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

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Biochemical Conversion

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Goal Statement

- 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.

- The engineered strains would 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, 2018
- Completion: 100%

Budget

	Total Costs Pre FY 17	FY 17 Costs	FY 18 Costs	Planned Funding (FY 19-)
DOE Funded	$519,569	$548,906	$163,673	-
Project Cost Share	$107,215 OSU: 66,409 GB: 22,235 UA: 18,571	$202,510 116,164 48,011 38,335	$5,648 2,560 0 3,086	-

Partners:

- OSU: Ohio State University (61.6%)
- GB: Green Biologics (19.6%)
- UA: University of Alabama (18.8%)

Barriers addressed

- Ct-D. Advanced Bioprocess Development

Objective

- Increasing butanol titer, rate, and yield in fermentation through metabolic engineering and process improvements to lower the production cost from cellulosic biomass

End of Project Goal

- Engineered strains that can be used in an integrated process for biobutanol production from cellulosic biomass at $2.25/gal or less than $3/gge

1. Project Overview

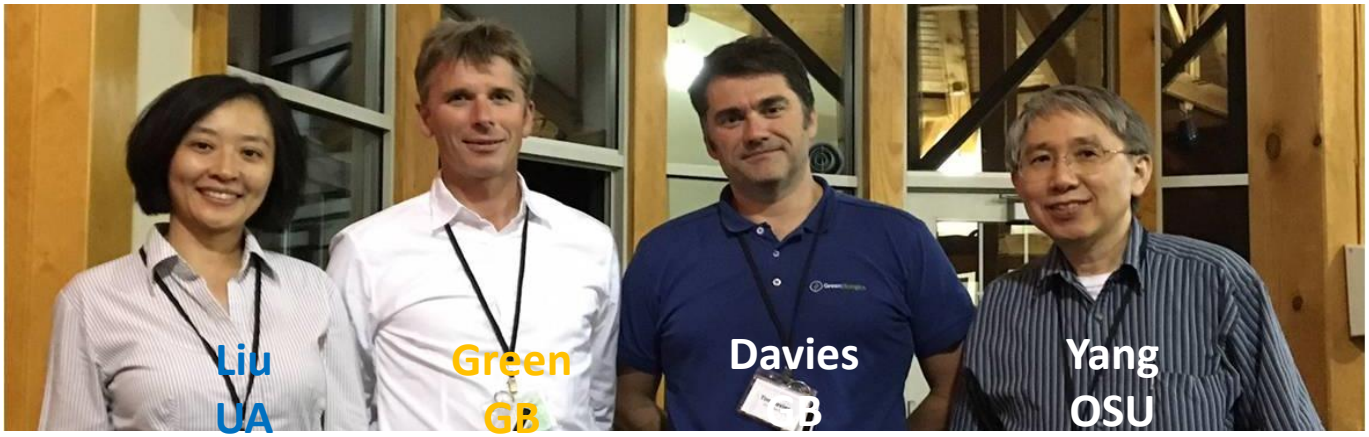
- This project had three partners - Ohio State University (OSU), Green Biologics (GB), and University of Alabama (UA) - with a long collaboration history working on biobutanol production.
- The proposal was submitted to DOE-EERE Biotechnology Incubator program in 2014 and funded in 2015 for 2 years (plus one-year no-cost extension for a total of 3 years).
- The project had 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 was managed by OSU sponsored research program office (Amy Dudley).
- Project was 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) (transferred to Univ. of Alabama at Birmingham (UAB) in the second year).
- PI and Co-PI's each led a major task:

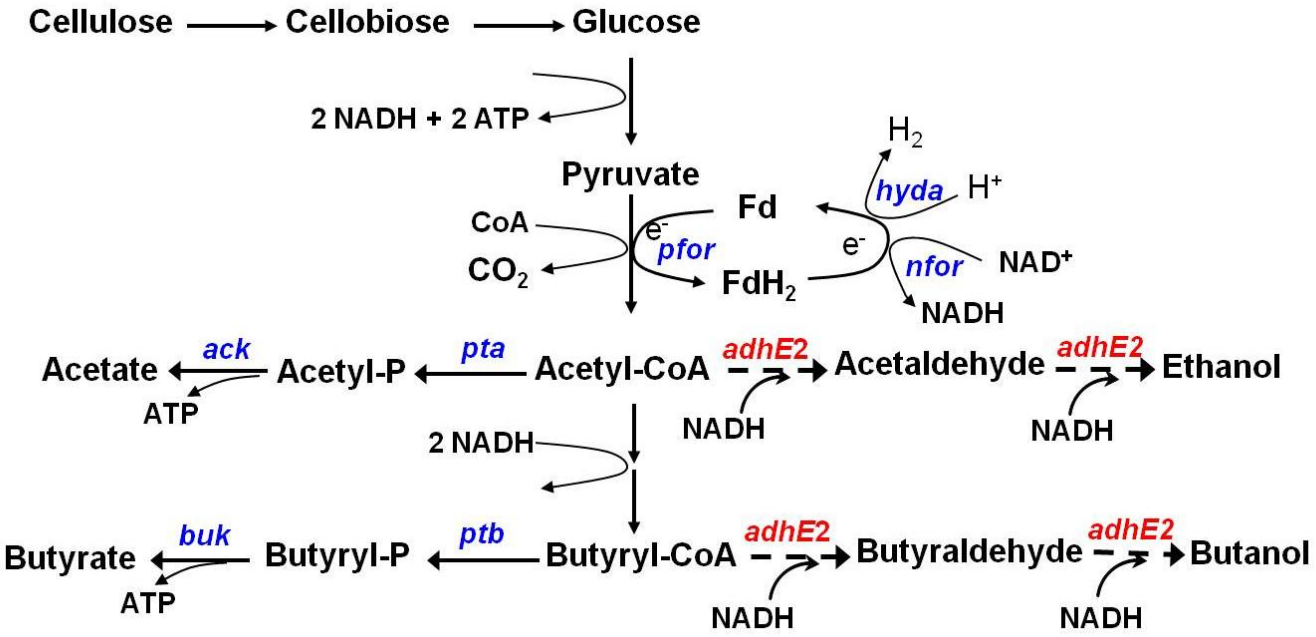
Task A	Task B	Task C	Task D
OSU	OSU / GB	UA	GB
Yang	Davies / Yang	Liu	Davies

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



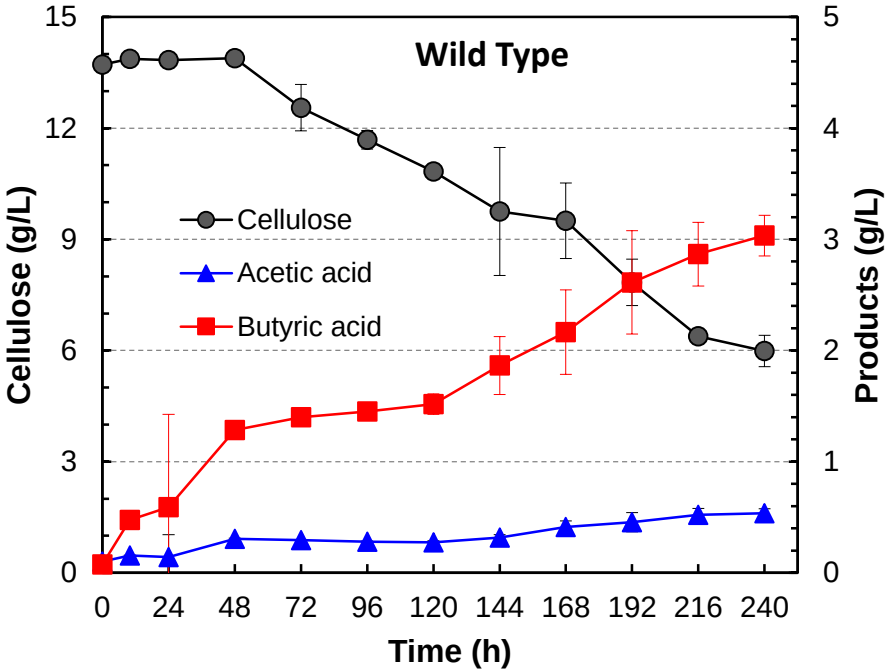
2 – Approach (Technical)

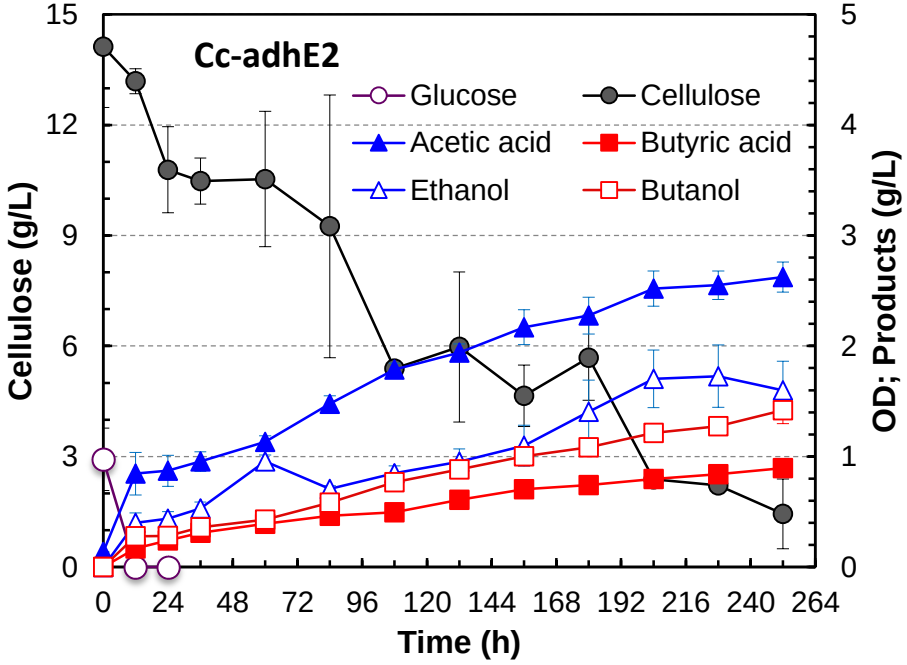
- Consolidated bioprocessing could save ~50% cost in using lignocellulosic biomass for biofuels production, but no organism naturally can produce *n*-butanol directly from cellulose.
- Engineering cellulolytic acidogen *Clostridium cellulovorans* to produce *n*-butanol and ethanol directly from cellulose 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 the Wood-Ljungdahl pathway

  Glucose → 3 Acetate

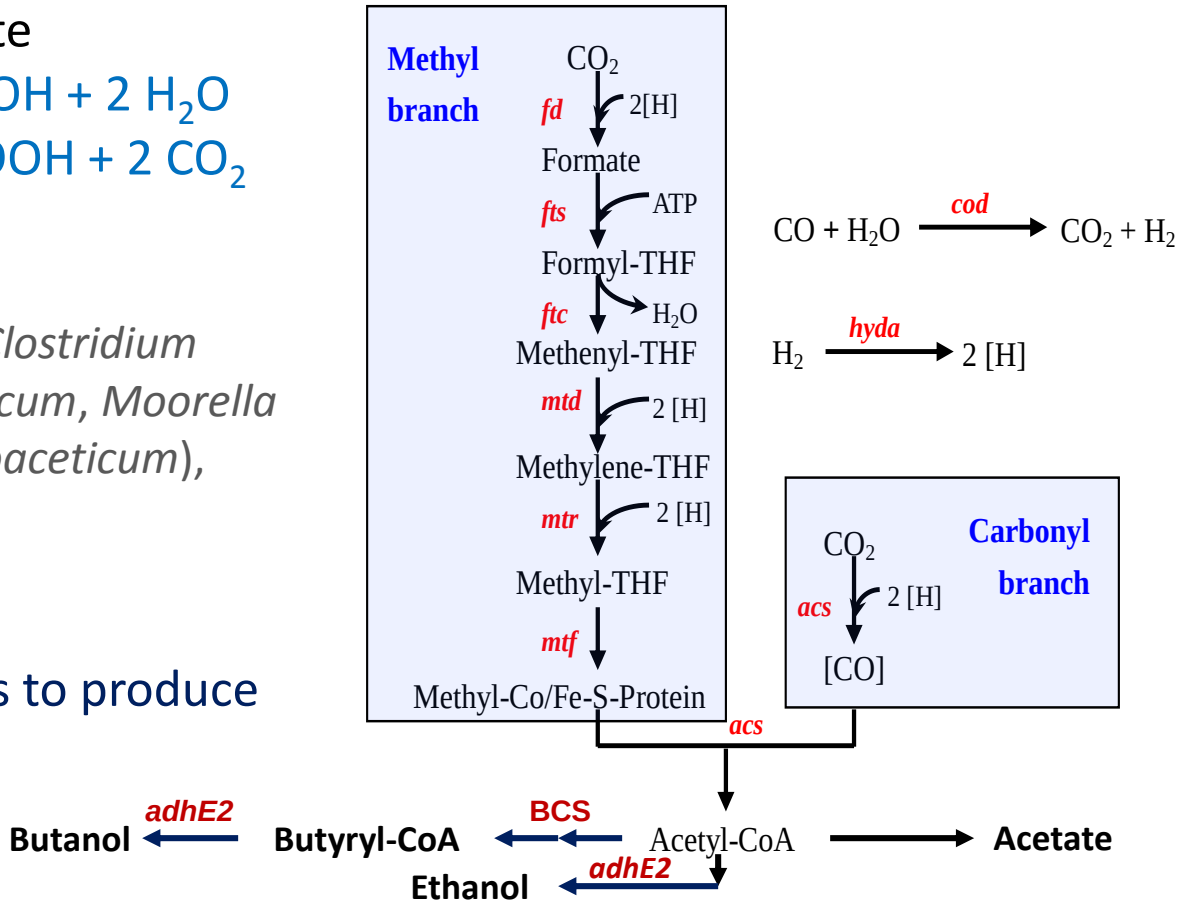
  $2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$

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

Homoacetogens:

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

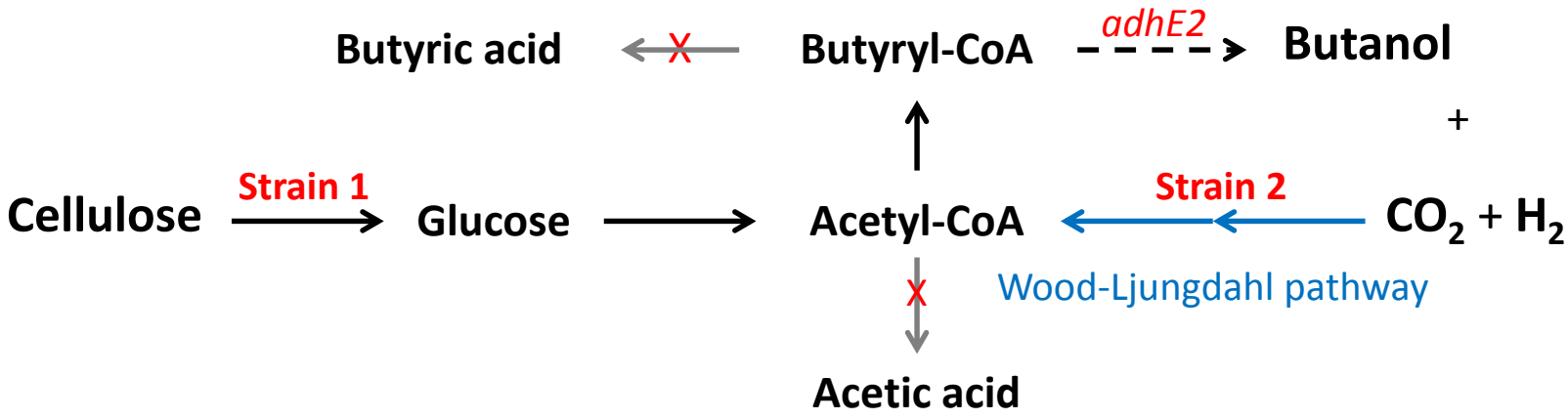
Engineering acetogens to produce ethanol and butanol



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
- Project Go/No Go decision point (Month 12 or 9/30/2016): obtaining engineered clostridial 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 was evaluated in batch fermentation with free cells in serum bottles. Samples were analyzed with HPLC and GC for sugars and fermentation products. Cellulose was analyzed after hydrolysis following the NREL protocol.
- Selected bacterial strains were further studied in 1-5 L bioreactors.

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

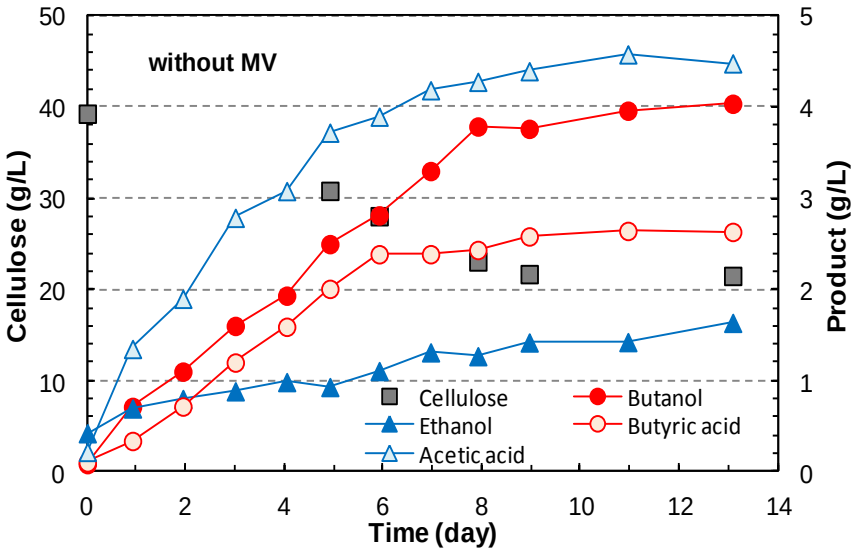
3 – Technical Accomplishments/Progress/Results

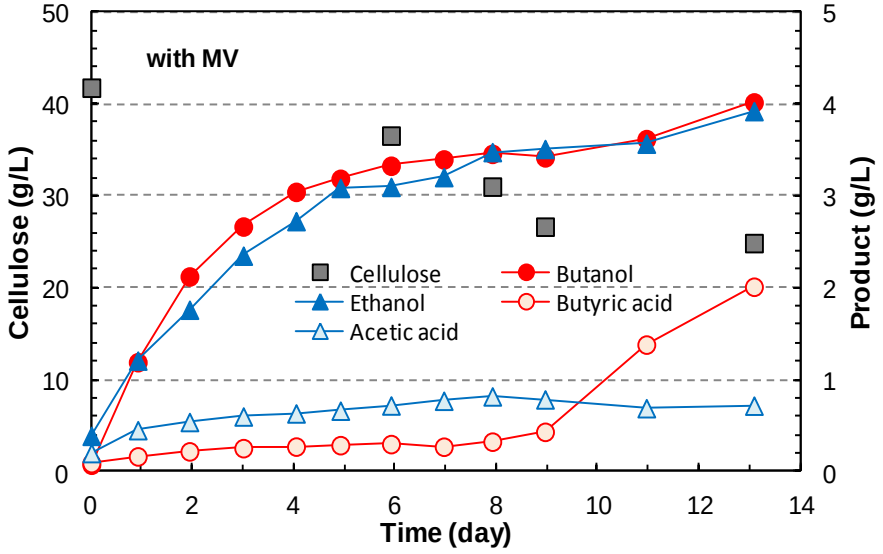
- Task A: Metabolic engineering of *C. cellulovorans* for butanol production from cellulose
  - Developed new cloning vehicles (plasmids) with better compatibility with the host cells that greatly increased transformation efficiency to facilitate metabolic engineering study and strain development
  - 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)	√
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)	√

