WHITE DOG LABS

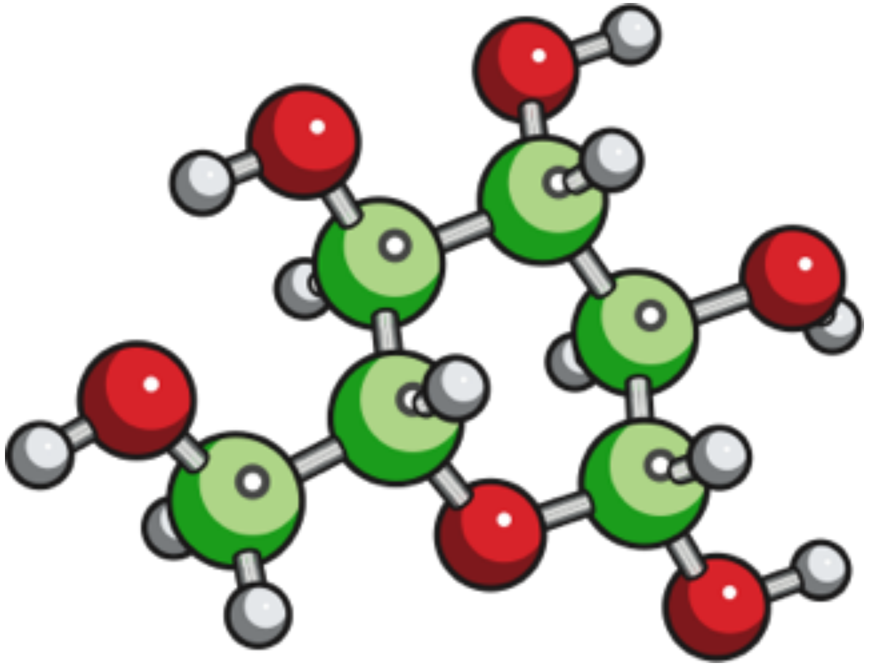
CO_2 Fixation by Anaerobic Non-Photosynthetic Mixotrophy for Improved Carbon Conversion

Emily E. Crawford, John R. Phillips, Pradeep C. Munasinghe, Carrissa A. Wiedel, Biniam Maru, Ilana Aldor, & Shawn W. Jones, Terry Papoutsakis & Bryan Tracy

