysis of identified proteins

- 624 proteins are grouped into cellular component, biological process, and molecular function based on gene ontology.
- Protein or metabolite changes can be related to their functional group.
- The effect of different conditions will be identified through global analysis.

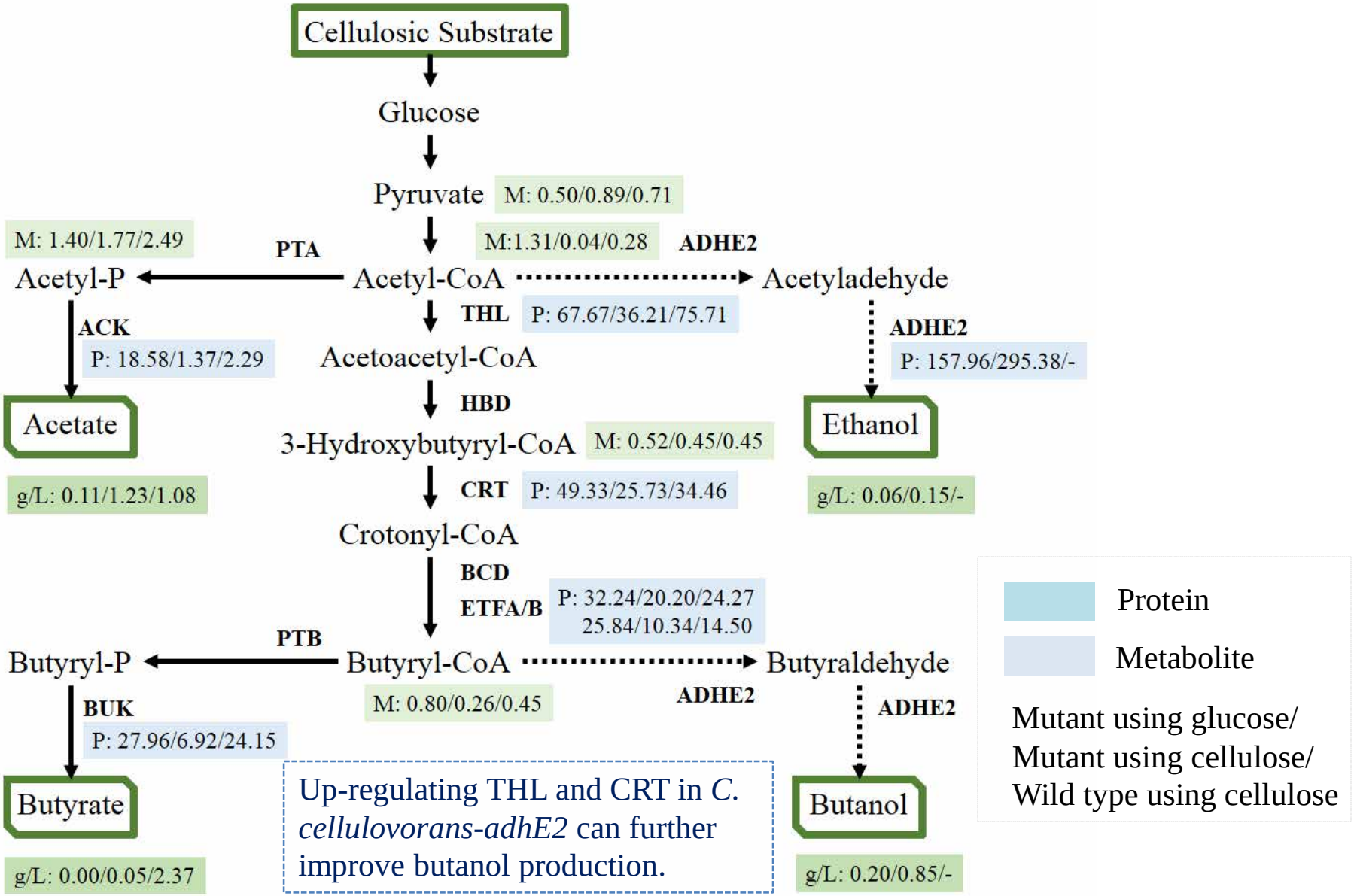
Metabolomics Analysis: Classification of Metabolites in *C. cellulovorans*