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

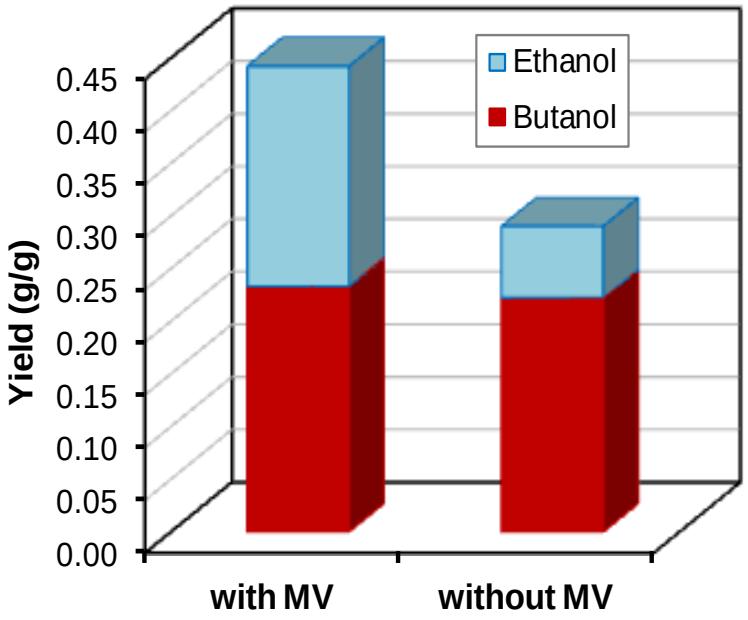


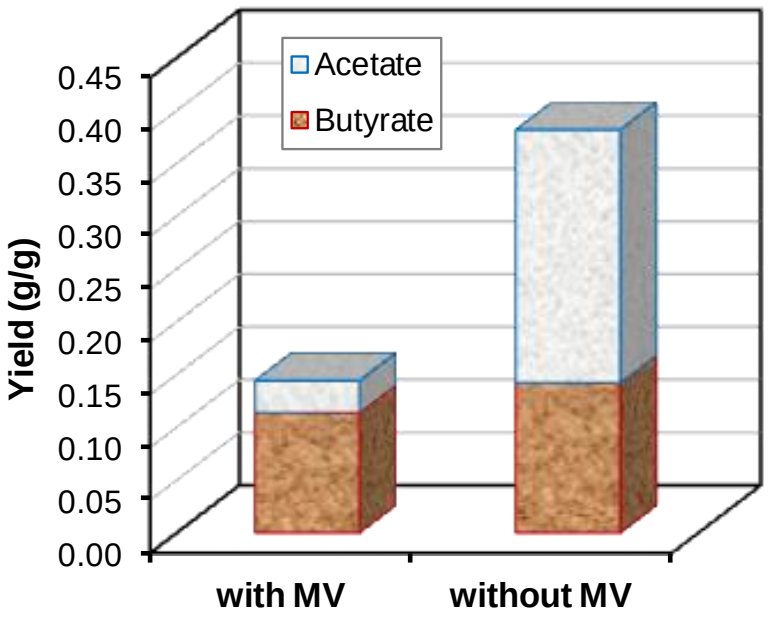


Yield (g/g)	Butanol	Ethanol	Butyrate	Acetate	Alcohols/Acids
Without MV	0.21	0.06	0.13	0.22	0.77
With MV	0.22	0.20	0.11	0.03	3.23

Butanol production from cellulose

Batch fermentation with high cell density

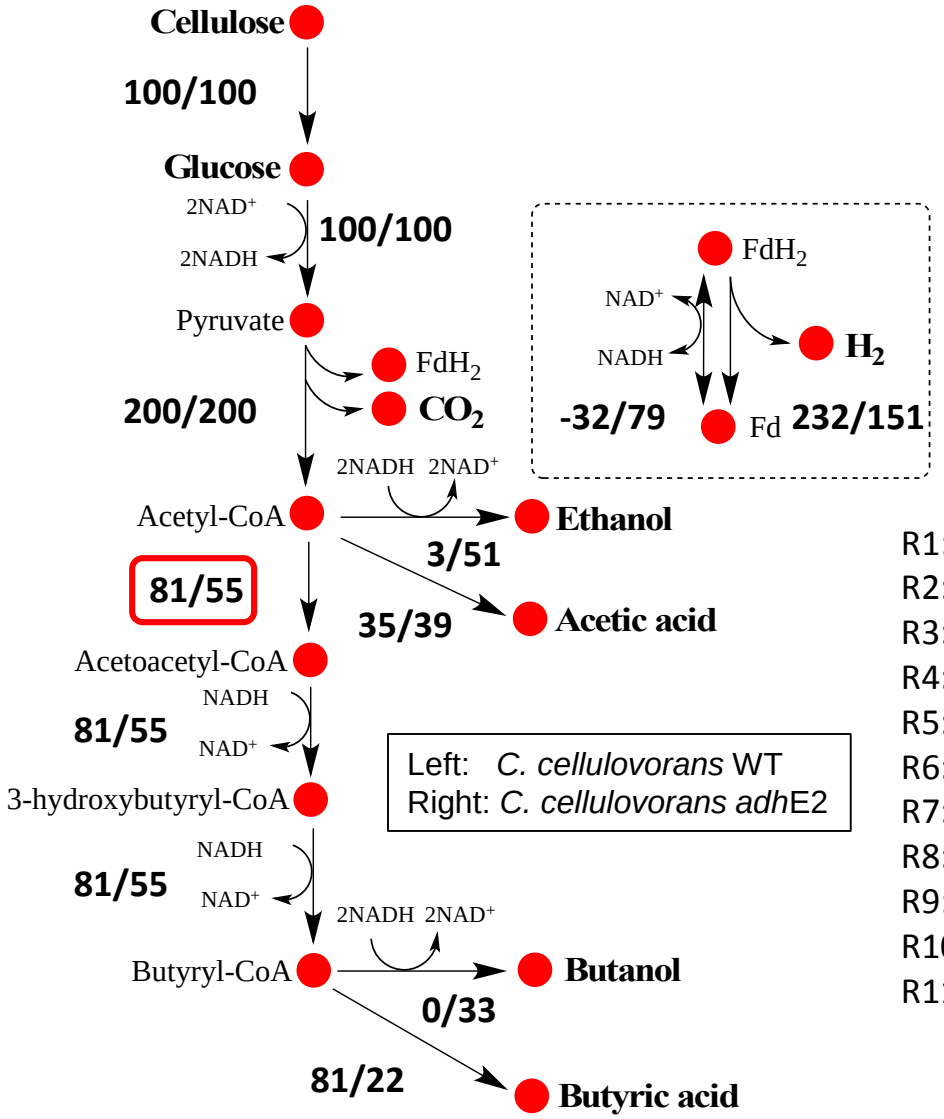




The total alcohol (butanol and ethanol) yield from cellulose consumed in the fermentation was 0.27 g/g without adding MV and 0.42 g/g with MV, meeting our **milestone M1.3** (butanol and ethanol as the main products at >0.35 g/g (Q6).

Challenges: 1. ME to increase butanol (vs. ethanol) production
2. ME to reduce acids (mainly butyric acid) production

Metabolic Flux Analysis



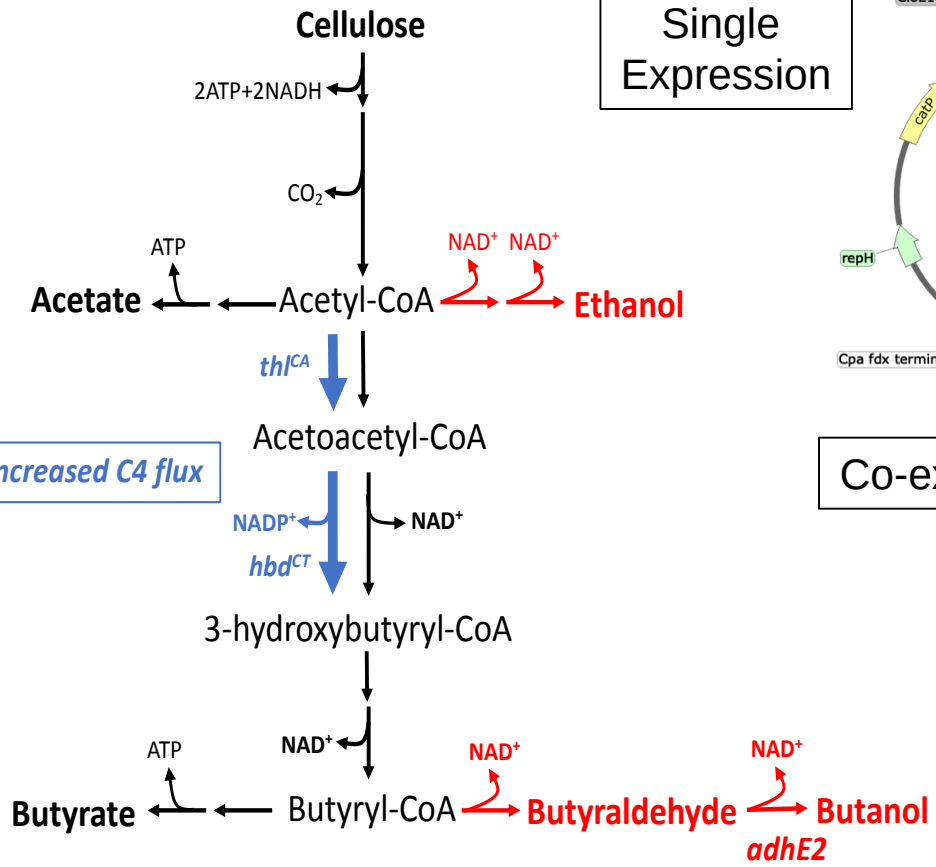
Rational Metabolic Engineering

- Increase carbon flux from C2 to C4
- Redistribute cofactor flux for alcohol biosynthesis
- Depress competitive acid biosynthesis pathways

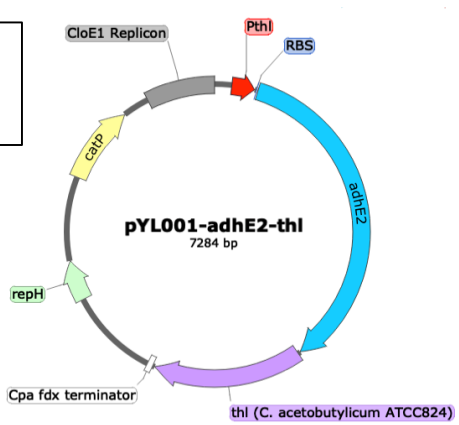
R1: Glucose → 2 Pyruvate + 2 NADH
R2: Pyruvate → Acetyl-CoA + FdH_2 + CO_2
R3: 2 Acetyl-CoA → Acetoacetyl-CoA
R4: Acetyl-CoA + 2NADH → Ethanol
R5: Acetyl-CoA → Acetic acid
R6: Acetoacetyl-CoA + NADH → 3-hydroxybutyryl-CoA
R7: 3-hydroxybutyryl-CoA + NADH → Butyryl-CoA
R8: Butyryl-CoA +2 NADH → Butanol
R9: Butyryl-CoA → Butyric acid
R10: FdH_2 → NADH
R11: FdH_2 → H_2

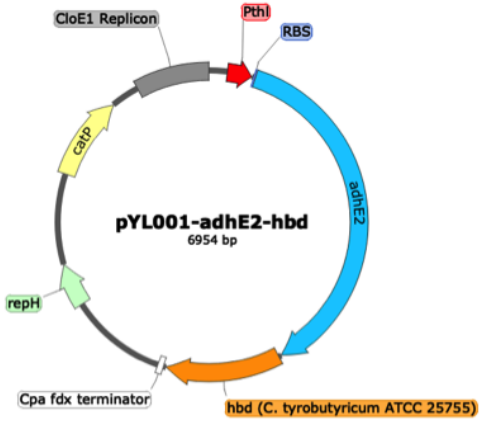
Metabolic Engineering

To increase C2 to C4 flux

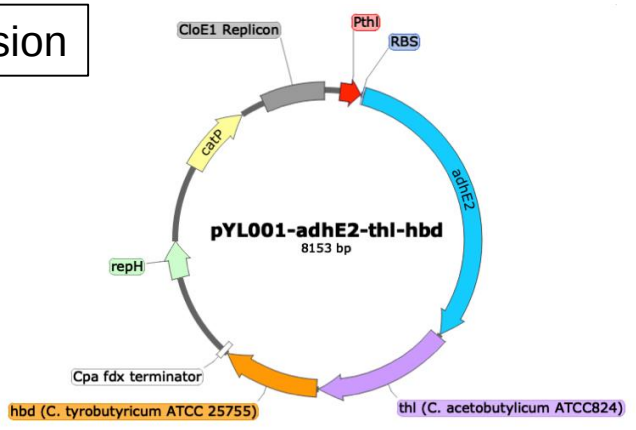


Single Expression





Co-expression



Metabolic Engineering

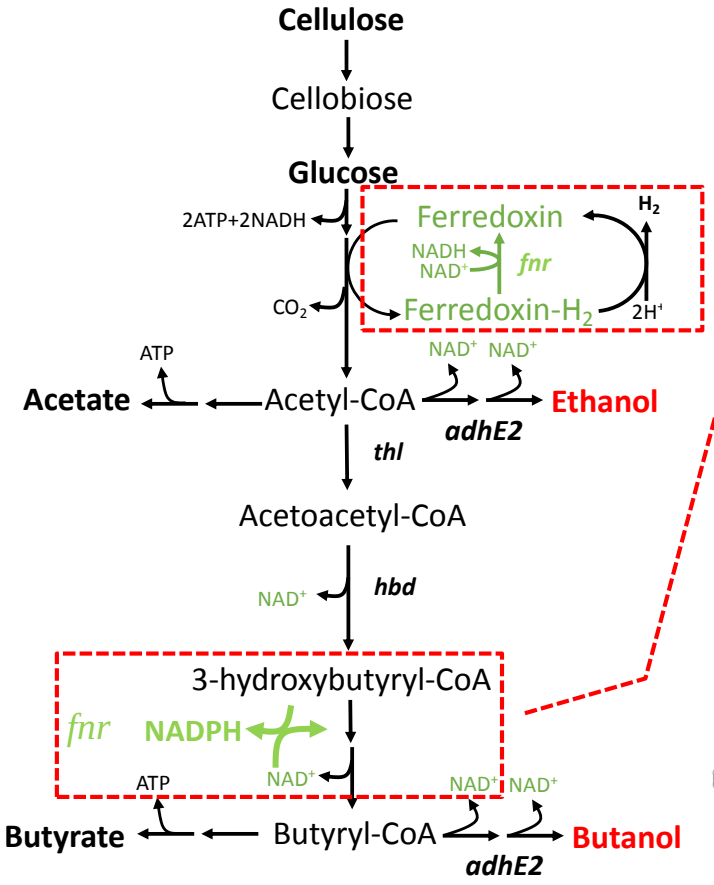
To increase C2 to C4 flux

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
Glucose as substrate									
*adh*E2	2.14	1.76	3.09	0.96	3.90	0.09	0.96	0.82	0.52
*adh*E2-*thl*^{CA}	0.62	0.94	2.11	4.23	1.56	0.06	0.25	1.53	**1.90**
*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**
Cellulose as substrate									
*adh*E2	2.01	2.00	2.00	1.57	4.01	0.11	1.12	0.99	0.89
*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**

- Over-expressing *thl* (thiolase) and/or *hbd* (hydroxybutyryl-CoA dehydrogenase) with *adh*E2 significantly increased C2-to-C4 carbon flux;
- However, these transformants produced more butyrate but less butanol due to insufficient NADH for the reduction of butyryl-CoA to butanol as indicated by the addition of MV.
- With MV, the mutant coexpressing thl and hbd with adhE2 produced mostly butanol with a high butanol/ethanol ratio of 12.18 and C4/C2 ratio of 5.87

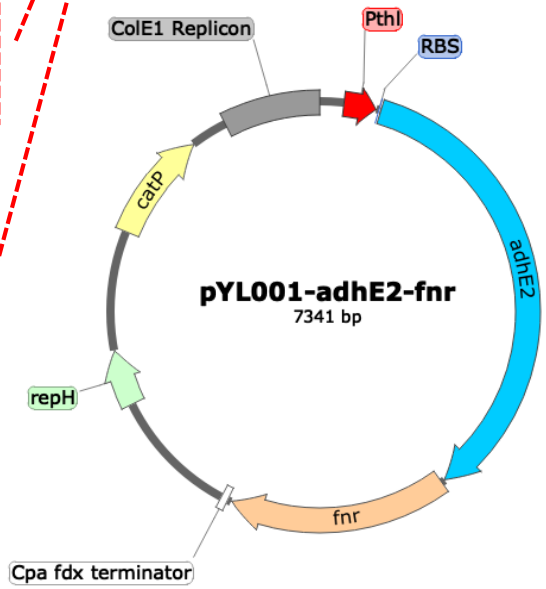
Metabolic Engineering

Redox Balance via Cofactor Engineering



NADH Regeneration System

Ferredoxin-H_2 + NAD^+ → Ferredoxin + NADH



Ferredoxin NAD(P)$^+$ reductases (*fnr*)

Redirect the electron flow from reduced ferredoxin toward the production of the NAD(P)H needed for butanol production

Metabolic Engineering

Redox Balance via Cofactor Engineering

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
Glucose as substrate									
*adh*E2	2.14	1.76	3.09	0.96	3.90	0.09	0.96	0.82	0.52
*adh*E2-*fnr*	0.35	**2.36**	1.99	4.61	2.70	0.15	0.41	**6.81**	**2.99**
Cellulose as substrate									
*adh*E2	2.01	2.00	2.00	1.57	4.01	0.11	1.12	0.99	0.89
*adh*E2-*fnr*	0.96	**3.06**	2.37	2.24	4.01	0.16	4.62	**3.18**	**1.59**
*adh*E2-*fnr* (MV)	1.93	**5.28**	0.63	0.73	7.22	**0.33**	5.30	**2.74**	**2.35**

- Overexpression *fnr* increased both butyrate and butanol production, resulting in increased butanol/ethanol ratio and C4/C2 ratio.
- With methyl viologen (MV), *n*-butanol production from cellulose further increased and reached a high final concentration of 5.28 g/L in 24 days, with a yield of 0.33 g/g.