ARPA-E Carbon Optimized Bioconversion

Sept 26, 2019

Bio-economy Conundrum



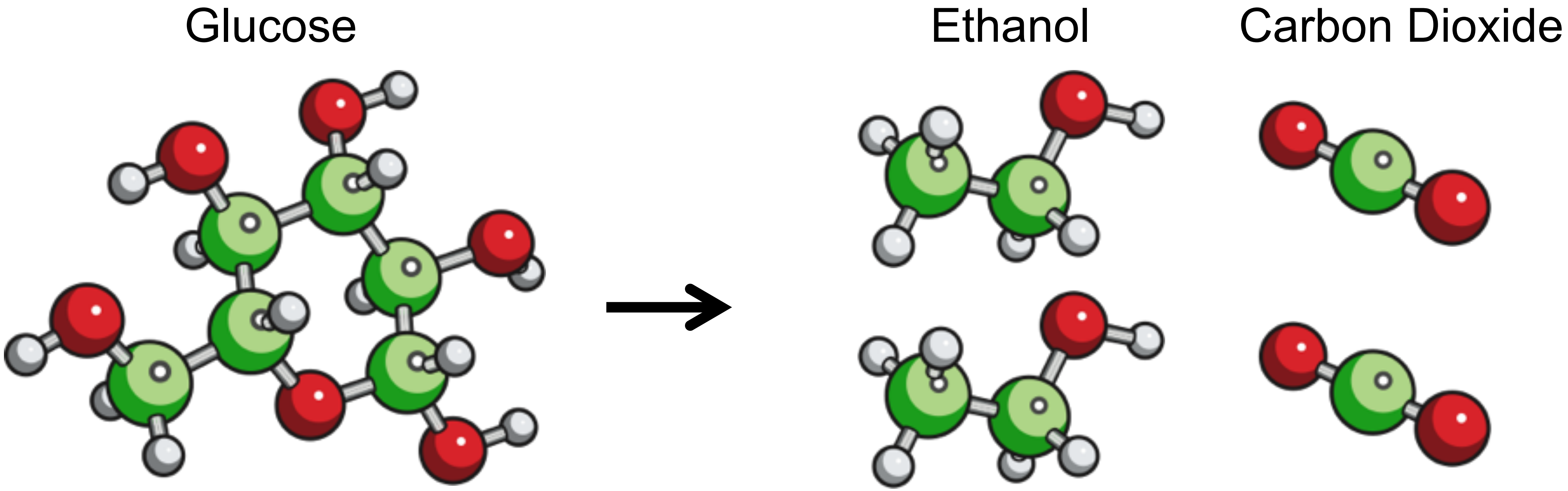
Glucose is

- ~50% Carbon
- ~50% Oxygen

- Carbon =  = $$$
- Oxygen =  = -$$$

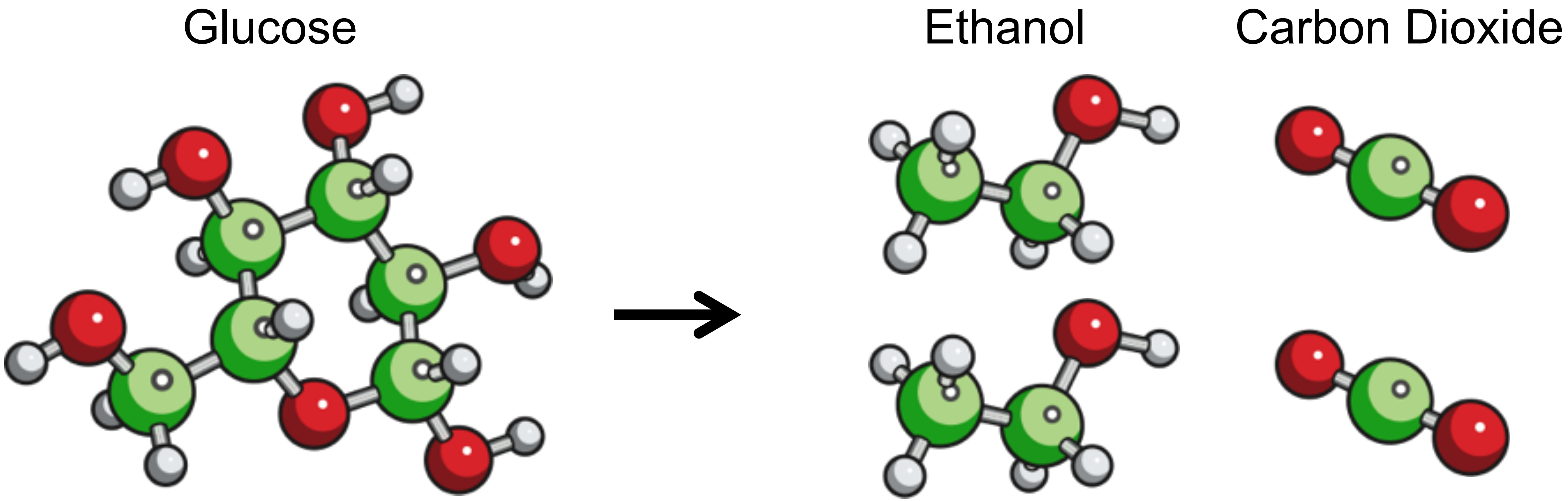
Nearly all molecules in industry have a carbon backbone with little to no oxygen. How does conventional fermentation deal with this?

Bio-economy Conundrum



Fermentation densifies energy and sacrifices 1/3 of the carbon to get rid of oxygen.