Classification analysis of identified metabolites

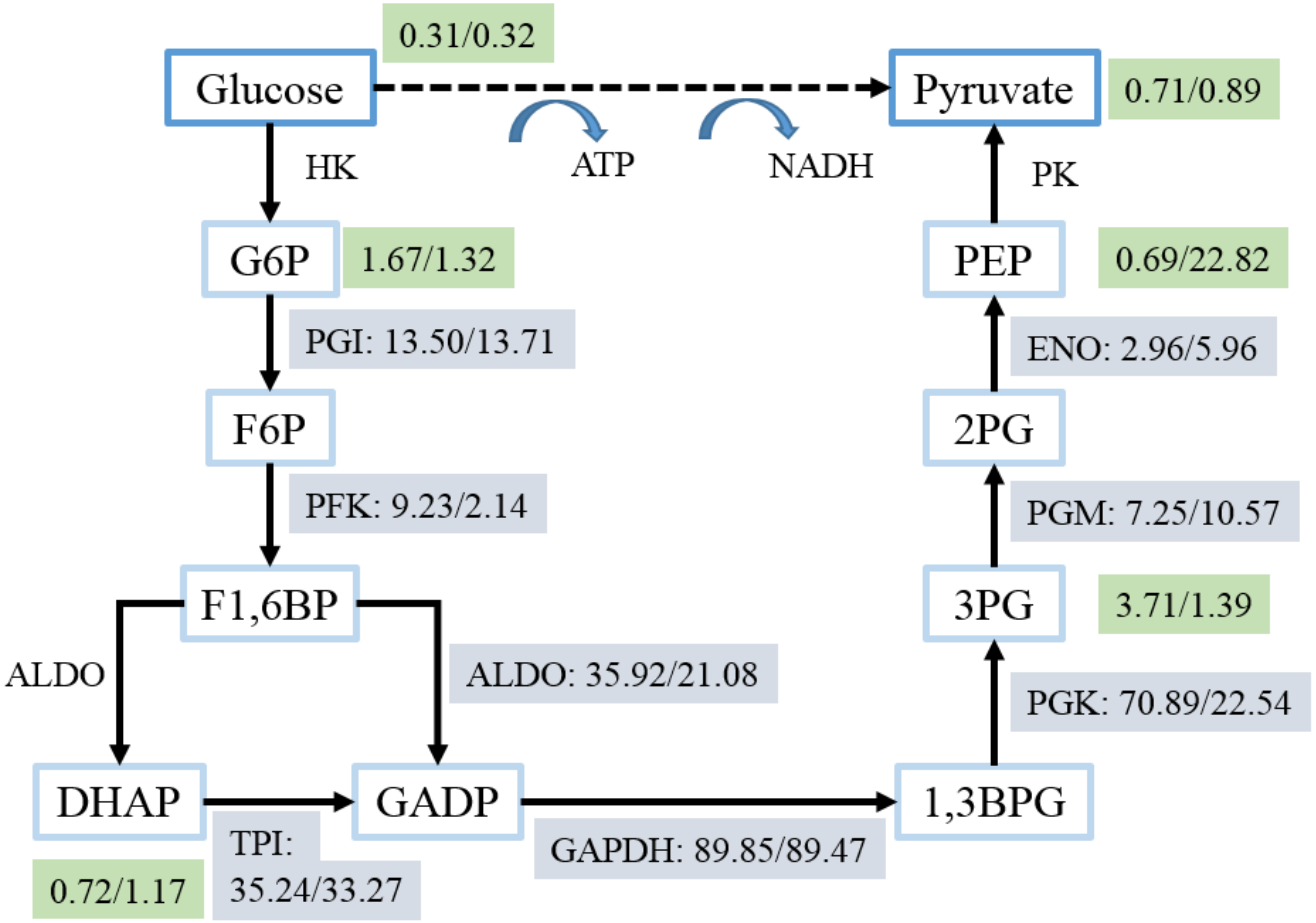
- Total of 474 intracellular metabolites were extracted and identified from *C. cellulovorans.*
- The metabolites are grouped as amino acid, carbohydrate, lipid, cofactor, nucleotide, peptide, and secondary metabolism.
- Unstable metabolites, such as cofactor and some peptide, were also quantified in our method.