ME *C. cellulovorans*

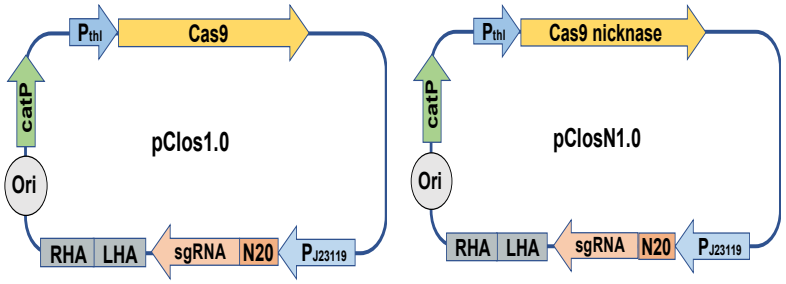
to increase butanol production

Strain	Ethanol (g/L)	Butanol (g/L)	Acetate (g/L)	Butyrate (g/L)	Total alcohol (g/L)	Butanol yield (g/g)	Alcohol yield (g/g)	Alcohol/ acid ratio	C4/C2 ratio
WT	0.25	0.00	2.07	6.40	0.25	0.00	0.01	0.03	2.77
adhE2	3.56	3.46	2.60	1.96	7.03	0.19	0.40	1.54	0.88
adhE1-bdhB	0.47	1.83	2.52	4.84	2.30	0.10	0.13	0.31	2.23
adhE2-aor	0.28	0.00	2.30	7.12	0.28	0.00	0.01	0.03	2.75
adhE2-bdhB	0.40	0.38	2.41	6.53	0.77	0.02	0.04	0.09	2.46
adhE2-fdh	0.58	1.13	2.70	5.75	1.71	0.06	0.09	0.20	2.10
adhE2-thl	2.29	3.73	2.42	3.73	6.02	0.20	0.32	0.98	1.58
adhE2-thl (MV)	4.74	4.31	0.68	0.89	**9.05**	0.30	**0.62**	5.75	0.96
adhE2-fnr	1.40	4.11	2.68	2.39	5.52	0.23	0.31	1.09	1.59
adhE2-fnr (MV)	1.93	5.28	0.63	0.73	7.22	**0.40**	0.54	5.30	2.35
adhE2-fnr (MV+)	2.66	**5.74**	0.65	0.60	8.40	**0.36**	0.53	6.72	1.92

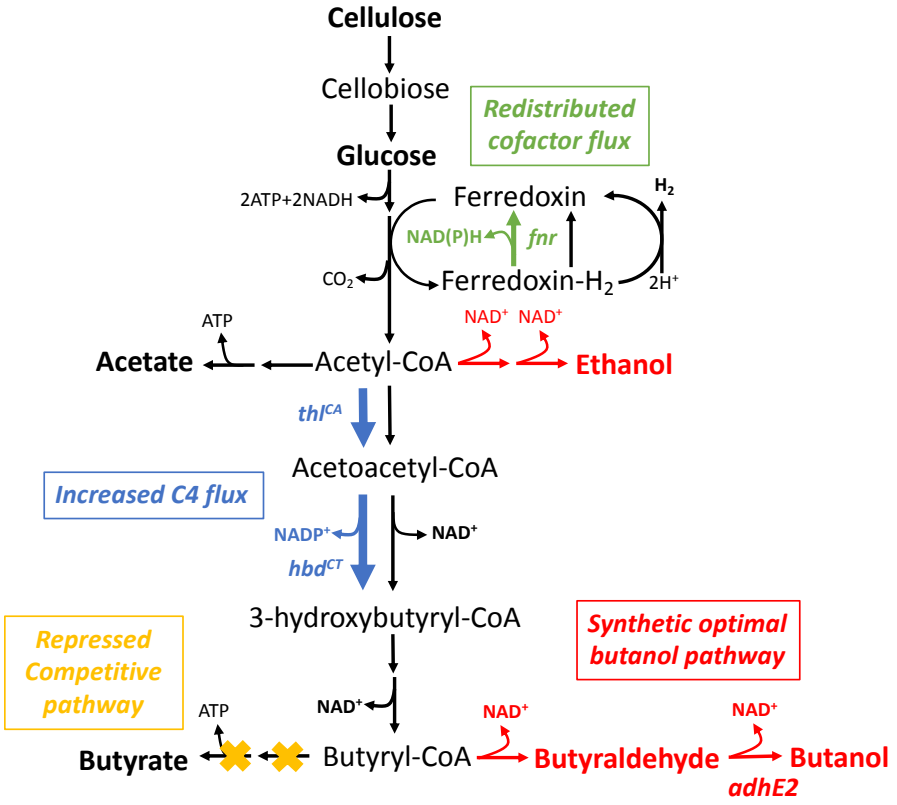
- Effects of overexpressing other genes such as *bdh*B, *aor*, *fdh*, *ctf*AB were also evaluated.
- Overall, *n*-butanol production from cellulose reached a high final concentration >5 g/L in 24 days, with a yield of 0.36 - 0.40 g/g.
- The highest total alcohol production of 9.05 g/L with a high yield of 0.62 g/g cellulose was achieved in batch fermentation.

Further ME Work

Knockout or knockdown *ptb* and *buk* to reduce butyrate biosynthesis for increased *n*-butanol production



CRISPR: Clustered regularly interspaced short palindromic repeats (segments of prokaryotic DNA for adaptive immune system)
PAM: protospacer adjacent motif (NGG)
Cas: CRISPR-associated system
Cas9: The Cas endonuclease from *Streptococcus pyogenes* for double-strand break (DBS)
sgRNA: small guide RNA to guide Cas9 to the target site
N20: 20 nucleotides upstream of PAM for complimentary targeting



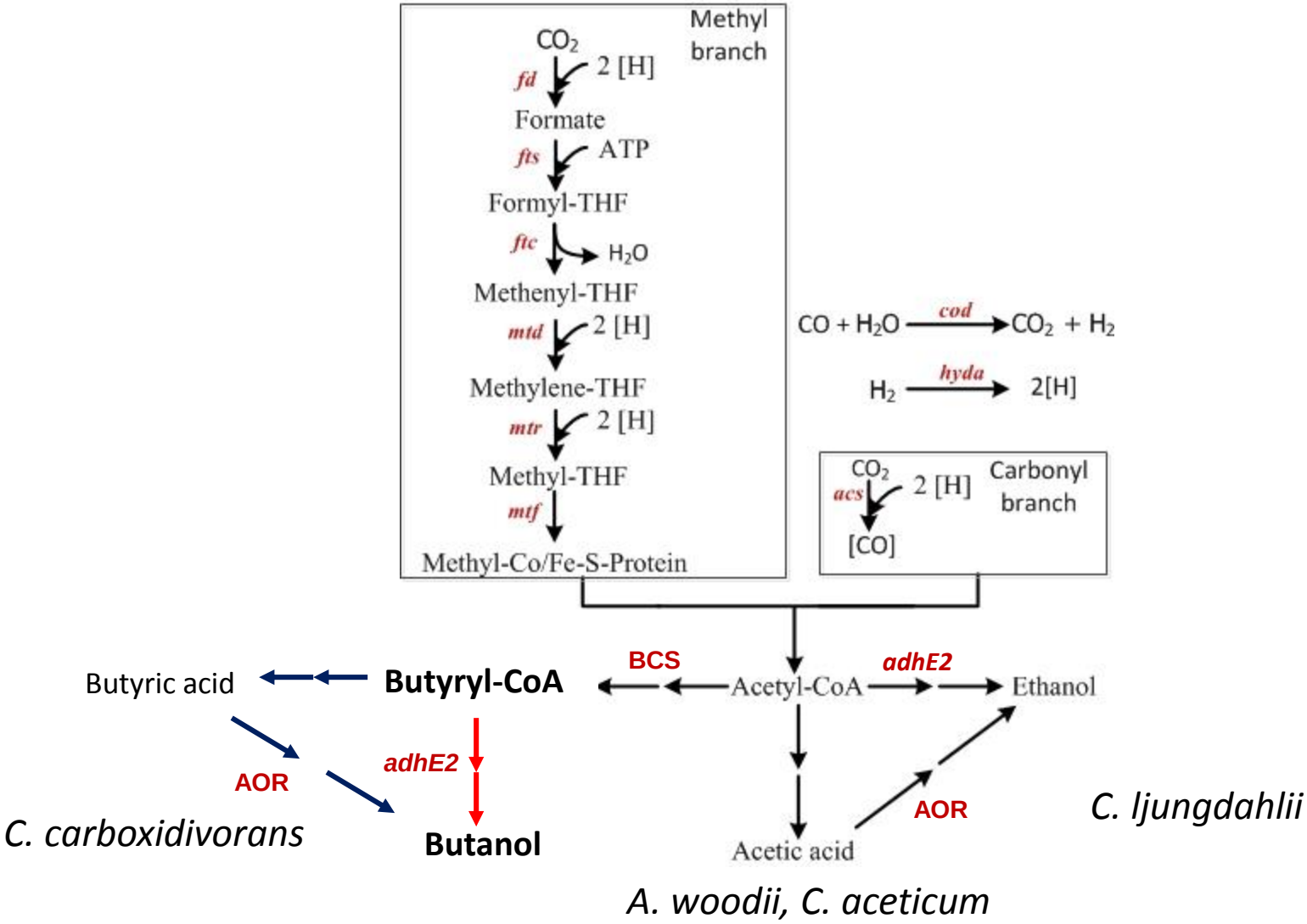
The development of a CRISPR-Cas9 system for genome editing of *C. cellulovorans* was not in the original proposal but would be beneficial to the development of stable engineered strains for industrial applications.

3 – Technical Accomplishments/Progress/Results

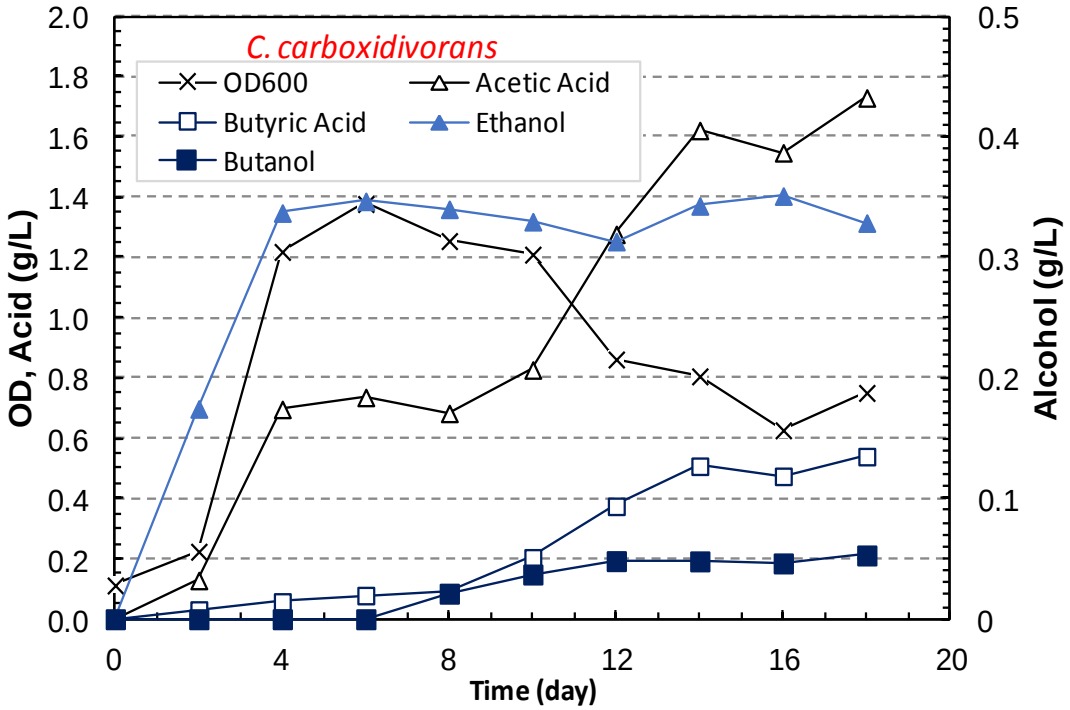
- Task A: Metabolic engineering of acetogens for butanol and ethanol production from CO_2 and H_2
  - All acetogens have very robust restriction modification (RM) systems, hindering effective transformation of recombinant plasmids into host cells.
  - Analyzed RM systems based on available genomic sequences 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)	√

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

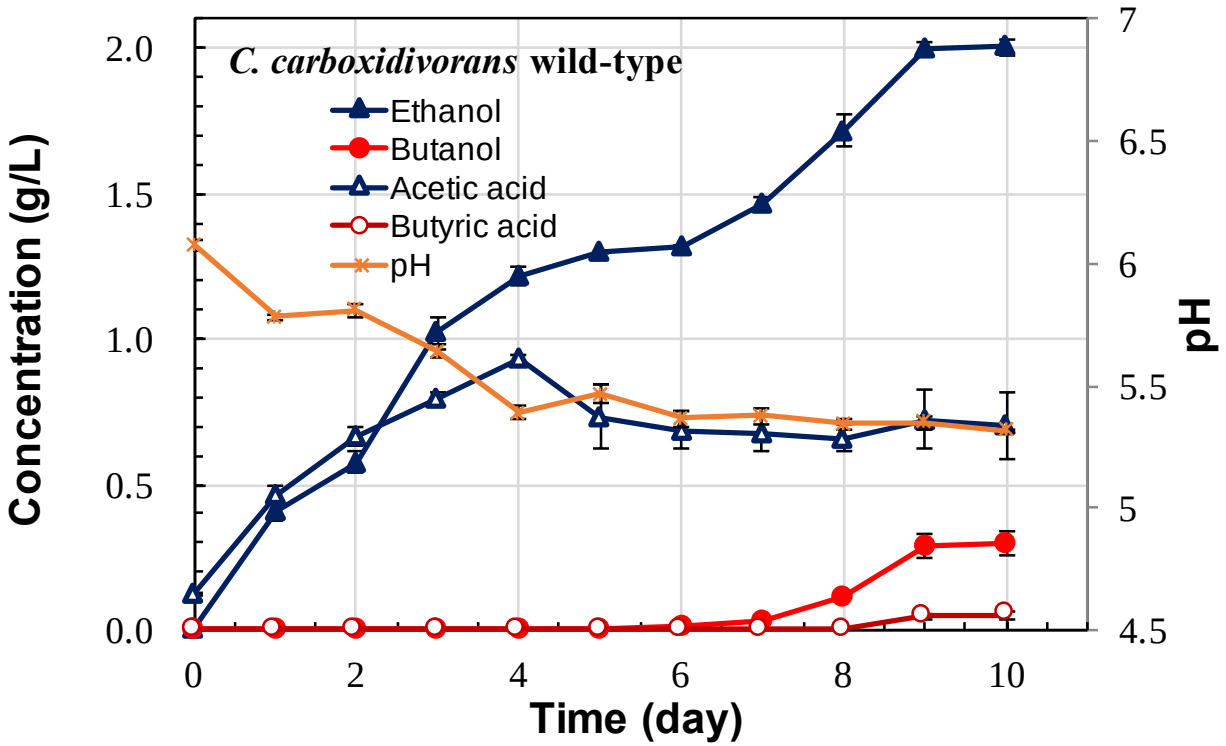


Alcohols production from CO_2 and H_2



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).

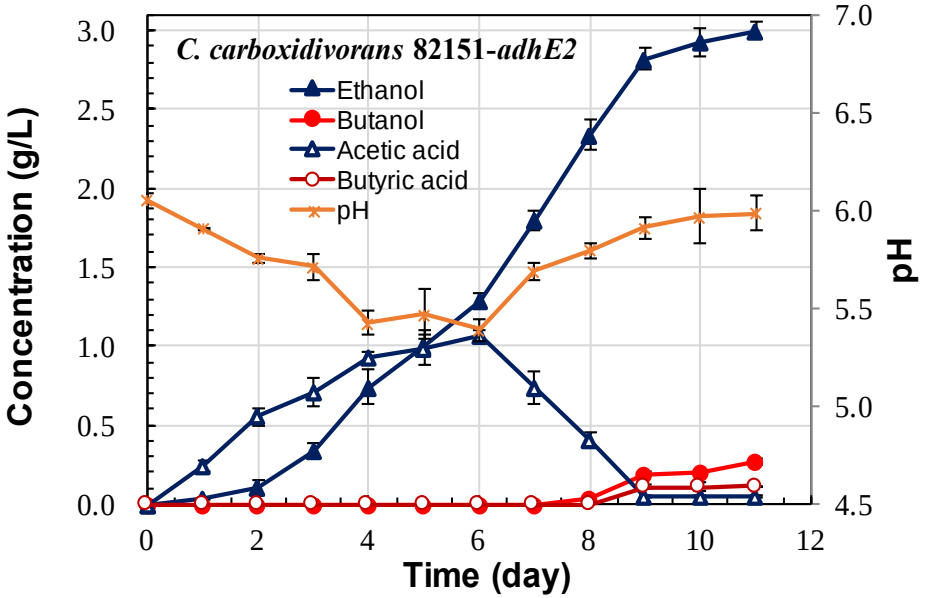
Alcohols production from CO_2, CO, and H_2

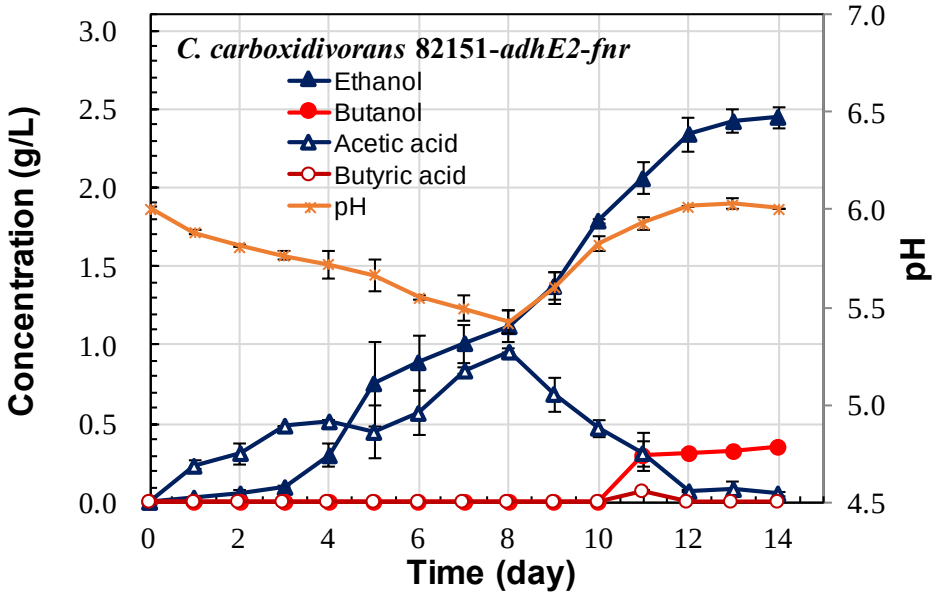


More ethanol and butanol were produced from syngas CO_2 /CO/H_2 (20%/40%/40%)

Gas composition	Acetic acid (g/L)	Ethanol (g/L)	Butyric acid (g/L)	Butanol (g/L)
CO_2/H_2	1.76	0.35	0.55	0.05
$CO_2/CO/H_2$	0.70	2.00	0.05	0.30