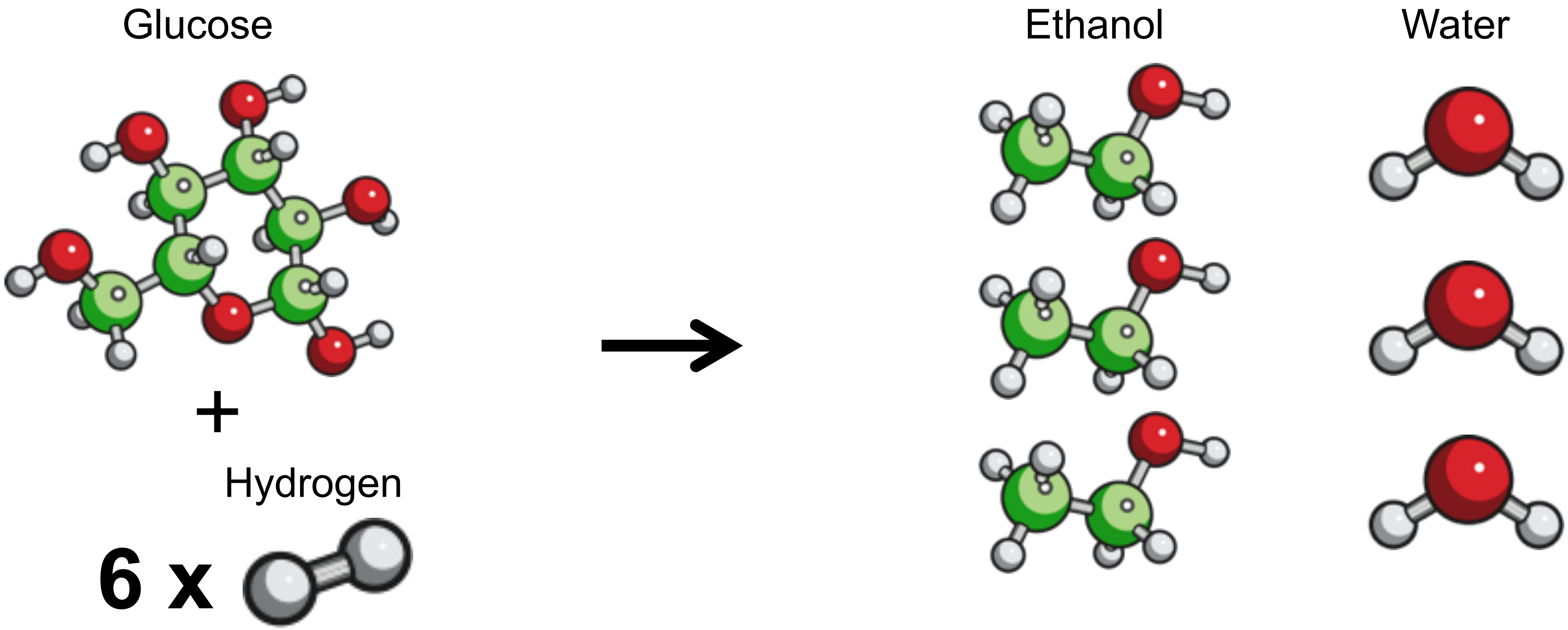
Conventional Ethanol



Fermentation sacrifices 33% of the carbon to get rid of oxygen and densify energy into ethanol.

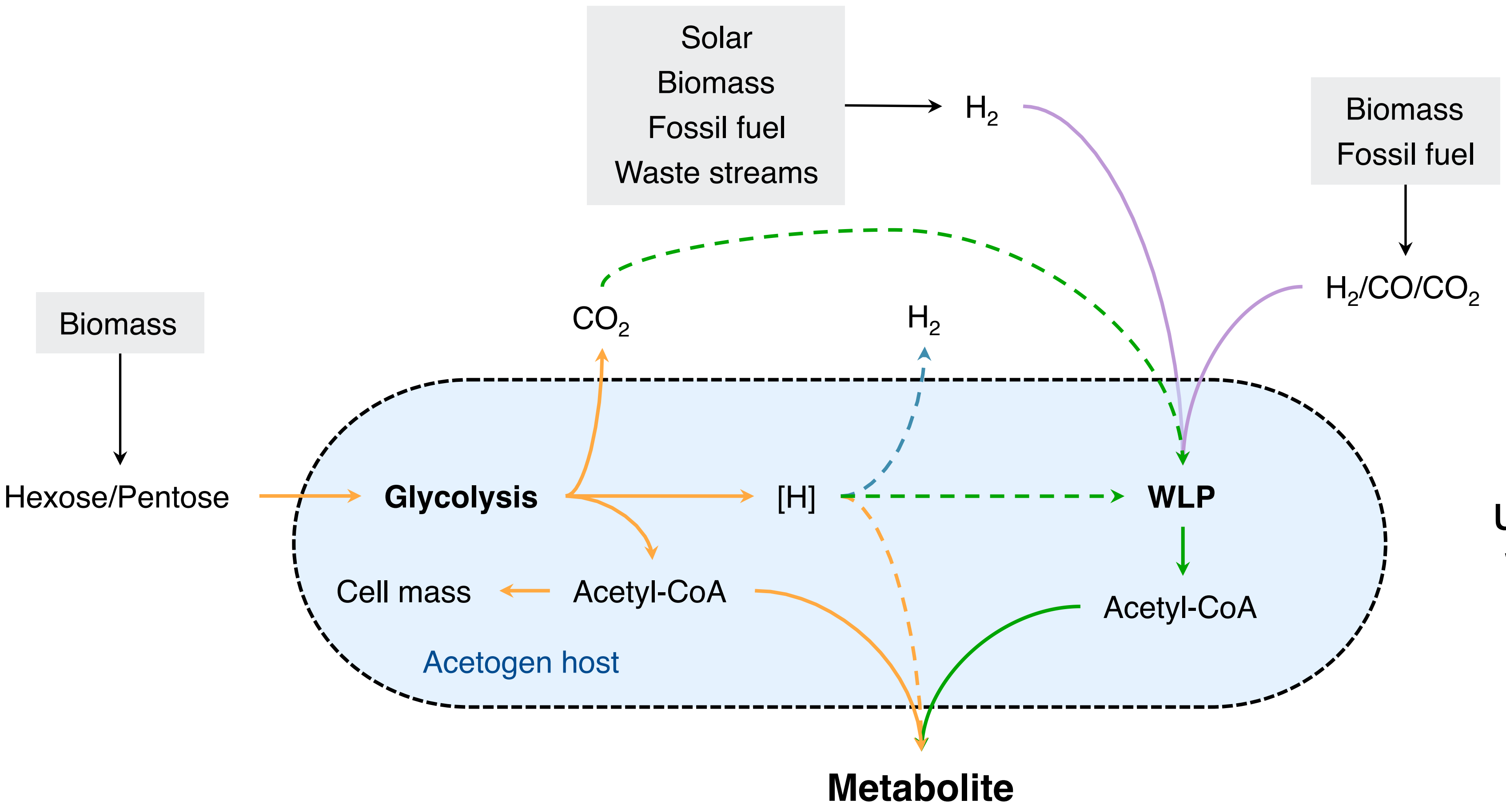
Is there a way to improve carbon yield?