Proteomics & Metabolomics Analyses



Proteomics & Metabolomics Analyses

Glycolysis Pathway



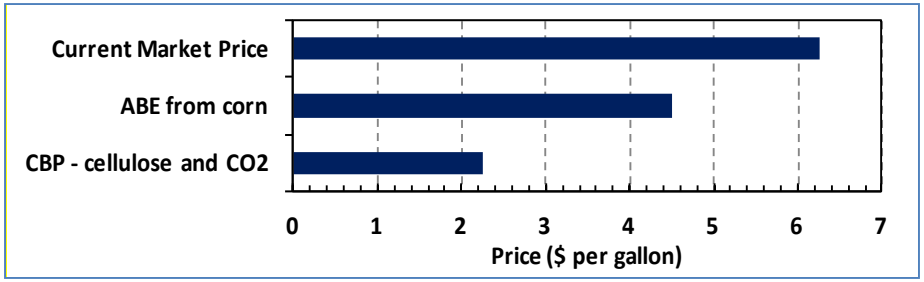
PFK and PGK up-regulation could improve the glycolysis efficiency in *C. cellulovorans-adhE2*.

4 – Relevance

Developing commercially viable bioenergy and bioproduct technologies

- Directly supports BETO's mission:
  "*Develop and demonstrate transformative and revolutionary bioenergy technologies for a sustainable nation*"
- Address BETO's 2022 target for a conversion cost of $3.0 per gallon of gasoline equivalent
  - Project fulfills a critical need for Conversion Enabling Technologies: "The need to develop the next generation of biocatalysts for conversion of biomass and … is critical in the advancement of biomass processing technologies.'
- Project metrics and technical targets are driven by TEA
- Reduction in conversion costs through improvements in: Direct cellulose conversion, CO_2 utilization, C efficiency/yield, process integration
- CBP integrated with engineered clostridia for butanol production from celluloses and CO_2 will be able to produce advanced biofuel at a competitive cost of $2.25/gal and reduce GHG emissions by >50%.

The technology will be validated at a pilot-scale plant and commercialized by ***Green Biologics***