ME of *C. carboxidivorans*

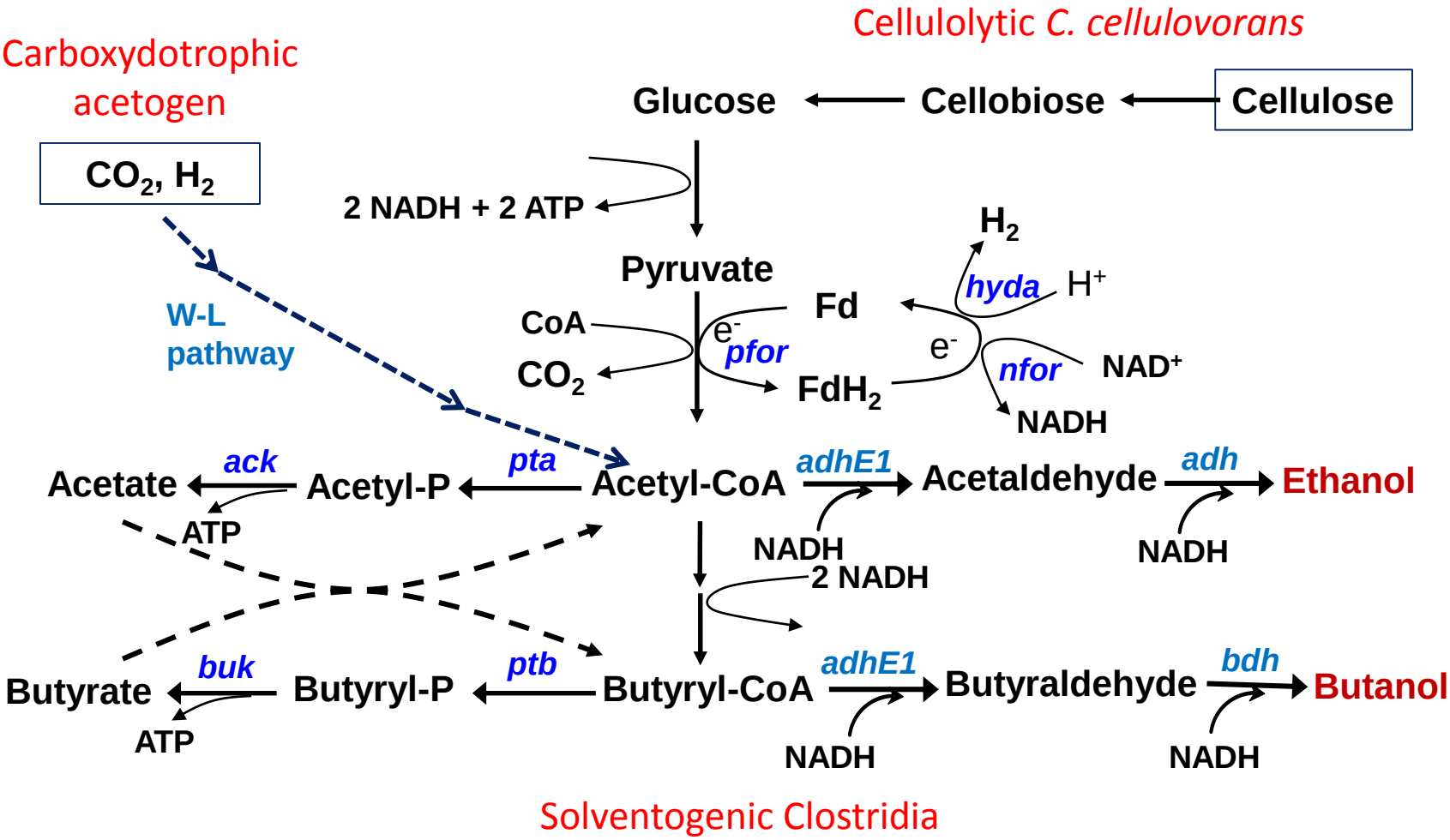




Strain	Acetic acid (g/L)	Ethanol (g/L)	Butyric Acid (g/L)	Butanol (g/L)
Wild-type	0.702±0.113	2.00±0.003	0.053 ±0.015	0.297±0.040
***aor* mutant**	0.865±0.090	2.38±0.133	0.069±0.002	0.202±0.030
***adhE2* mutant**	0.045±0.005	3.00±0.061	0.112±0.004	0.272±0.019
***aor-fnr* mutant**	0.600±0.035	1.62±0.056	0.052±0.008	0.057±0.029
***adhE2-fnr* mutant**	0.054±0.012	2.44±0.077	0	0.351±0.010

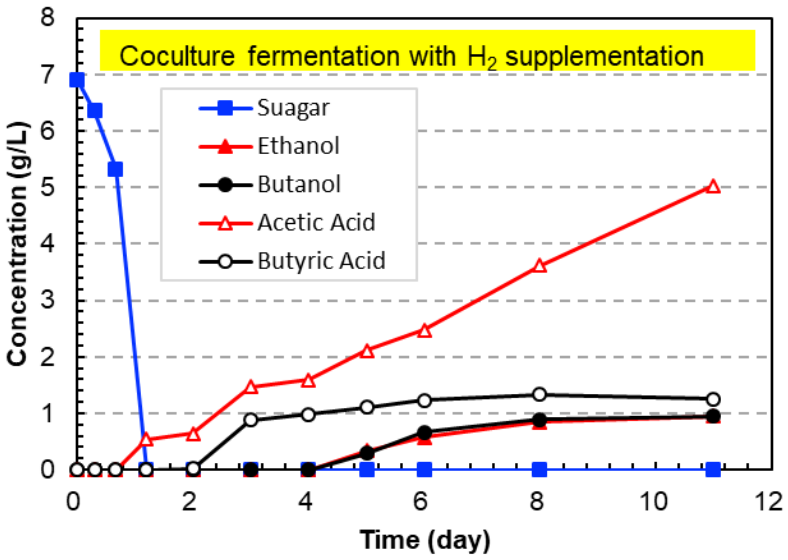
For the first time, metabolic engineering of *C. carboxidivorans* to overexpress genes for enhanced alcohols production was demonstrated, which laid the foundation for further engineering this carboxydotrophic clostridia for butanol production from syngas (CO, CO_2, and H_2).

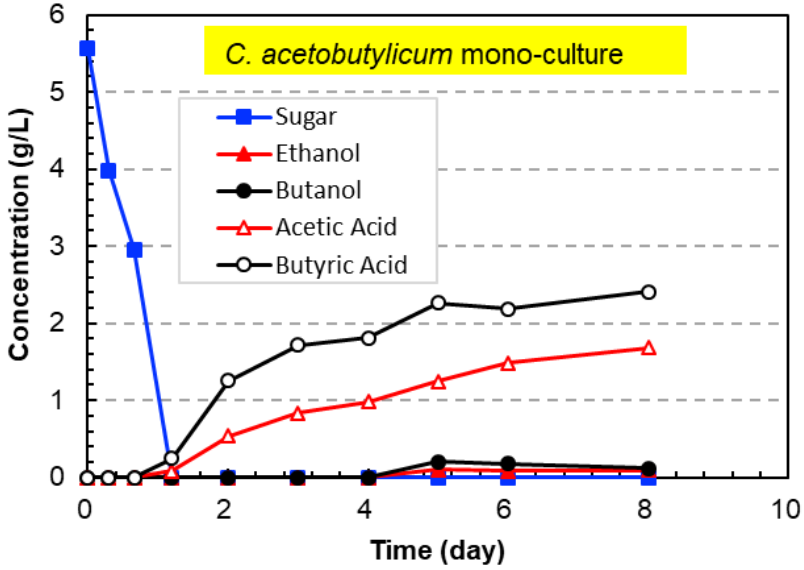
Mixed Culture Fermentation

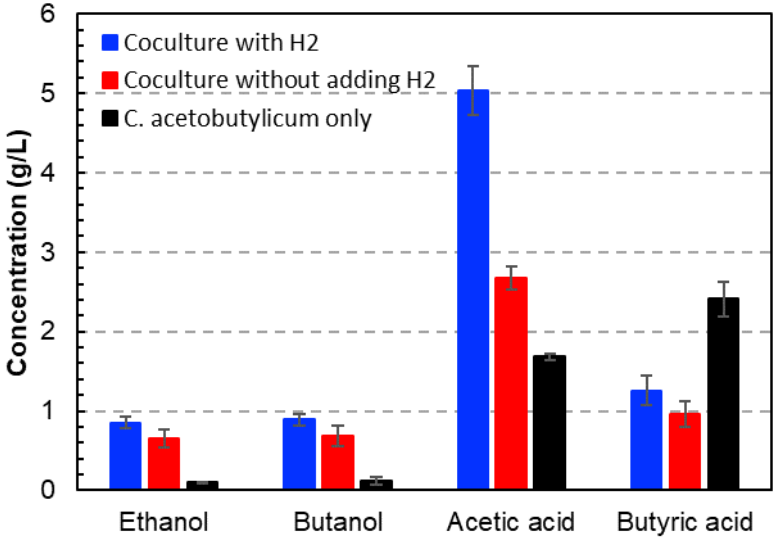


Butanol titer: >10 g/L, Yield: >0.3 g/g, Productivity: >0.2 g/L h

Mixed Culture Fermentation







C. acetobutylicum cocultured with a carboxydotrophic clostridia produced more alcohols and acetic acids because of the assimilation of CO_2 and H_2.

3 – Technical Accomplishments/Progress/Results

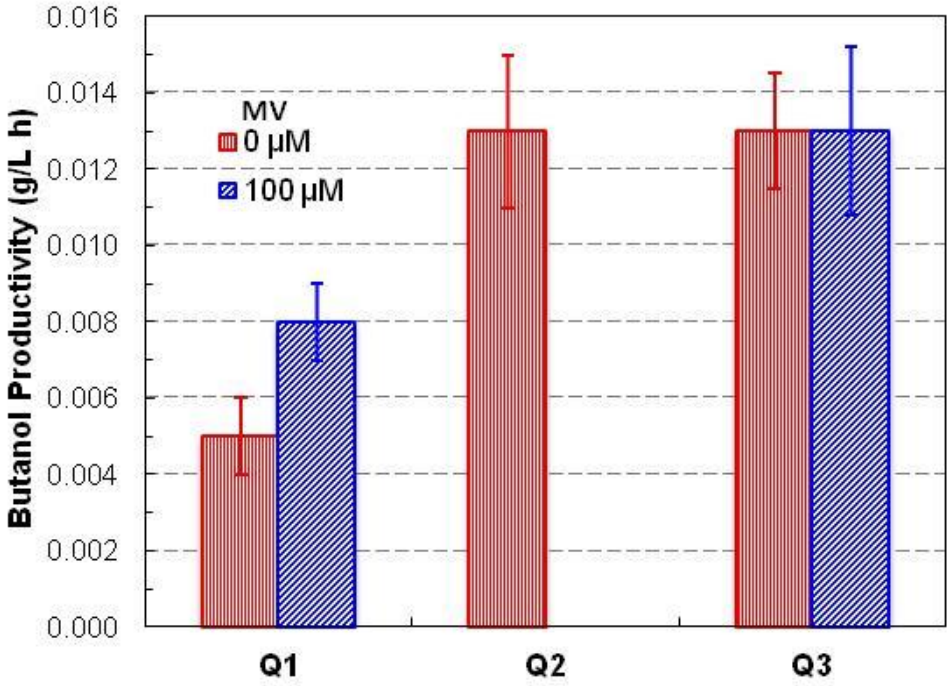
- Task B: Fermentation kinetics studies and process optimization
  - Cellulose fermentation in serum bottles and stirred-tank bioreactors (1-5 liters) with process parameters (pH, substrate concentration, etc.) optimized.
  - Gas fermentation in different types of bioreactors (stirred-tank, bubble column, packed bed) evaluated.
  - Medium optimization to increase cell density, activity, and productivity.
  - Different pretreatment methods for enhancing cellulose degradability evaluated.
  - *In situ* product recovery by adsorption and gas stripping to alleviate butanol toxicity demonstrated with butanol >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)	√
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	√

Butanol production from cellulose

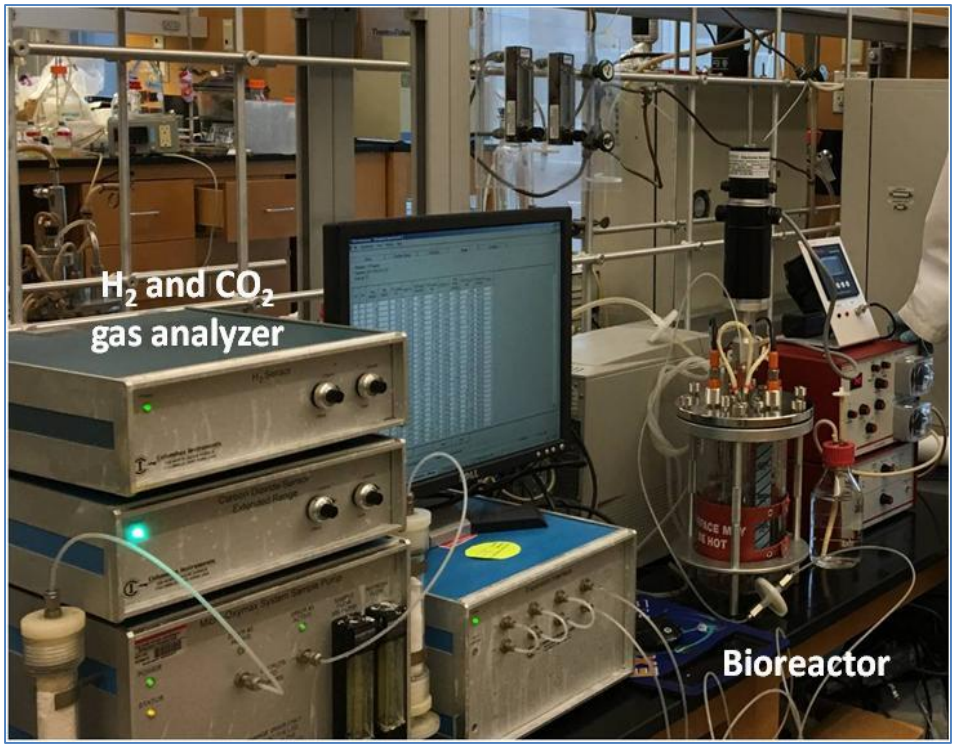
- Compared to glucose, butanol productivity from cellulose is low due to low cell density/activity and slow cellulose degradation

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

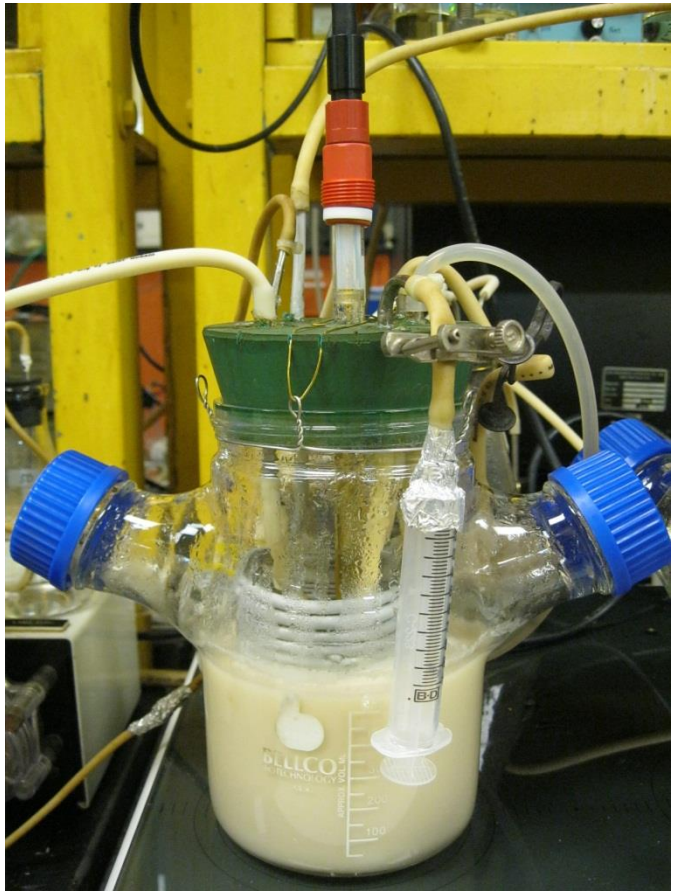


- Productivity can (will) be improved (>10-fold) in bioreactor with process optimization and by better pretreatment of cellulose to increase its accessibility to cells for degradation

Fermentation kinetics studies and process optimization



Bioreactor for gas fermentation with H_2 and CO_2 gas analyzer



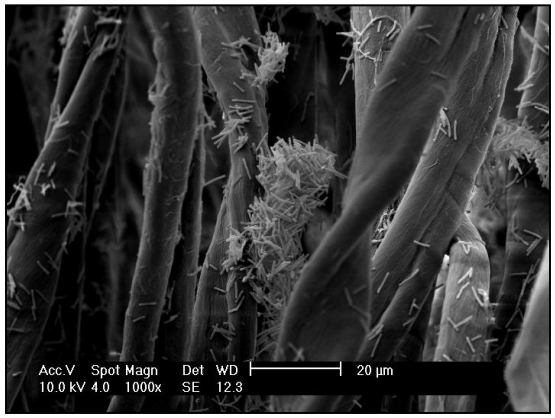
Cellulose fermentation

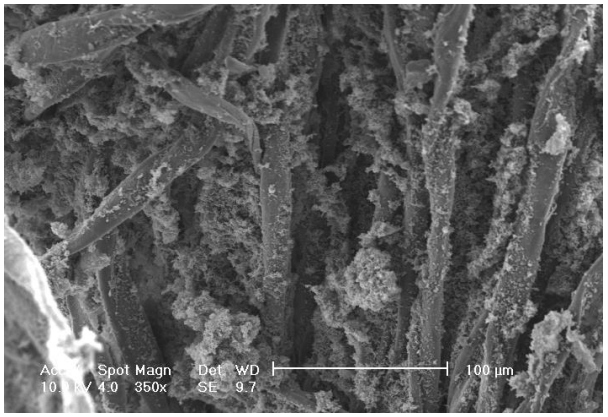
High cell density is achieved with cell recycle and/or immobilization in bioreactor

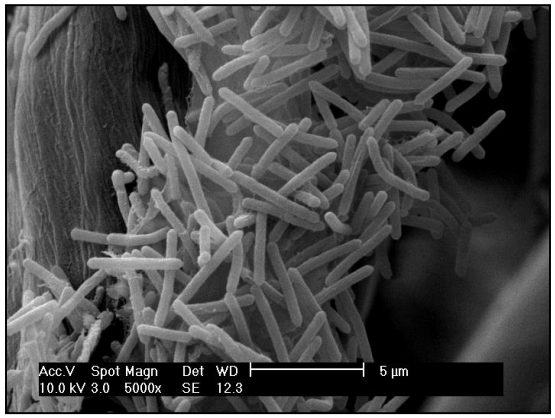
Immobilized Cell Fermentation

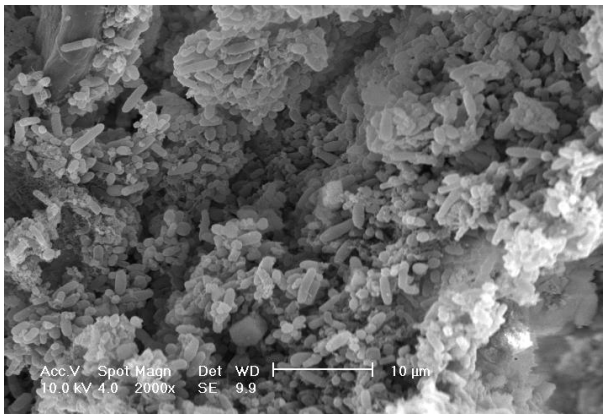
Fibrous-Bed Bioreactor

(U.S. Patent 5,563,069)