Fermentation Plus Chemical Reductant



100% carbon yield with water as only by-product

Clostridial Mixotrophy (MixoFerm) at its Core



Reducing equivalents are used to fix CO_2 through the Wood-Ljungdahl Pathway (WLP)

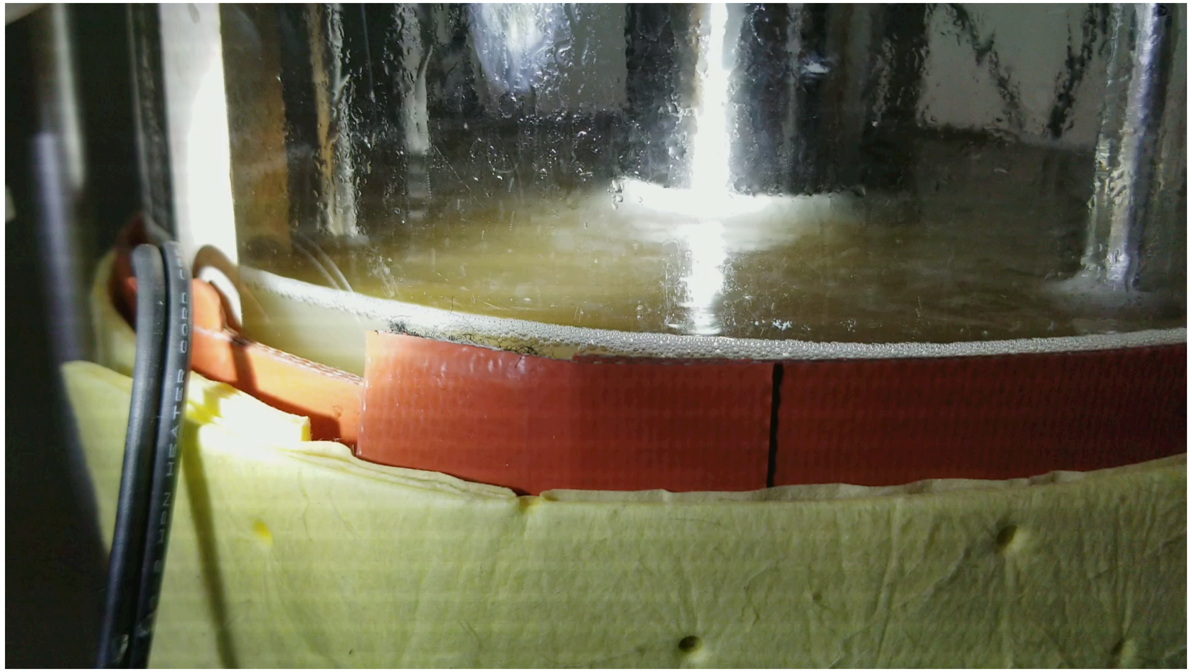
Fast, AG, Schmidt, ED, Jones, SW, & Tracy, BT. 2015. *Curr Opin Biotechnol* 33:60-72.