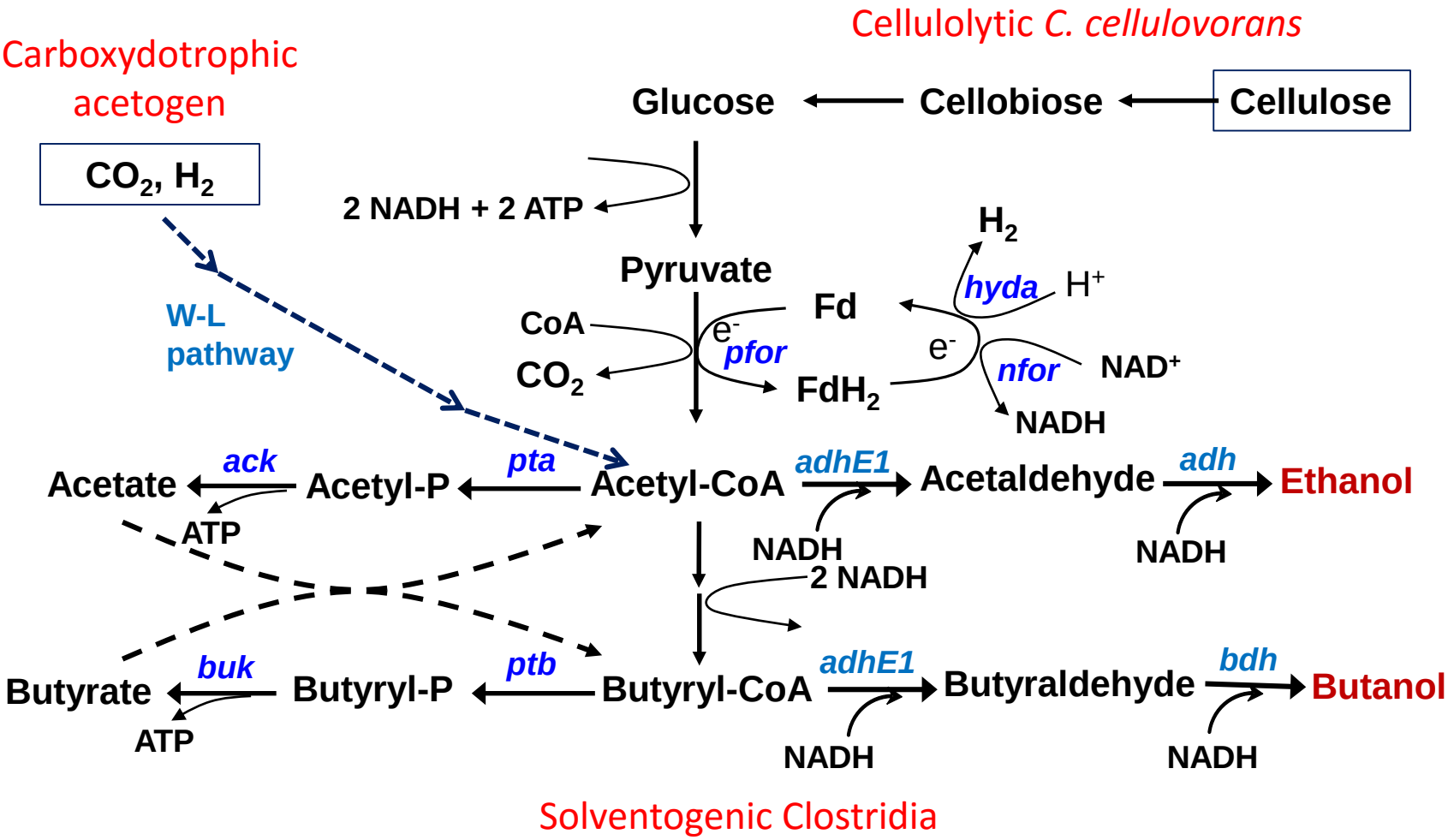
5 – Future Work

- Engineering *C. cellulovorans* to knockout acetate and butyrate biosynthesis using CRISPR-Cas9 genome editing technique
- Engineering acetogens for butanol and ethanol biosynthesis from CO_2 and H_2
- Continue proteomics and metabolomics analyses to aid ME and process design
- Mixed-culture fermentation process development and optimization
- Integrated fermentation process with in situ product recovery
- Life cycle and cost analyses

Milestone	Description	Month
M2.3	High cell density fermentation with OD >20, productivity >0.1 g/L·h	21
M2.4	Mixed fermentation process with cellulosic and gaseous substrates producing butanol and ethanol at >5 g/L	21
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	23
M4.1	Pre-treatment process selected. The process and conceptual plant design with outline butanol production costs achieved	21
M4.2	Process and conceptual plant design of advanced fermentation defined	22
M4.3	Process and conceptual plant design for CBP and co-culture completed	23
M4.4	A conceptual plant for butanol production from lignocellulosic biomass at $2.25/gal with 90% reduction in GHG emissions	24