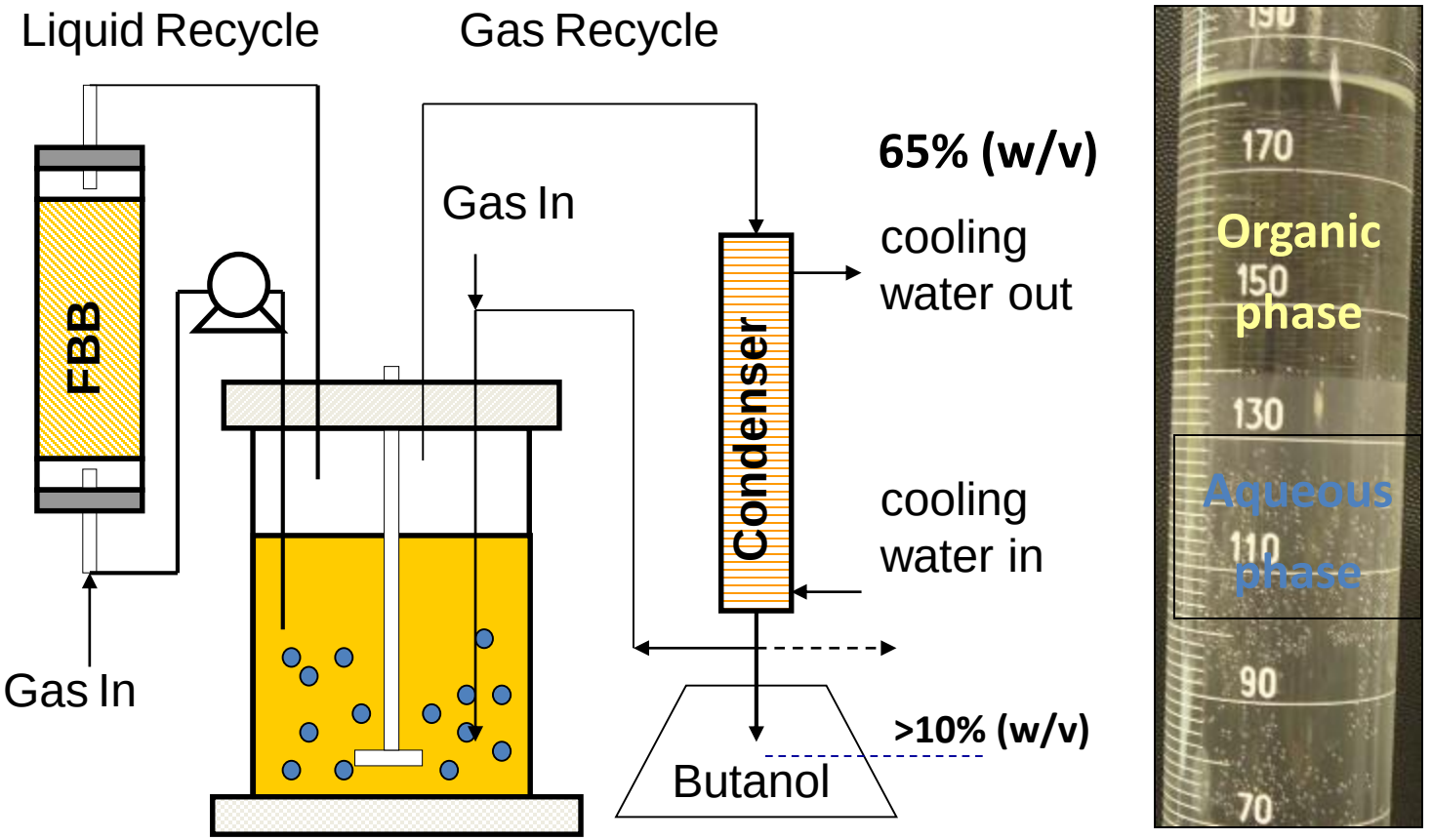




C. acetobutylicum *C. tyrobutyricum*

High cell density: 35 – 100 g/L

Fermentation with Gas Stripping



In situ separation of butanol by gas stripping with fermentation-produced off-gases can efficiently recover butanol from a ~1% broth to a product containing ~65% butanol (after phase separation)

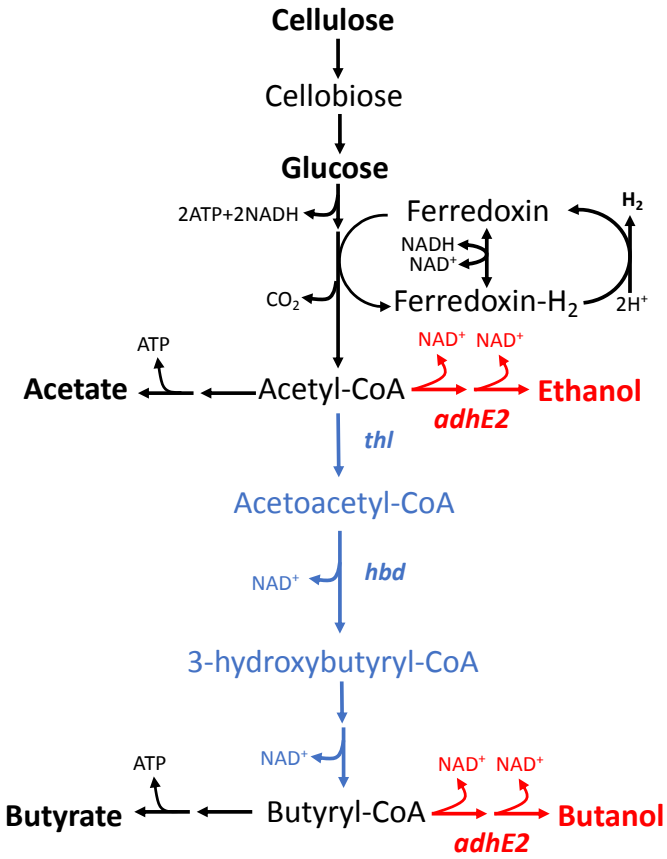
Xue et al., Biotechnol. Bioeng., 109: 2746-27 (2012)

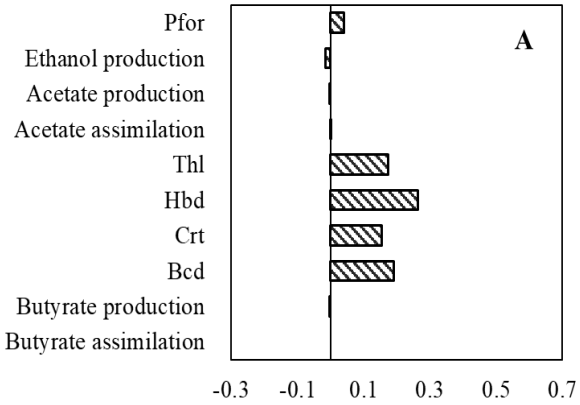
3 – Technical Accomplishments/Progress/Results

- Task C: 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) were identified for metabolic engineering to improve butanol production from cellulose

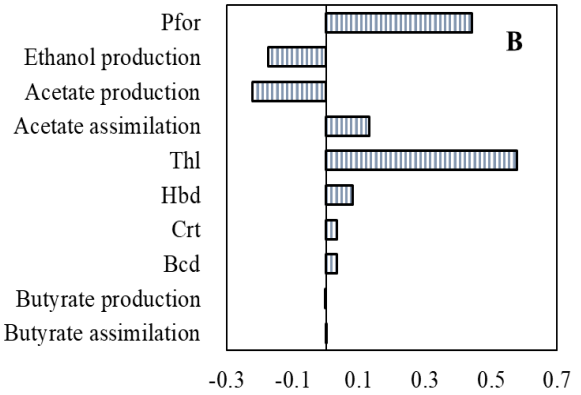
Milestone	Description (Targeted Quarter to Meet)	Status
M3.1	Proteomics profiling of mutants generated and suitable cell engineering strategy identified (Q4)	√
M3.2	Core metabolites responsible for carbon, energy and redox balance identified to assist process development and scale-up (Q7)	√

Metabolic Flux Control Analysis





Substrate: Glucose



Substrate: Cellulose

- In **glucose** fermentation, ***hbd***, ***thl***, *bcd*, and *crt* are limiting the flux; In **cellulose** fermentation, the flux is mainly limited by ***thl***. In addition, the negative values of ethanol and acetate indicated the necessity to reduce their production.

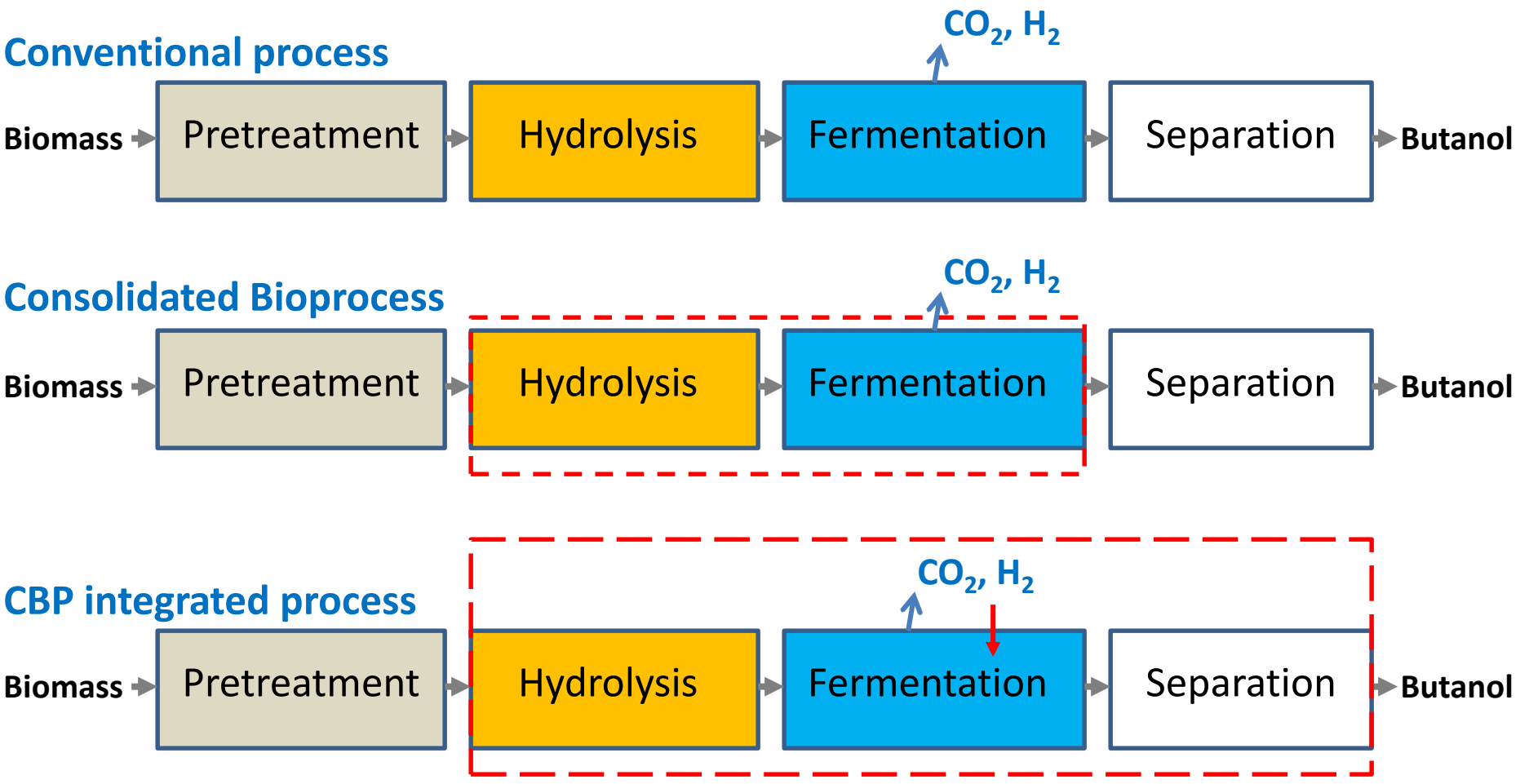
Butanol production is limited by the carbon flux from C2 to C4

3 – Technical Accomplishments/Progress/Results

- Task D: Process design and cost analysis
  - Mixed-culture fermentation process development and optimization
  - Integrated fermentation process with in situ product recovery
  - Life cycle and cost analyses

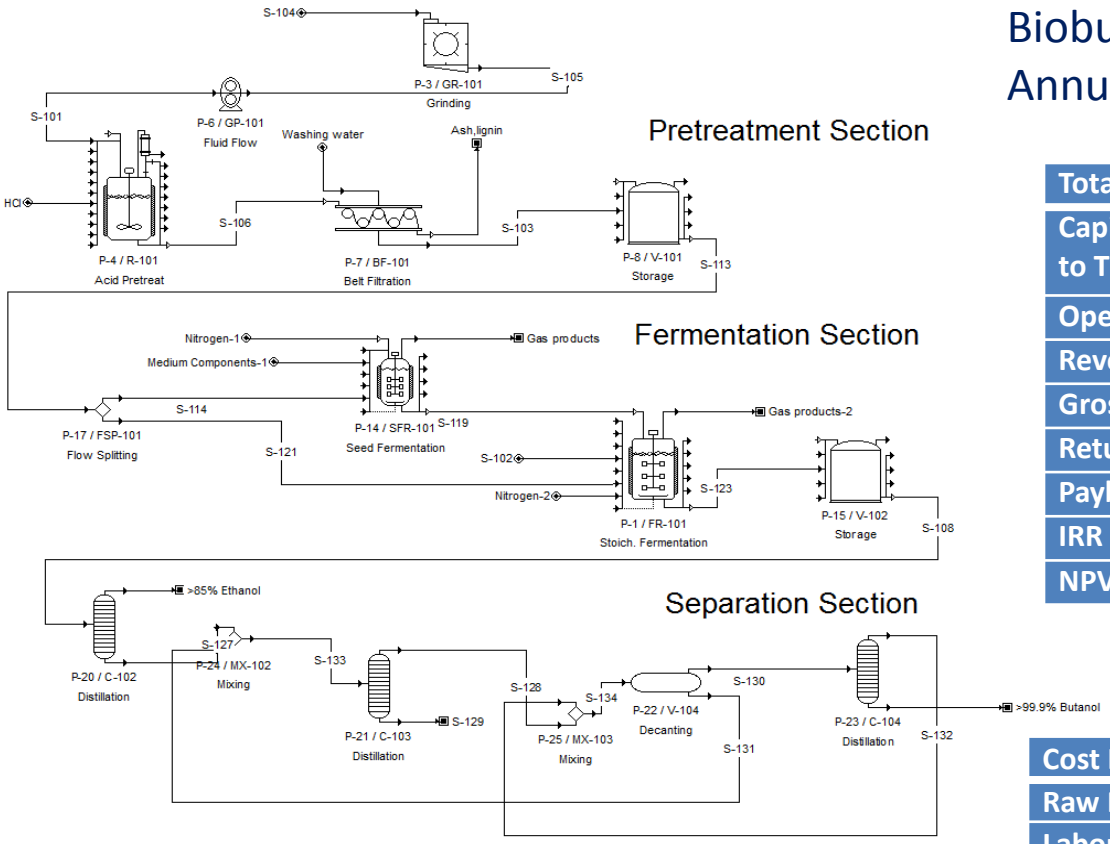
Milestone	Description	Status
M4.1	Pre-treatment process selected. The process and conceptual plant design with outline butanol production costs achieved	√
M4.2	Process and conceptual plant design of advanced fermentation defined	√
M4.3	Process and conceptual plant design for CBP and co-culture completed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

Process Integration and Consolidation



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

Process Cost Analysis



Biobutanol production from corn cob

Annual production: 50,000 metric tons

Total Capital Investment	**$ 41,632,000**
Capital Investment Charged to This Project	$ 41,632,000
Operating Cost	$ 34,374,000
Revenues	$ 80,197,000
Gross Margin	57.14 %
Return on Investment	74.55 %
Payback Time	1.34 year
IRR (After Taxes)	50.55 %
NPV (at 7.0% Interest)	$ 281,798,000

Annual production cost:

Cost Item	$	%
Raw Materials	13,068,000	38.02
Labor-Dependent	6,284,000	18.28
Facility-Dependent	7,031,000	20.46
Utilities	7,677,000	22.33
TOTAL	34,374,000	100.00

Product cost: $0.71/kg or $2.13/gal.
Selling price: $1.55/kg for chemical, $2.5/gal ($0.83/kg) for fuel

Basis

1. Feedstock cost: corn cob $ 50 per dry ton
2. Corn cob: 40% cellulose, 30% hemicellulose, and 20% lignin
3. Fermentation: production of ~5.5 g/L butanol with a butanol yield of 0.4 g/g cellulose/hemicellulose and productivity of 0.05 g/L·h.

Life Cycle Analysis

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

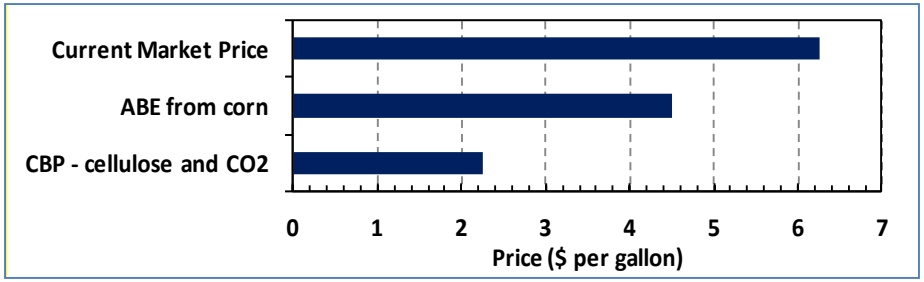
The well-to-pump life cycle analysis using Argonne National Laboratory's **GREET** Model shows that biobutanol production by ABE fermentation has a GHG reduction of ~**24%** from corn (GHG from farming is considered) and **62%** from corn stover (excluding GHG from farming since corn stover is a waste from corn farming) compared to the traditional chemical process. 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 at least **75%** compared to the traditional chemical process.

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/H_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 need to be validated at a pilot plant before commercialization.



5 – Future Work

- Further metabolic engineering using CRISPR-Cas9 genome editing technique to knockout acetate and butyrate biosynthesis pathways in *C. cellulovorans*
- Genome engineering to develop stable strains for industrial fermentation
- Further engineering carboxydotrophic acetogens for butanol and ethanol biosynthesis from CO_2 and H_2
- Mixed-culture fermentation process optimization
- Integrated fermentation process with *in situ* product recovery

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
- The integrated biobutanol production process with *in situ* separation can produce *n*-butanol at $2.25/gal ($3.0 gge) and reduce GHG emissions by >50%

Responses to Previous Reviewers' Comments

- The PIs present a novel approach for direct conversion of biorefinery cellulose to biofuels through genetic engineering. Targeting cellulose as the substrate is a worthwhile goal, and if productivity issues can be developed, this might be a nice alternate approach to mixed alcohols. They further plan to improve carbon utilization by developing organisms that can consume CO_2 generated during fermentation and convert it into butanol. This is an interesting and potentially promising approach, but the team needs to update their preliminary economics in the short term to evaluate whether the overall process has industrial viability.
  - **Response:** Further development and commercialization decisions will be based on the results of TEA and life-cycle analysis studies, which have shown very promising results so far.
- For the targets set, the team has made great progress, but there is still a long way to commercialization with a lot of challenges, both biological and engineering.
  - **Response:** We understand that there is a long way toward eventual process scale-up and commercialization of the technology. Nevertheless, to demonstrate the technology concept and its feasibility and economical and environmental benefits in 2 years would meet the goal of this incubator program.
- This is a well-organized project and is making good progress towards converting both biomass and "waste" CO2 to fuel molecules in a CBP-like process. I personally favor the co-fermentation approach over asking one CBP organism to do everything. With similar strains, there is a reasonable chance of developing a robust single tank co-culture during both growth and production. Scale-up will be exciting!
  - **Response:** Regarding engineering the cellulolytic strain to uptake hydrogen, this would be very difficult to do, as uptake hydrogenases are complicated and difficult to express in a heterologous host. In contrast, we are taking the approach to engineer the strain with minimal CO_2 and H_2 production, so most substrate carbon will be in the final product, butanol. Any CO_2 and H_2 released from the cellulolytic strain will then be captured and used by the carboxydotrophic strain.

Publications, Presentations, and Commercialization

Publications:

- 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).
- 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, in press (2018). doi: 10.1016/j.ygeno.2018.11.020
- 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., in review (2019).
- 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., in review (2019).
- 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., in review (2019).

Presentations:

- 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.
- 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.
- 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.
- 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.
- 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.
- 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.