Jones, SW, *et al*. 2016. *Nat Comm* 7:12800.

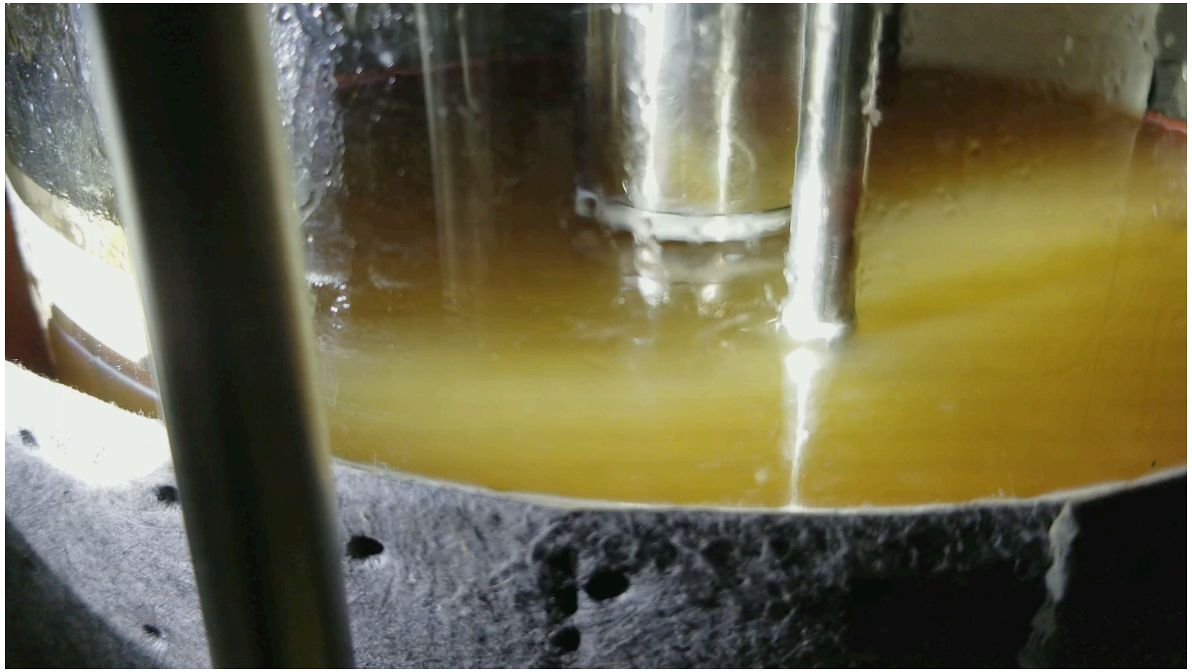
Clostridial Mixotrophy *In Video*

MixoFerm organisms consume sugar and CO_2 thus increasing yield