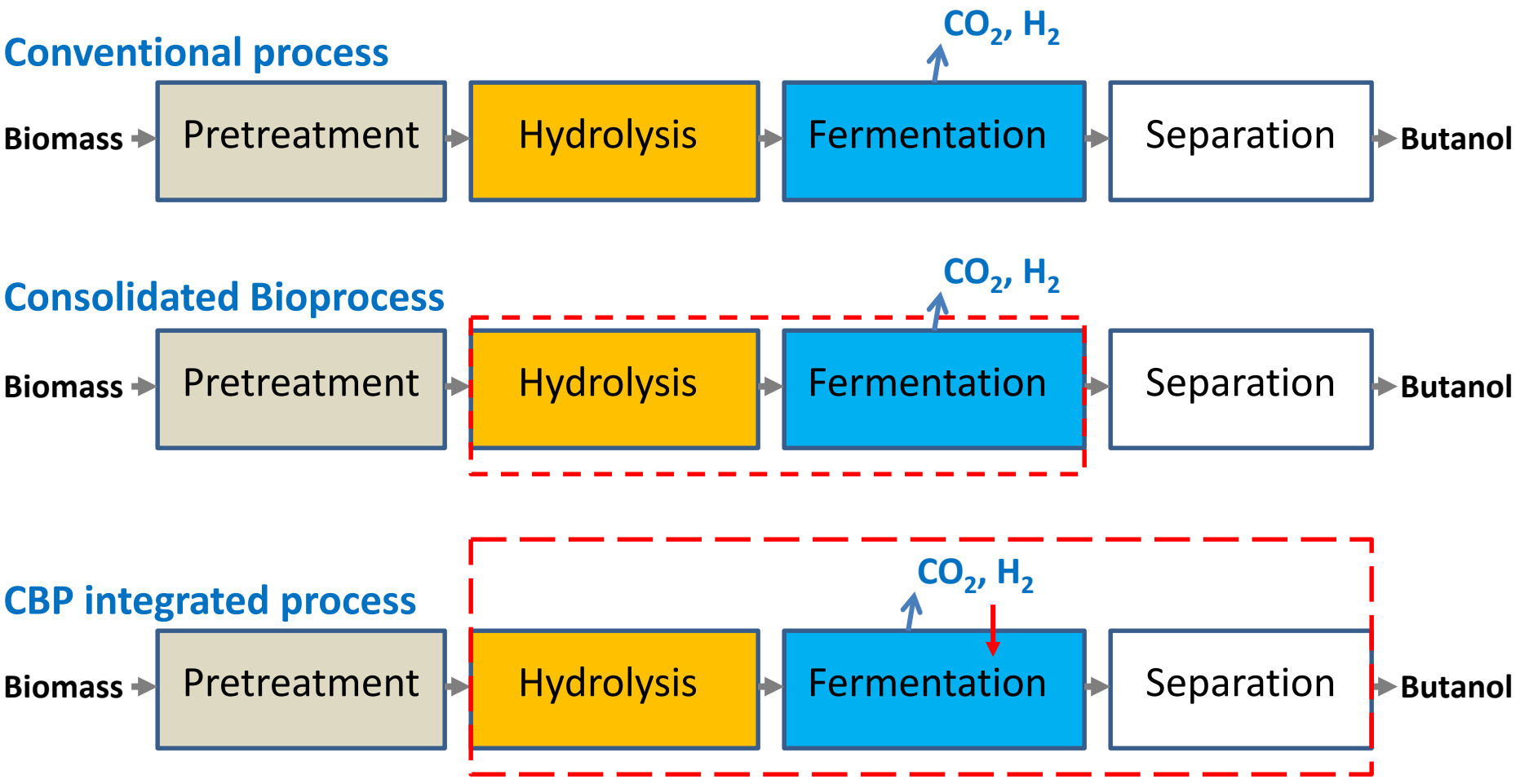
- The remaining budget ($606,460) is sufficient to complete the remaining work

Mixed Culture Fermentation



Butanol titer: >10 g/L, Yield: >0.3 g/g, Productivity: >0.2 g/L h

Process Integration and Consolidation



Conceptual design, Process simulation, Cost analysis, Life cycle analysis

Summary

- Consolidated BioProcessing with engineered clostridia for *n*-butanol production from cellulose and CO_2
- *C. cellulovorans* engineered to express genes for directly converting cellulose to *n*-butanol at a high yield (>0.4 g/g); CO_2 is further converted to acids and alcohols by acetogens in a co-cultured fermentation
- Metabolic and process engineering are aided with proteomics and metabolomics analyses to meet milestones and reach project goal
- The process integrated with *in situ* separation is expected to produce *n*-butanol at $2.25/gal ($3.0 gge) and reduce GHG emissions by >50%
- Strain engineering and process optimization will be continued to support the development of a co-cultured fermentation process with economical and life cycle analyses performed by industrial partner