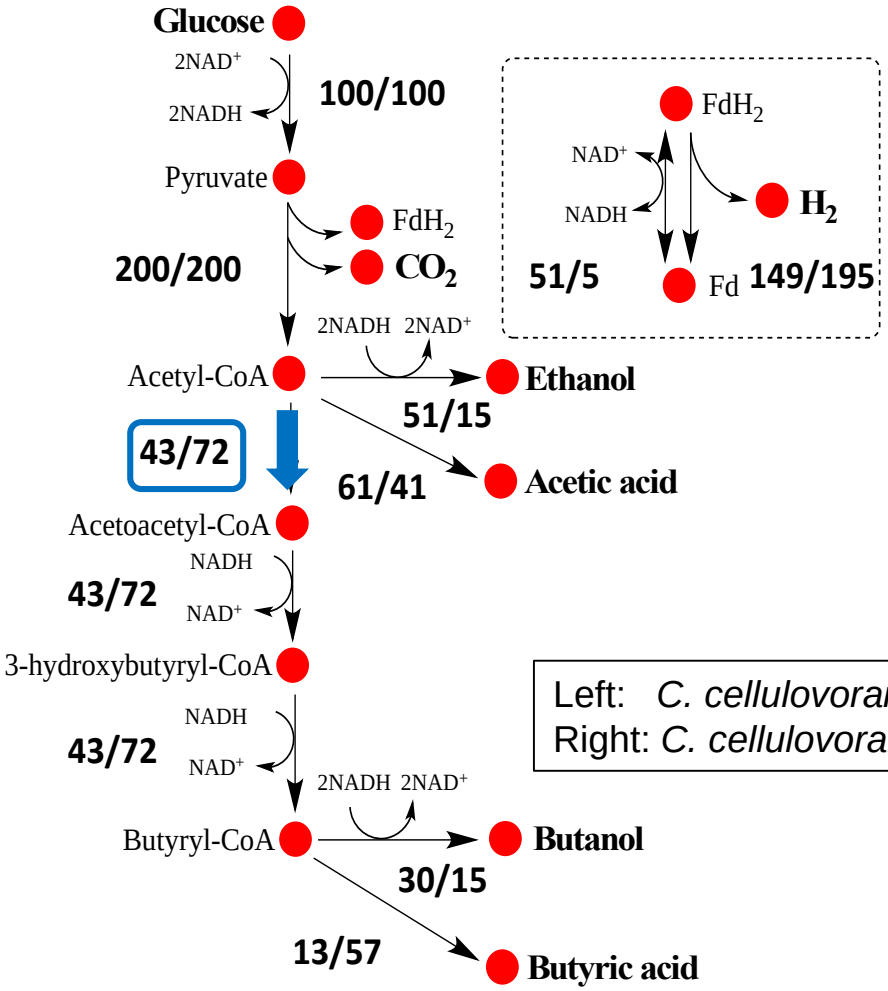
GreenBiologics

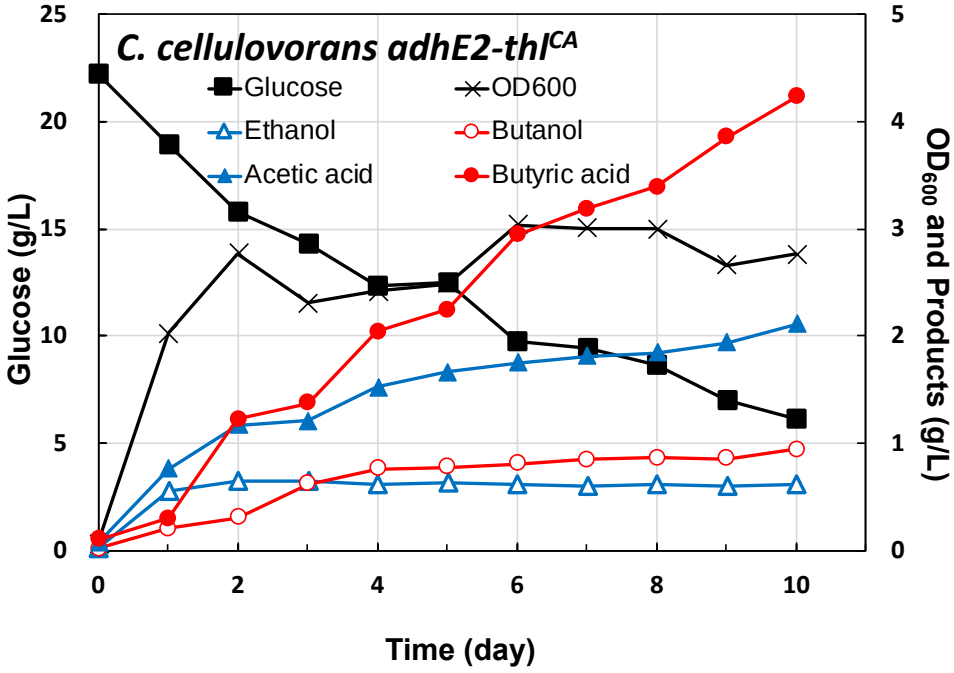
Additional / Supporting Data

- ME fermentation kinetics data and flux analysis
- Proteomics and metabolomics analysis of C. cellulovorans
- Fermentation data with original strain (shown in last project review report)
- Technical & Economic Metrics used in feasibility and cost evaluation