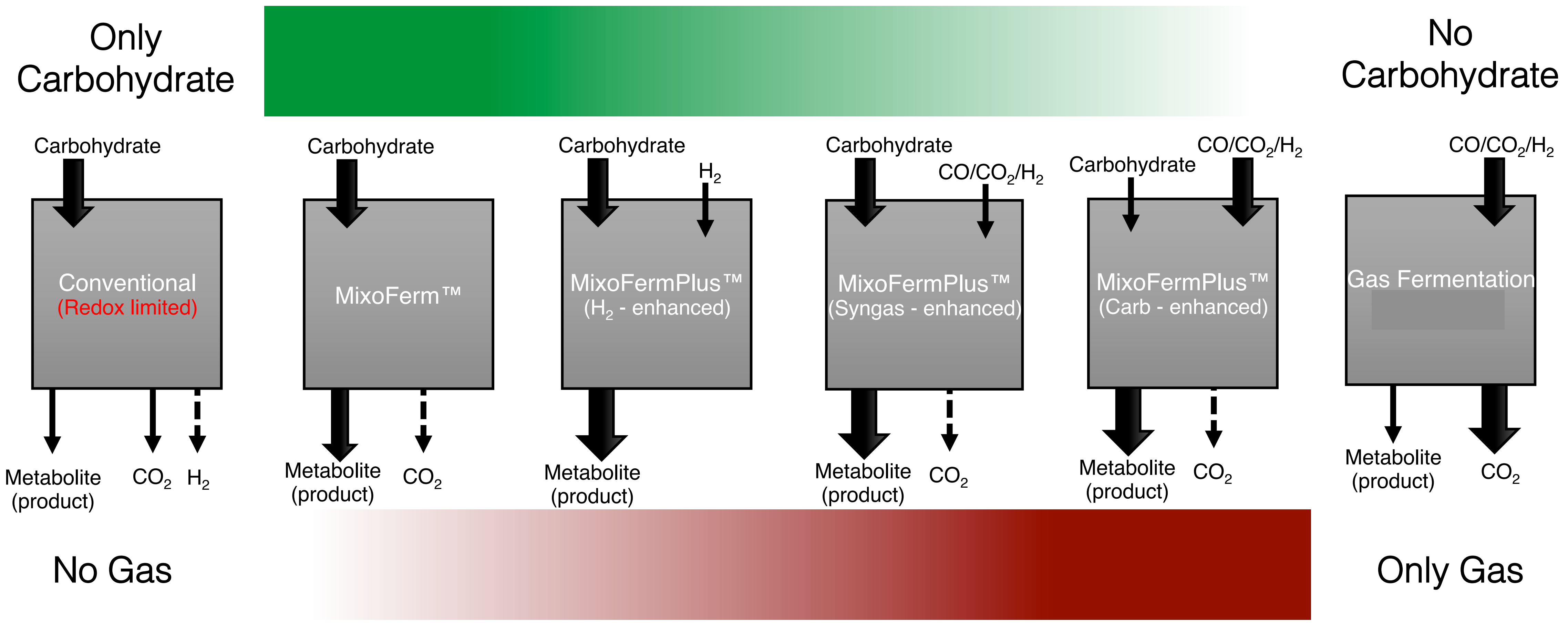
Conventional fermentation – note the bubbles due to gas evolution



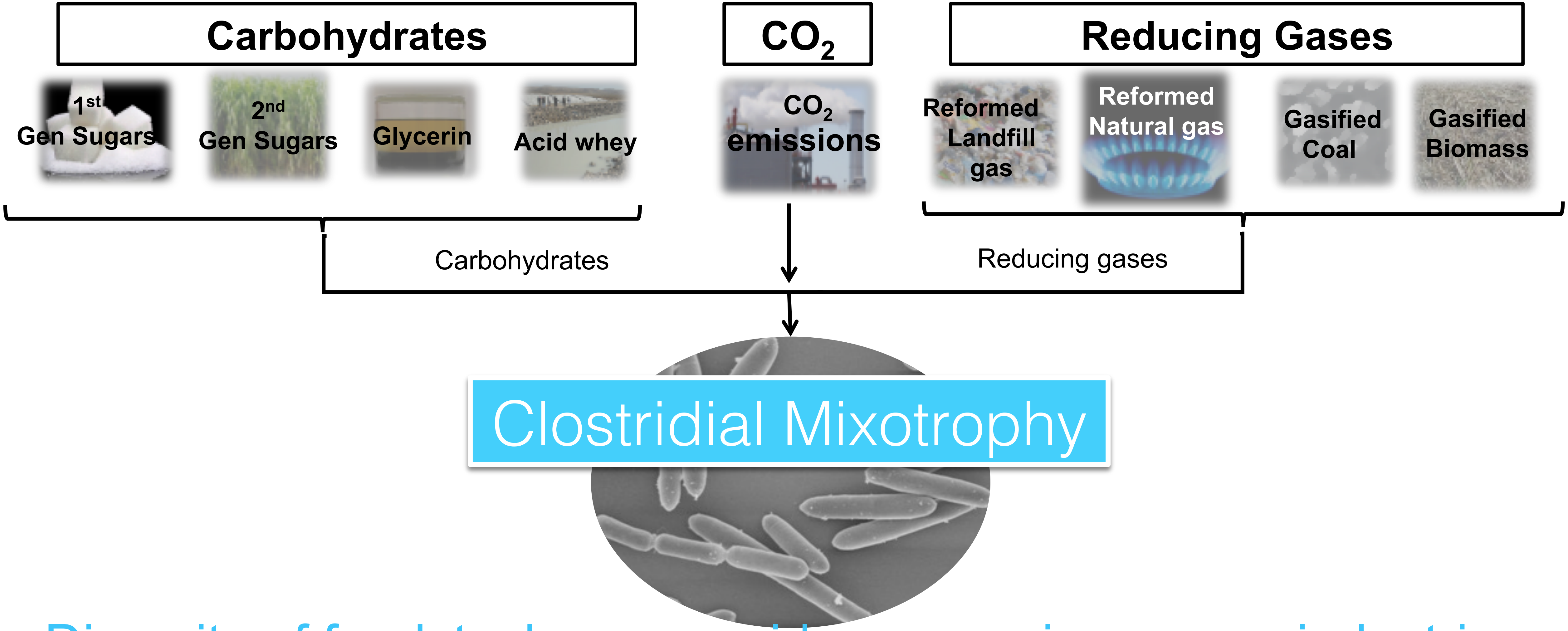
MixoFerm™ fermentation – note the lack of bubbles