Metabolic Engineering

To increase C2 to C4 flux

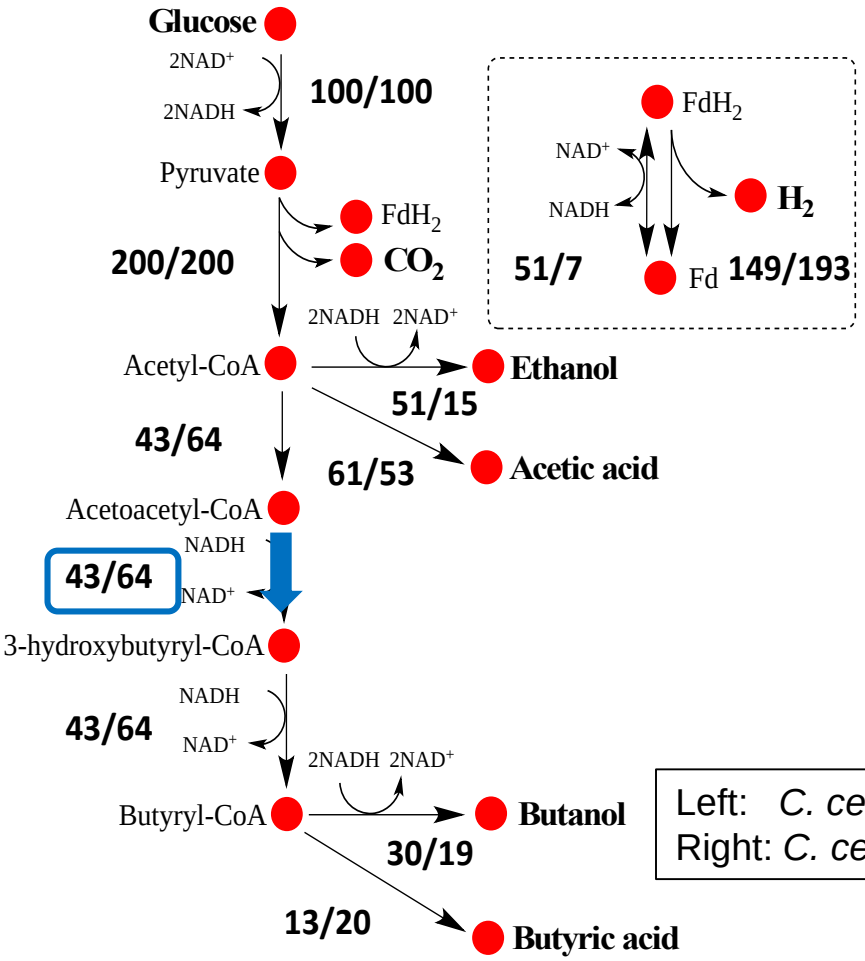


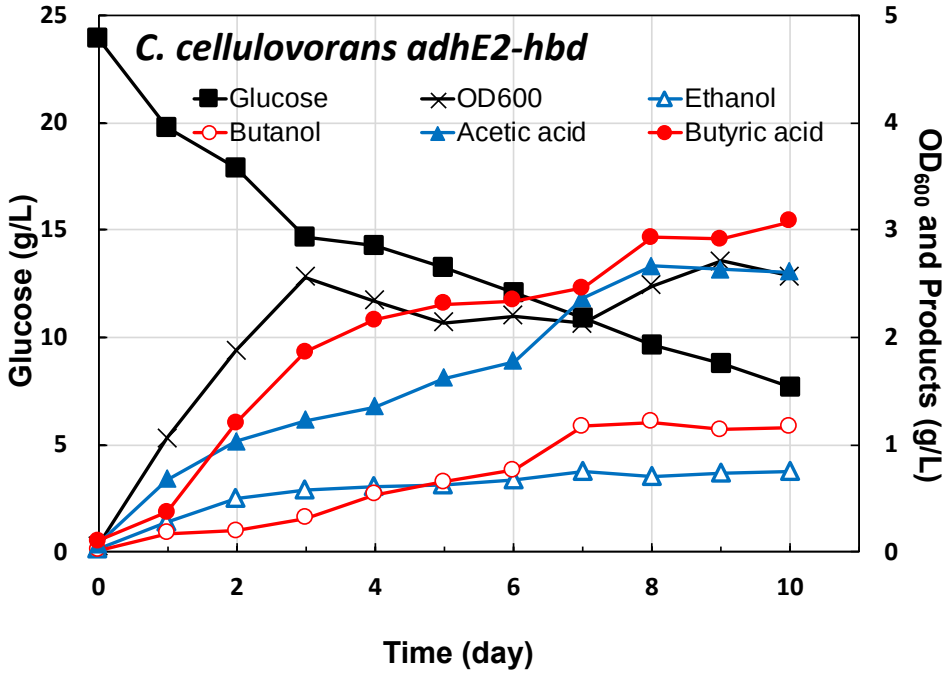


Left: *C. cellulovorans adh*E2
Right: *C. cellulovorans adh*E2-*thl*^{CA}

Metabolic Engineering

To increase C2 to C4 flux

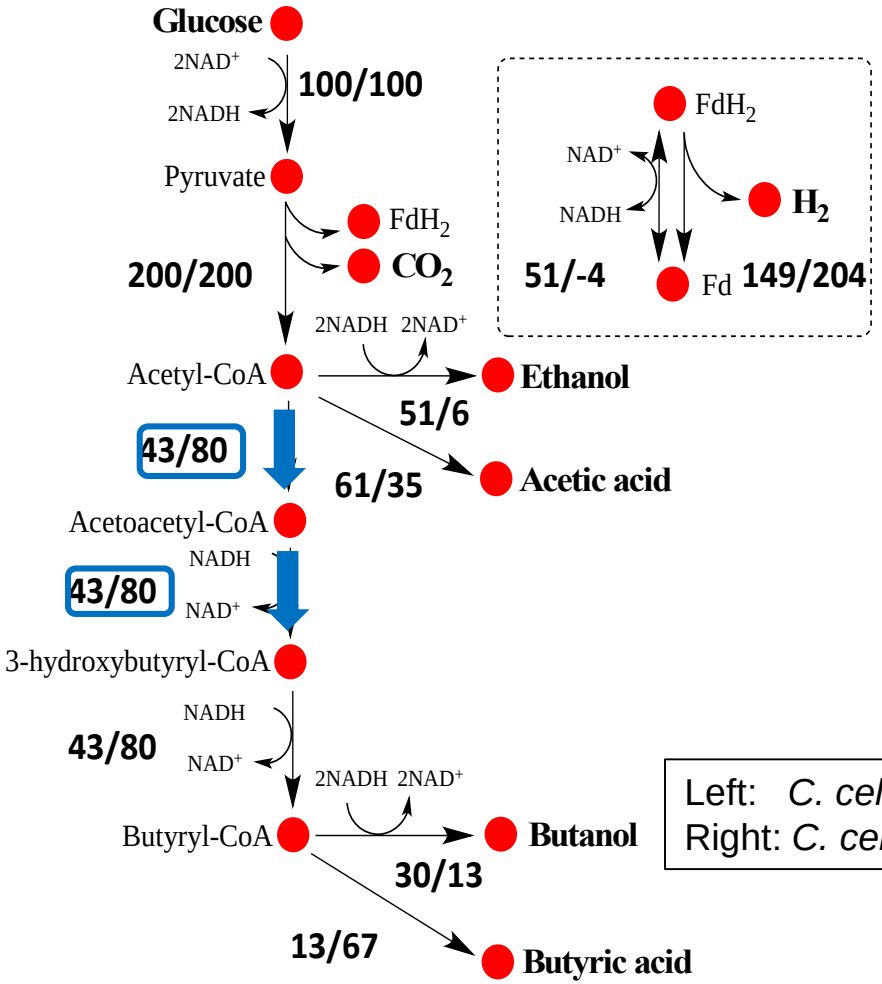


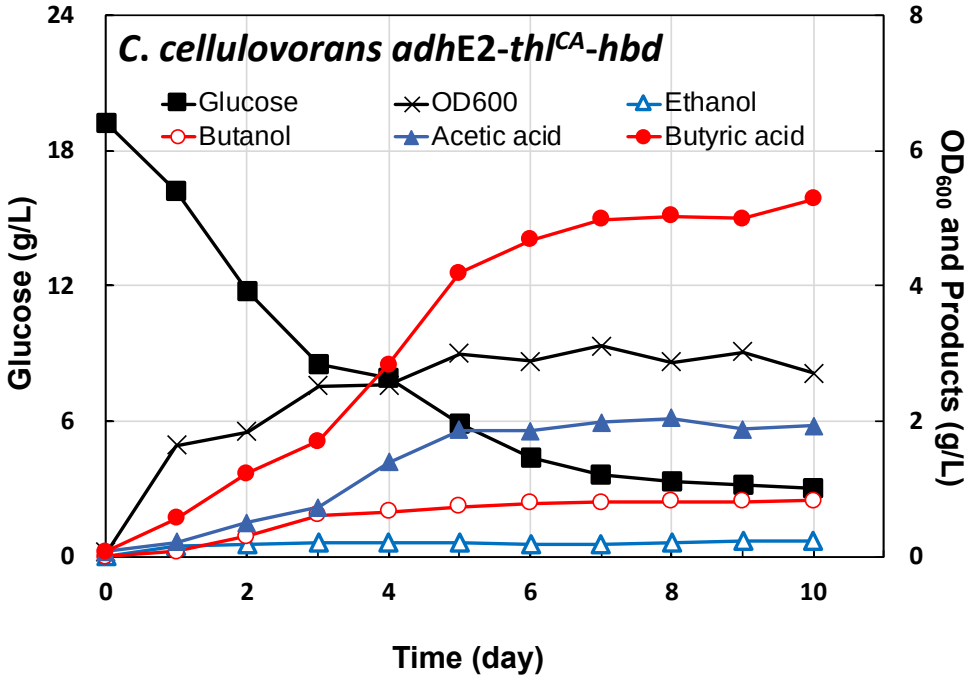


Left: *C. cellulovorans adh*E2
Right: *C. cellulovorans adh*E2-*hbd*

Metabolic Engineering

To increase C2 to C4 flux

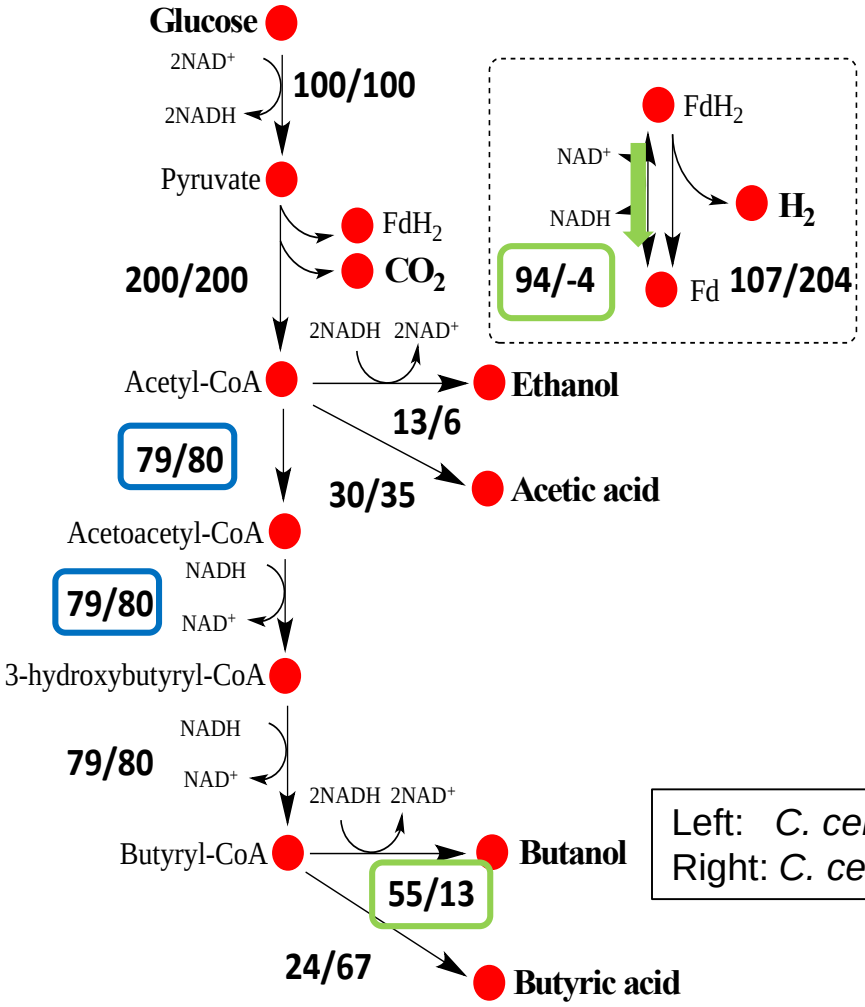


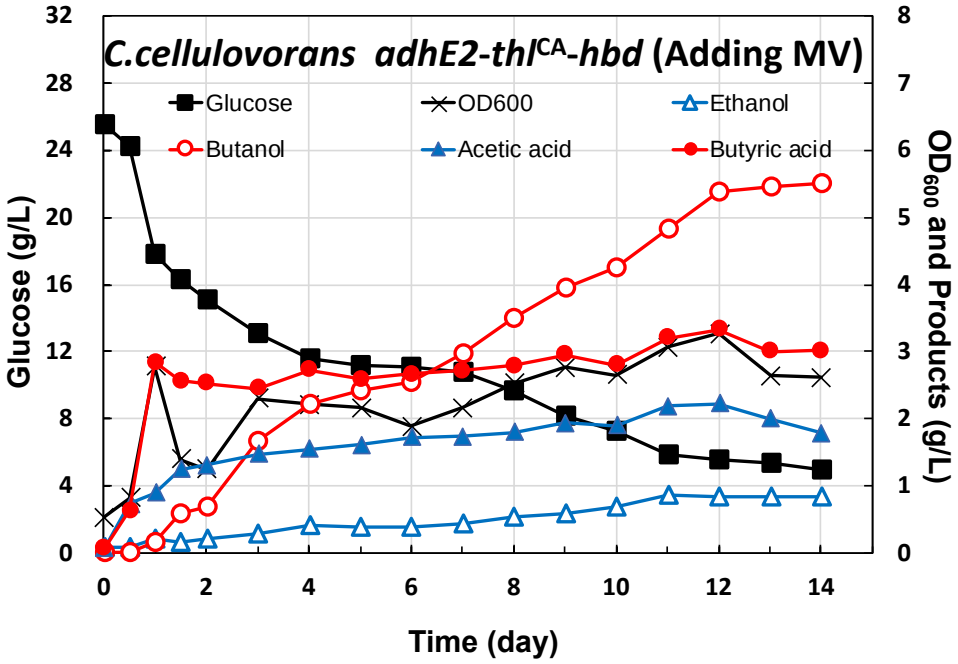


Left: *C. cellulovorans adh*E2
Right: *C. cellulovorans adh*E2-*thl*^{CA}-*hbd*

Metabolic Engineering

To increase C2 to C4 flux

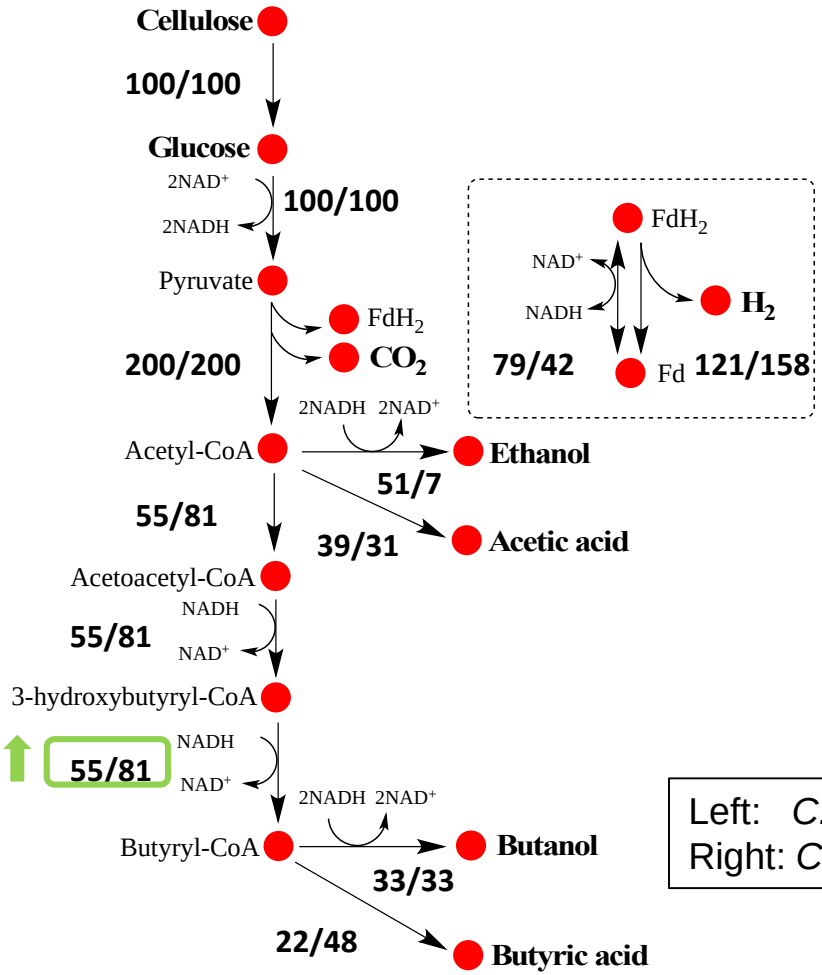


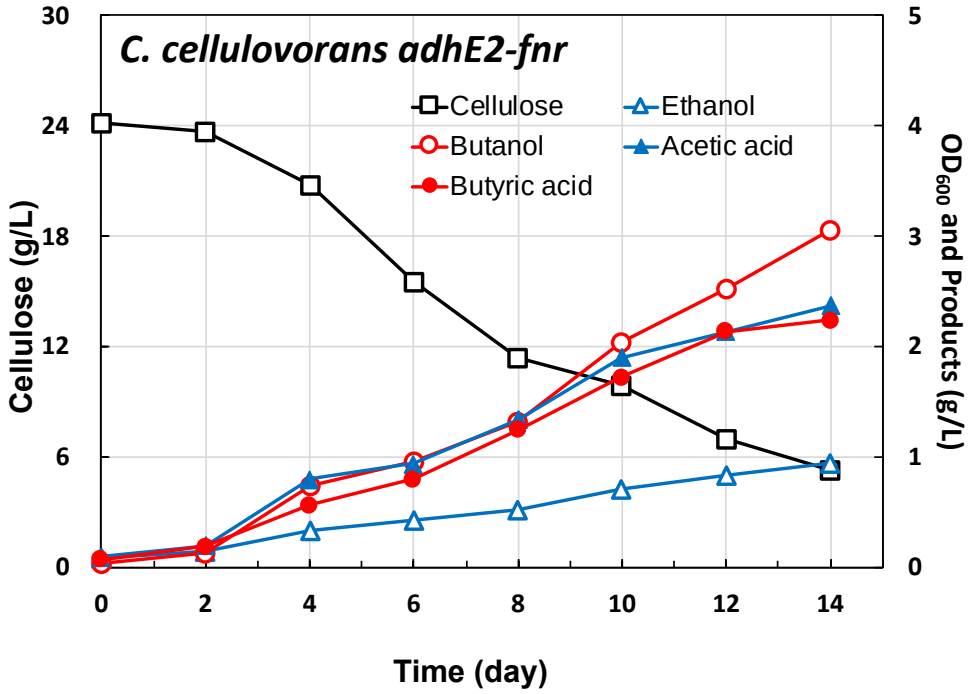


Left: *C. cellulovorans adh*E2-*thl*^{CA}-*hbd* (MV)
Right: *C. cellulovorans adh*E2-*thl*^{CA}-*hbd*

Metabolic Engineering

Redox Balance via Cofactor Engineering

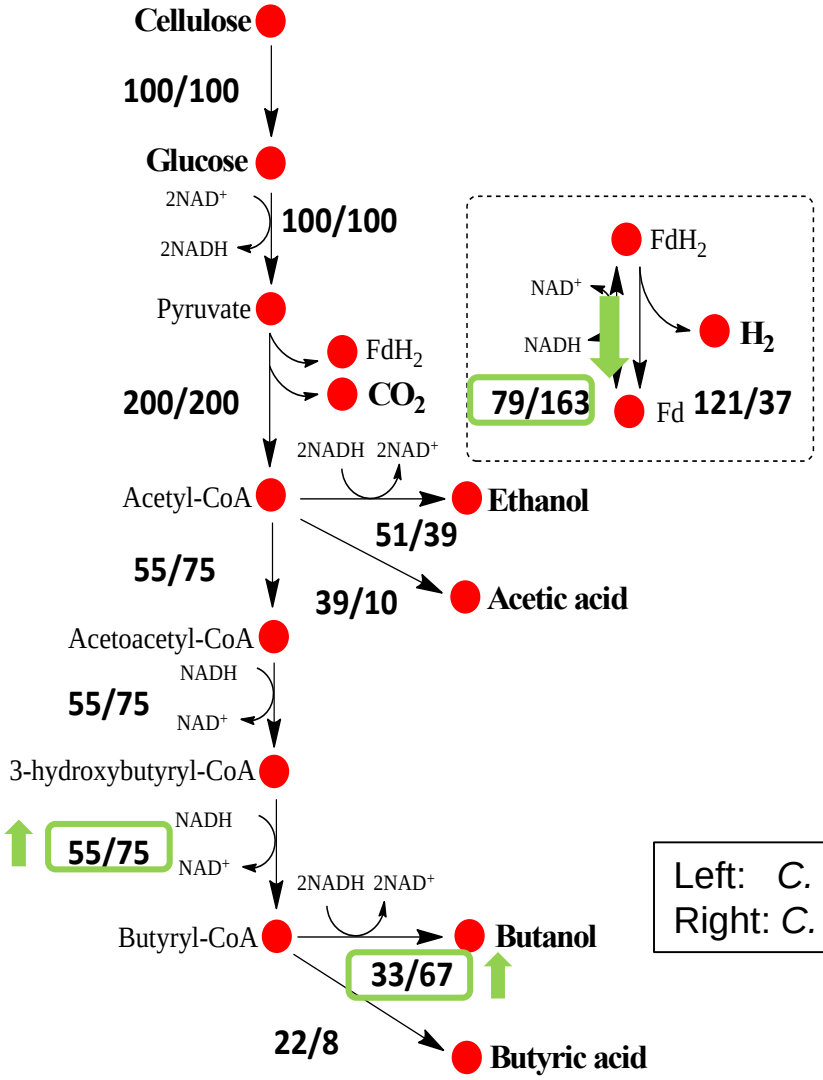


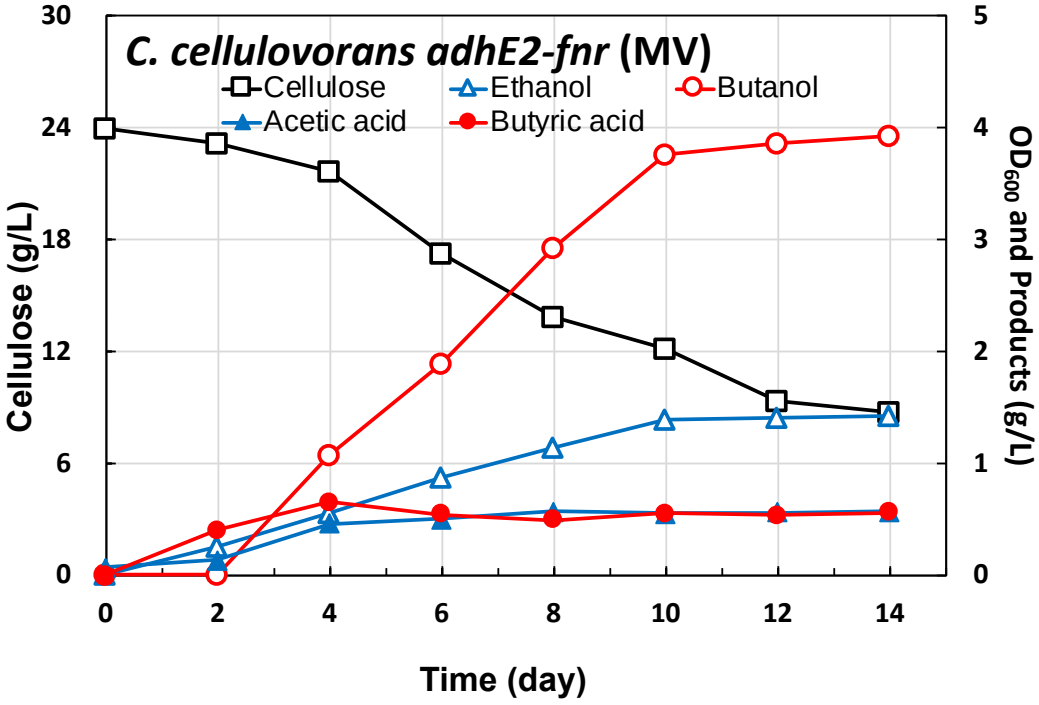


Left: *C. cellulovorans adh*E2
Right: *C. cellulovorans adh*E2-*fnr*

Metabolic Engineering

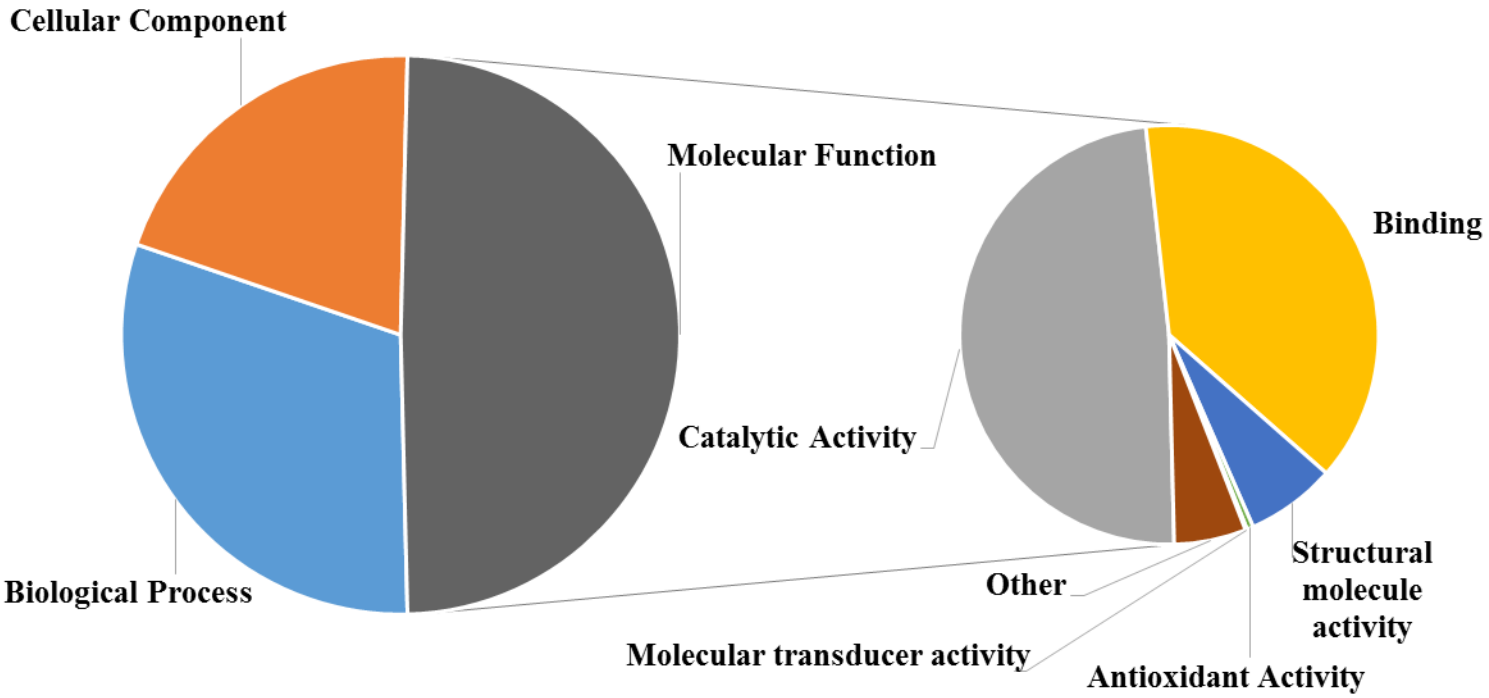
Redox Balance via Cofactor Engineering





Left: *C. cellulovorans adh*E2
Right: *C. cellulovorans adh*E2-*fnr* (MV)

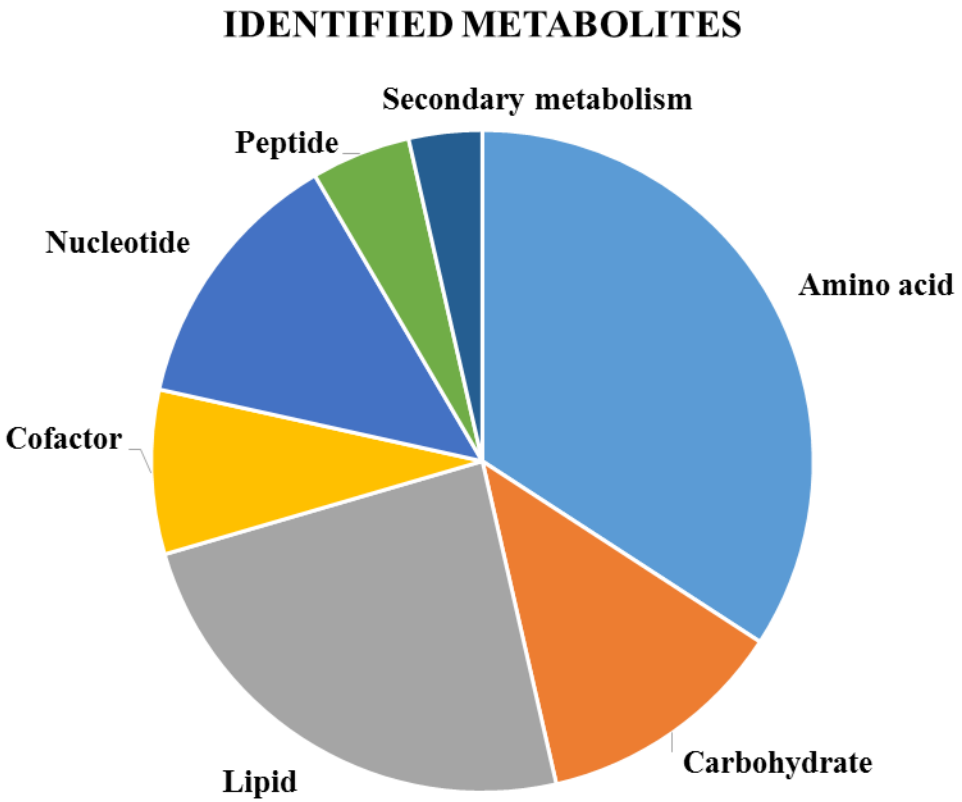
Proteomics Analysis: Classification of Proteins in *C. cellulovorans*



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.

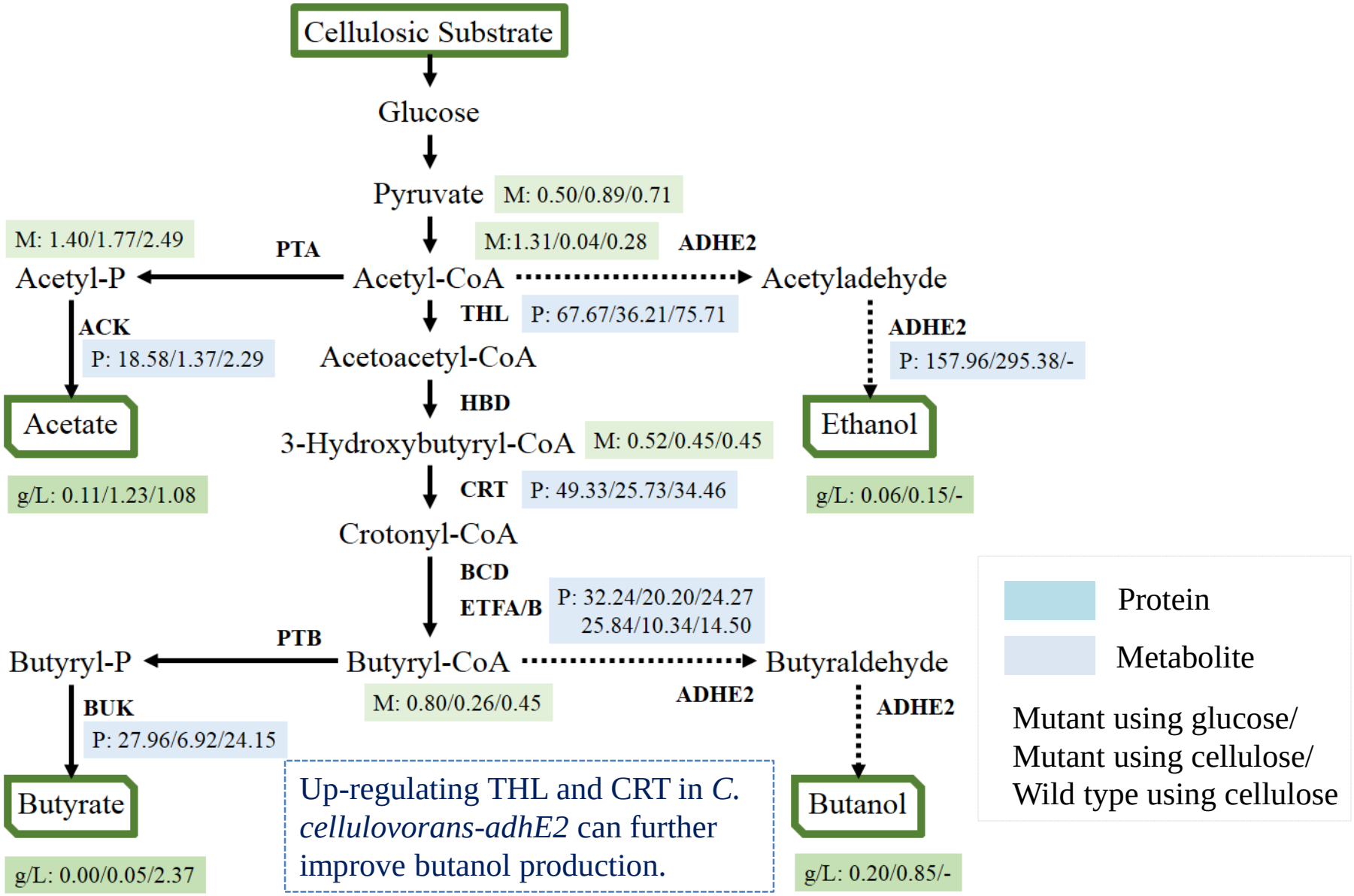
Metabolomics Analysis: Classification of Metabolites in *C. cellulovorans*



- 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.

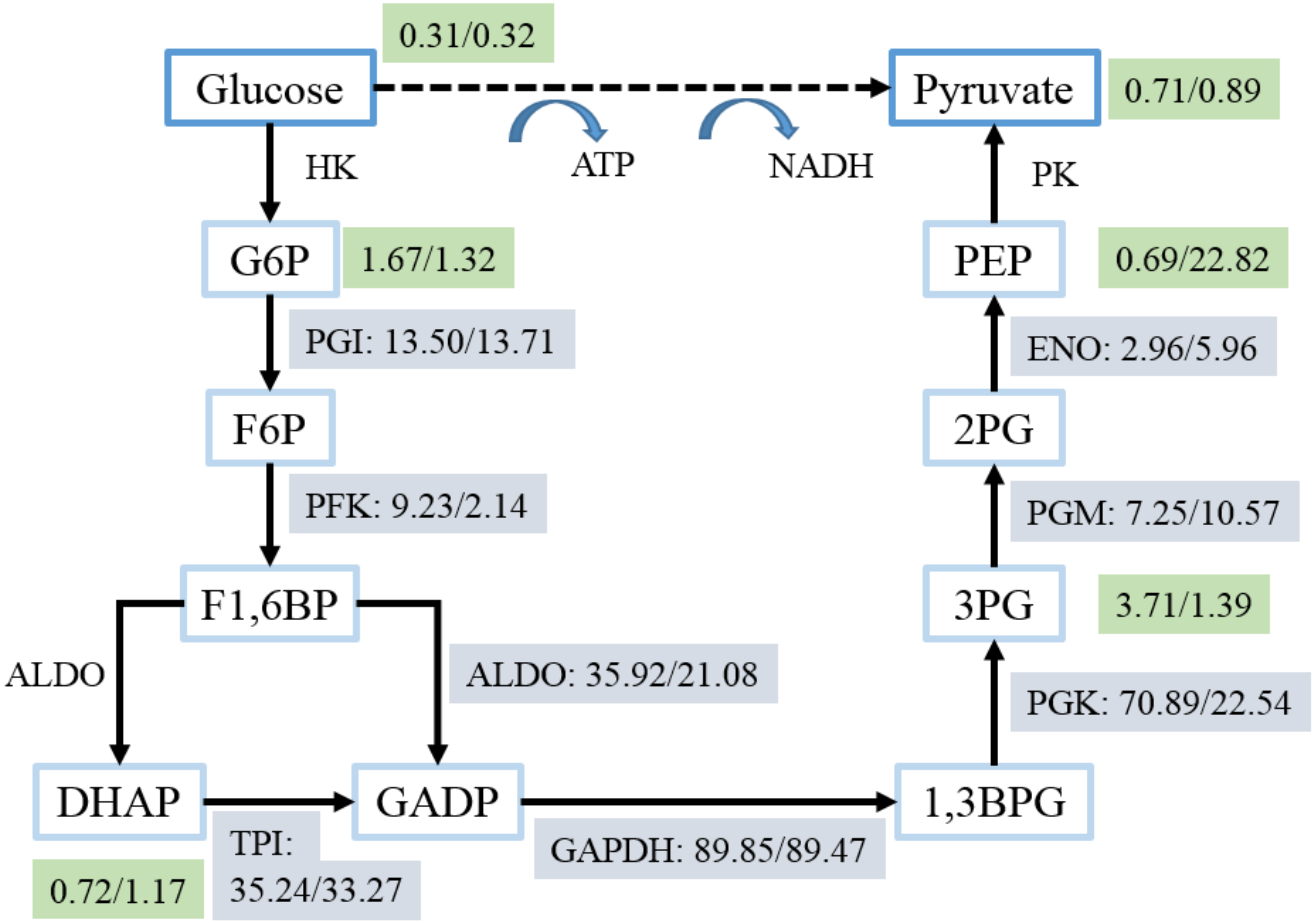
Classification analysis of identified metabolites

Proteomics & Metabolomics Analyses



Proteomics & Metabolomics Analyses

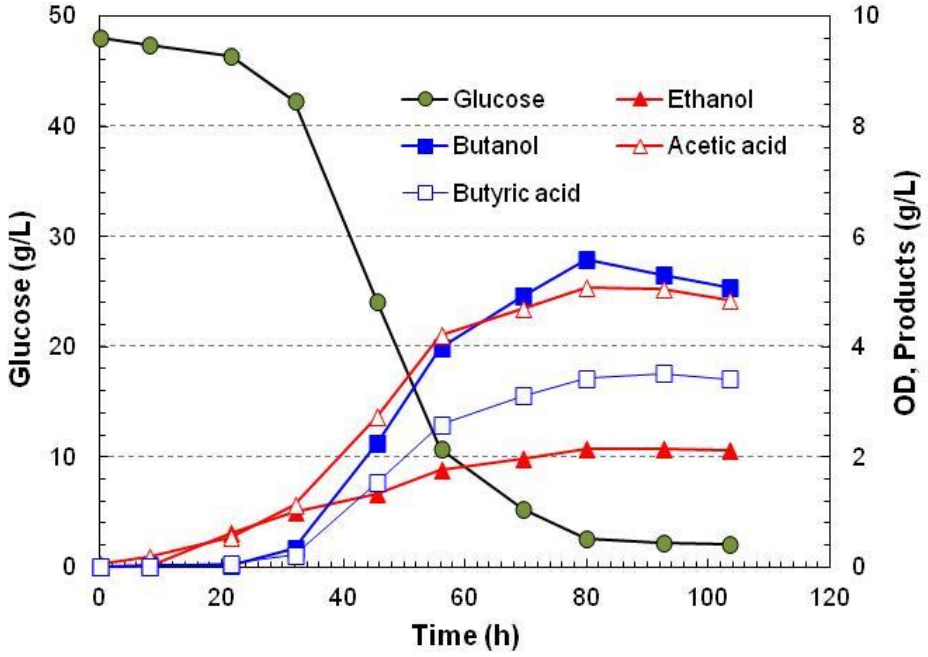
Glycolysis Pathway

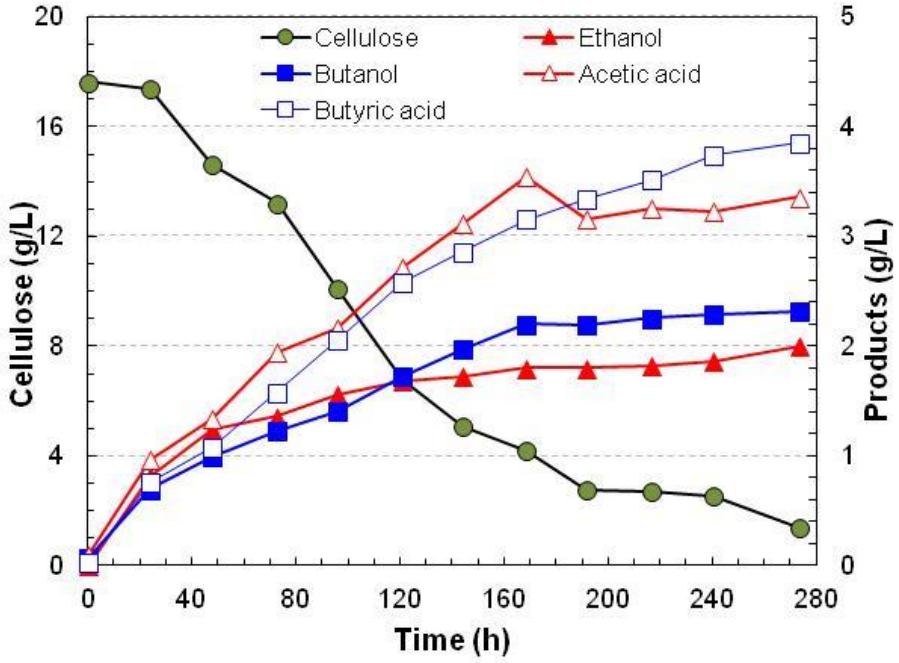


PFK and PGK up-regulation could improve the glycolysis efficiency in *C. cellulovorans-adhE2*.

C. cellulovorans – adhE2

Batch fermentation in serum bottles – Cellulose vs. Glucose

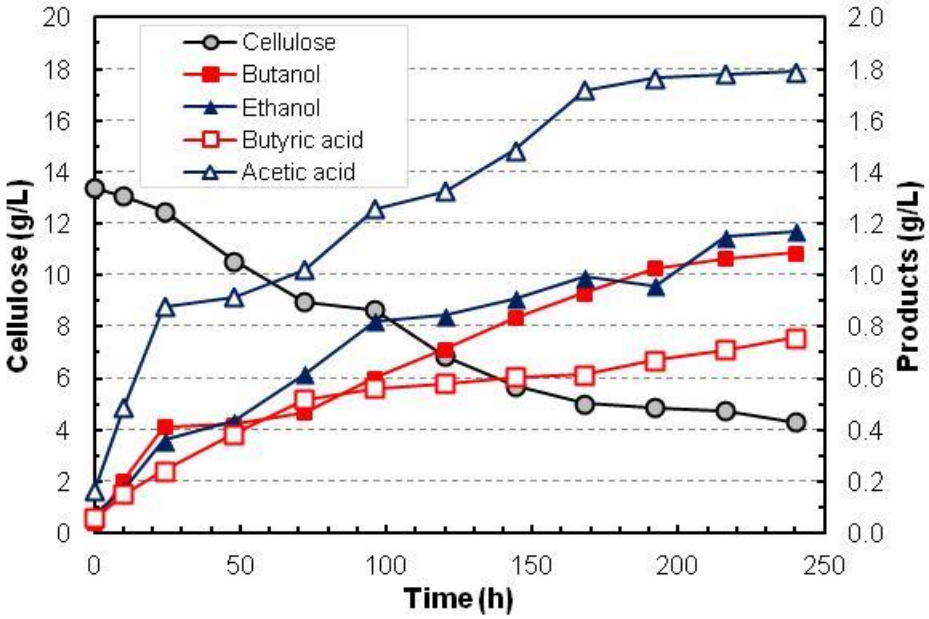


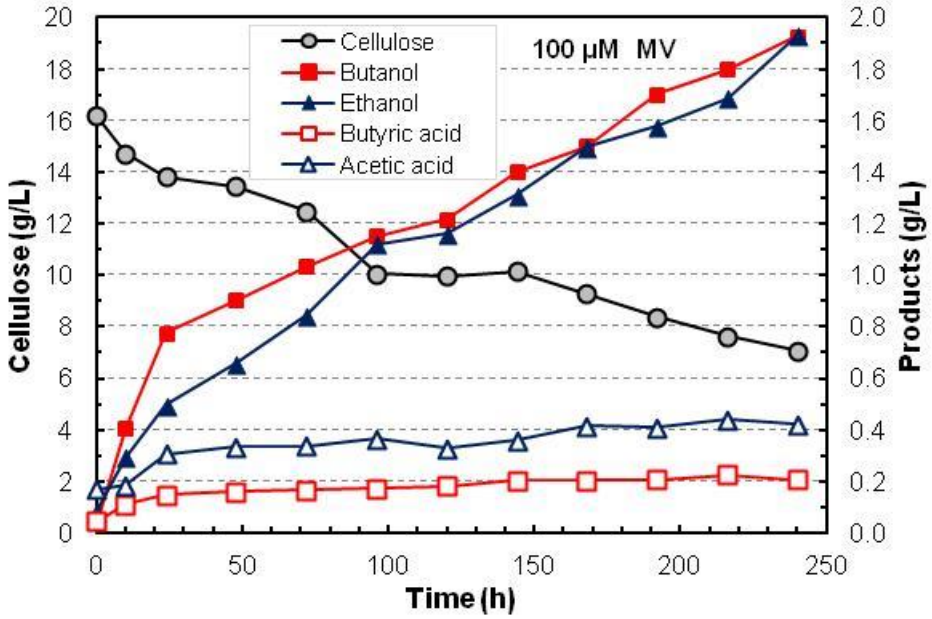


Substrate	Butanol Yield (g/g)	Ethanol Yield (g/g)	Butanol Productivity (g/L h)
Glucose	0.122	0.046	0.070
Cellulose	0.164	0.141	0.013

C. cellulovorans – adhE2

Batch fermentation in serum bottles – Effects of MV



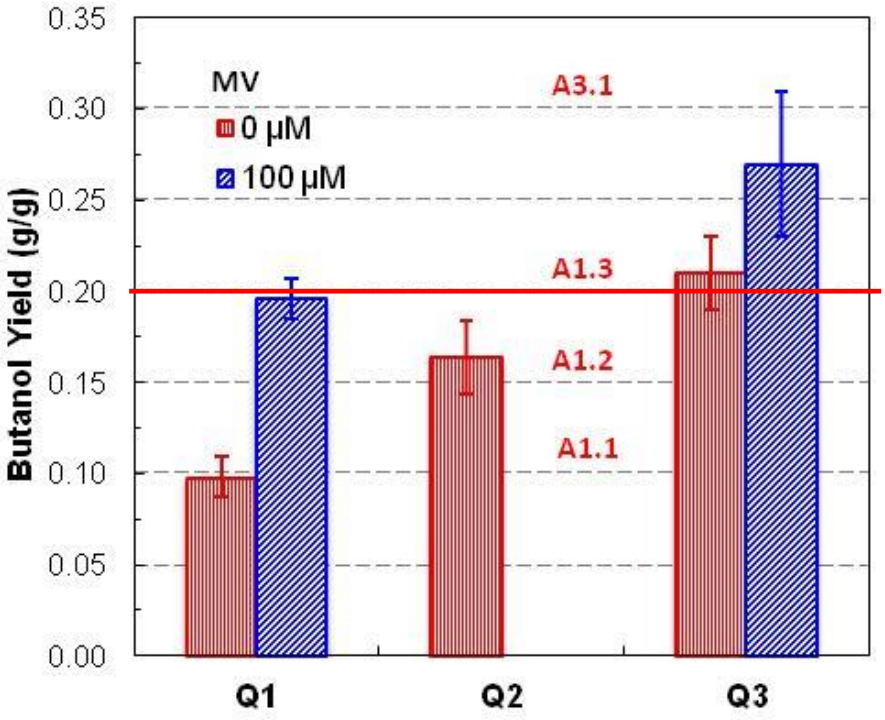


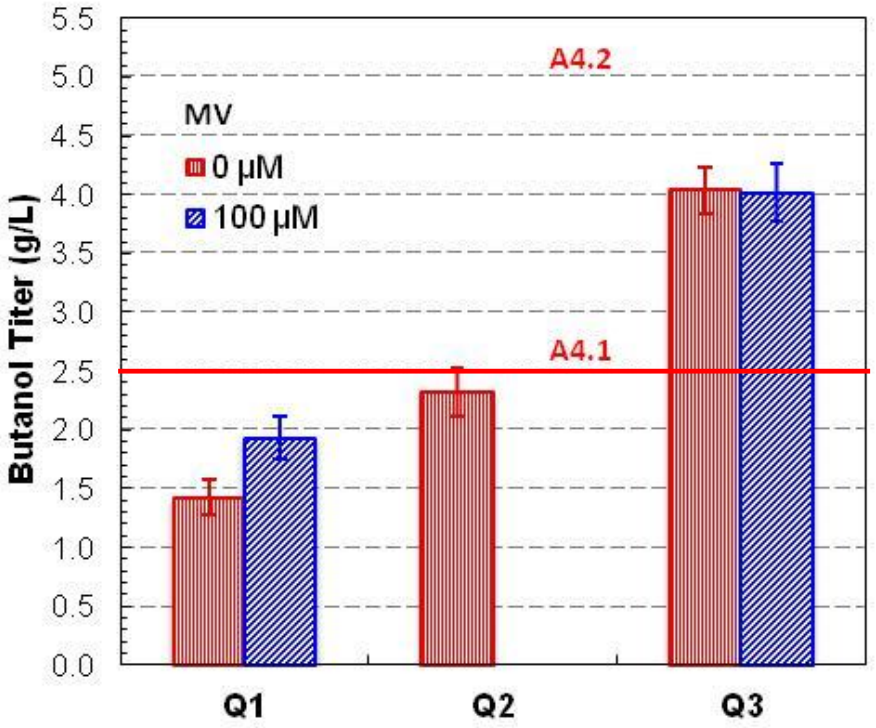
Methyl viologen (MV) as an artificial electron carrier was added to shift metabolic flux to increase NADH availability for alcohol production

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

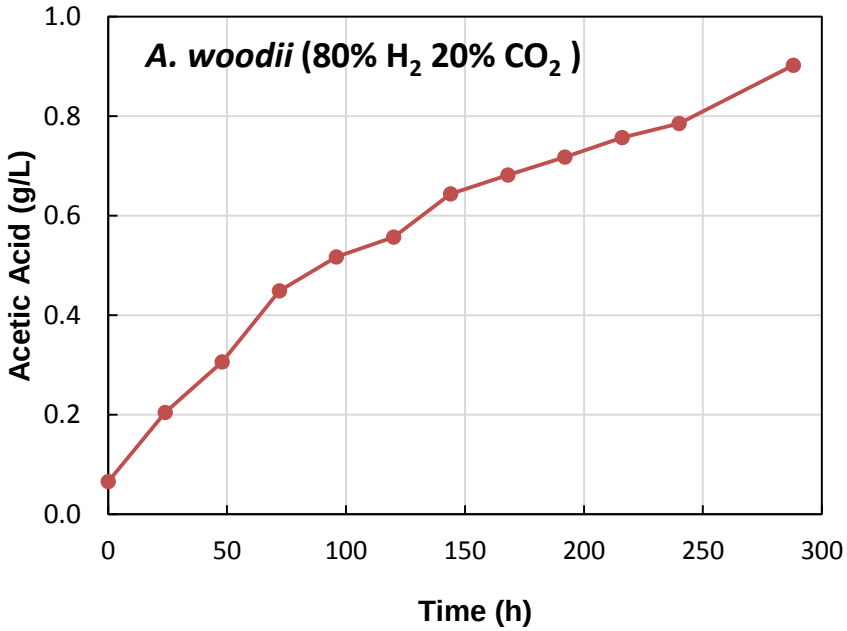
Batch fermentation with high cell density

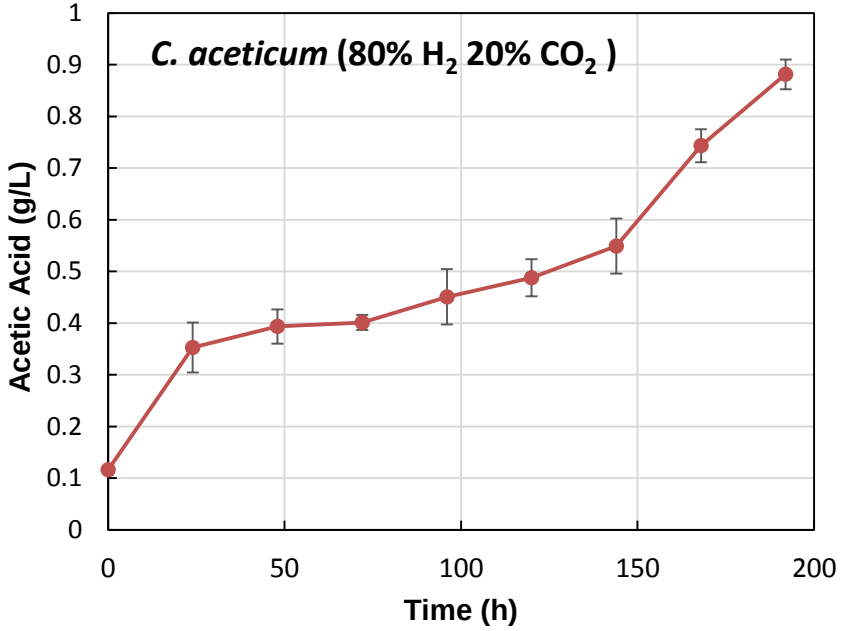




C. cellulovorans mutant overexpressing *adhE2* meets the Go criteria:
Butanol yield >0.2 g/g cellulose, Butanol titer >2.5 g/L

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

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