Spectrum of Mixotrophic Processes

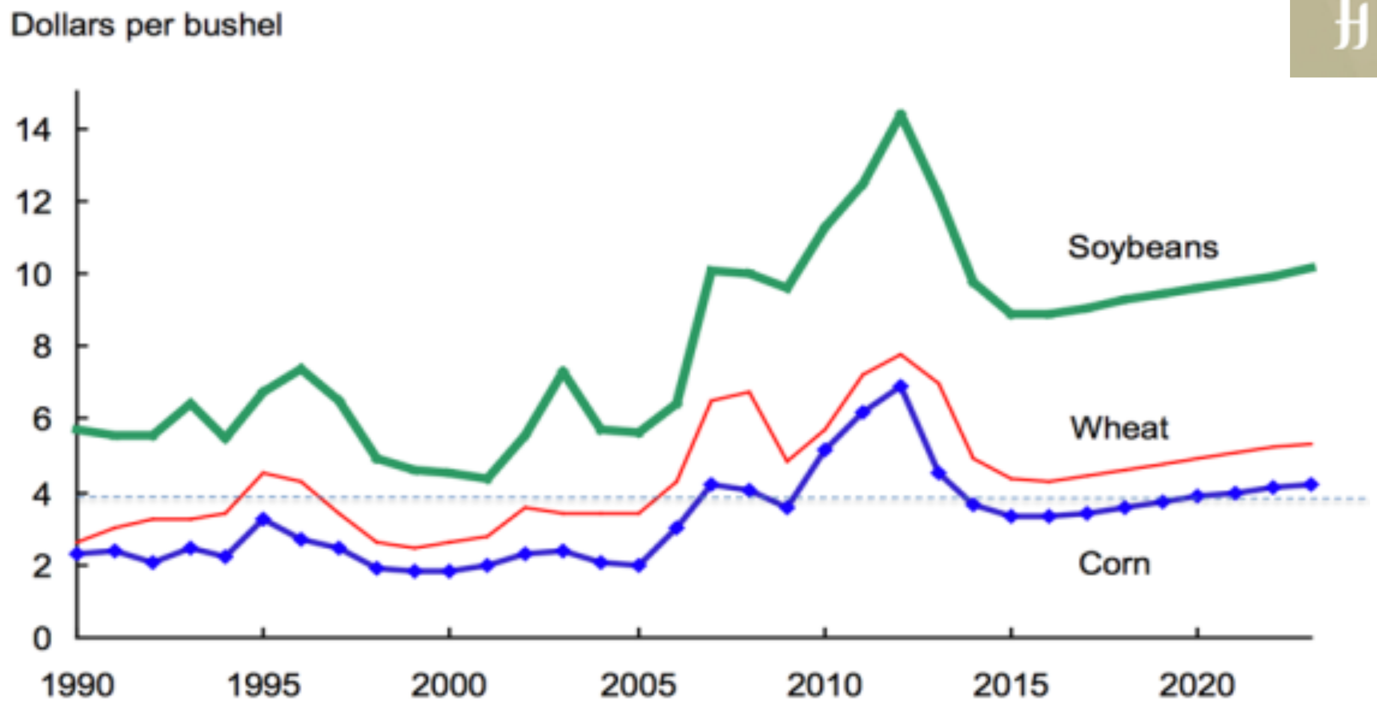


Array of Feedstock Combinations

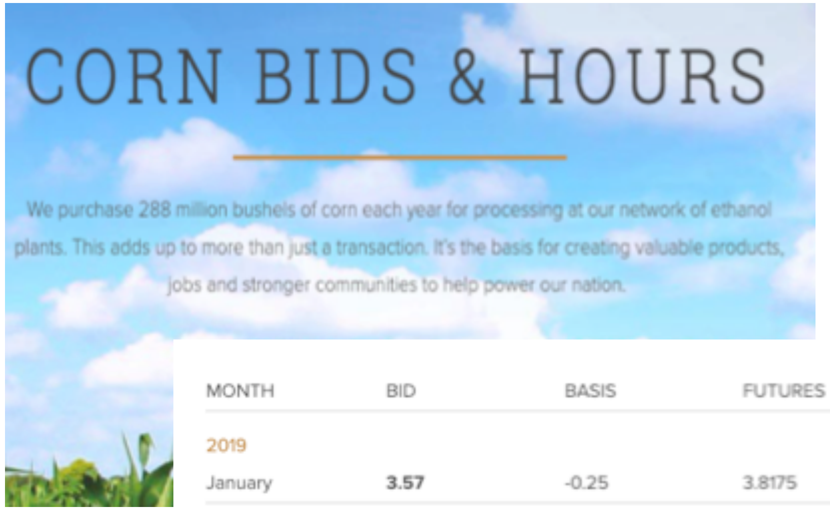


Diversity of feedstock use provides synergies across industries

Starch Industry Motivation for Mixotrophy



USDA Agricultural Projections to 2023 http://www.ers.usda.gov/publications/oce-usda-agricultural-projections/oce141.aspx



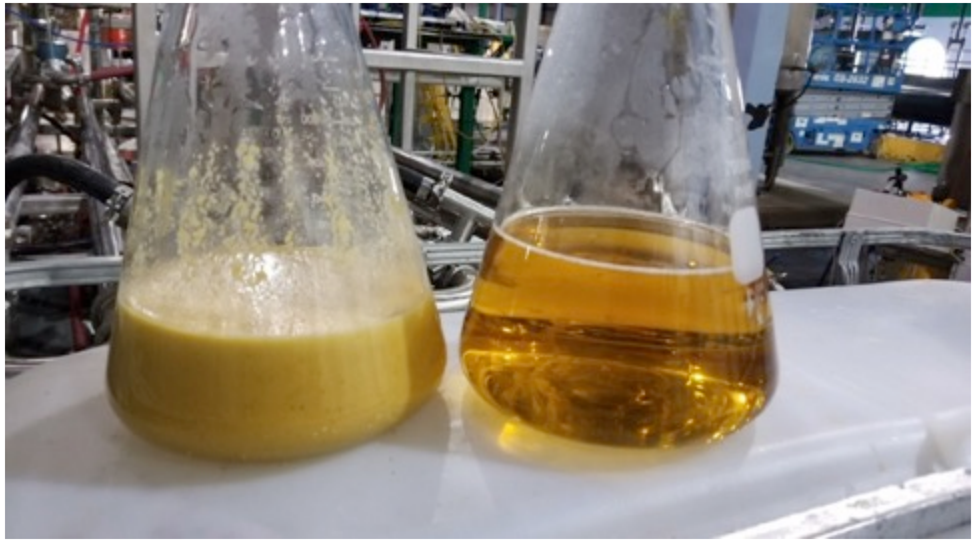
MONTH	BID	BASIS	FUTURES
2019			
January	3.57	-0.25	3.8175
February	3.57	-0.25	3.8175
March	3.57	-0.25	3.8175
April	3.60	-0.30	3.9000
May	3.60	-0.30	3.9000
June	3.65	-0.32	3.9725
July	3.65	-0.32	3.9725
August	3.65	-0.35	3.9975
September	3.65	-0.35	3.9975
October	3.67	-0.37	4.0375
November	3.67	-0.37	4.0375
December	3.77	-0.27	4.0375
2021			
January	3.91	-0.30	4.2100

ClearMash™ Technology

WDL adopted an existing production technology

Vertically installed, multi-pass membrane modules in a multi-stage system design. Vertical configuration conserves floor space.

Applied the technology to corn mash clarification



Built pilot system – 3 years of operation

Low CapEx clarification technology

- <$3M project cost for production of >50,000 MT clarified starch per year
- Sterilizable, readily customized
- Starch generation upwards of 30wt% solids
- Resistant to major process disruptions

ClearMash™ - enabling <$0.08/lb starch production

Starch Industry Motivation for Mixotrophy

- Attractive sugar economics today
- Robust supply and highly supportive supply chain with strong lobbying capabilities
- Trillions of dollars of already invested capital

However

- Sacrifice of biodiversity
- Through practices that are not so carbon neutral (corn itself exhibits 25% of the petro-CO_2 intensity)
- Still mandated markets that lead to tremendous business uncertainty
- Even at $0.08/lb sugar, it still struggles to compete with $60/barrel oil

2nd Generation Sugar Motivation for Mixotrophy

Cellulosic Hydrolysis

Biomass

Pre-treatment
liberates cellulose from the lignin "seal" technical issues & expensive

Cellulose

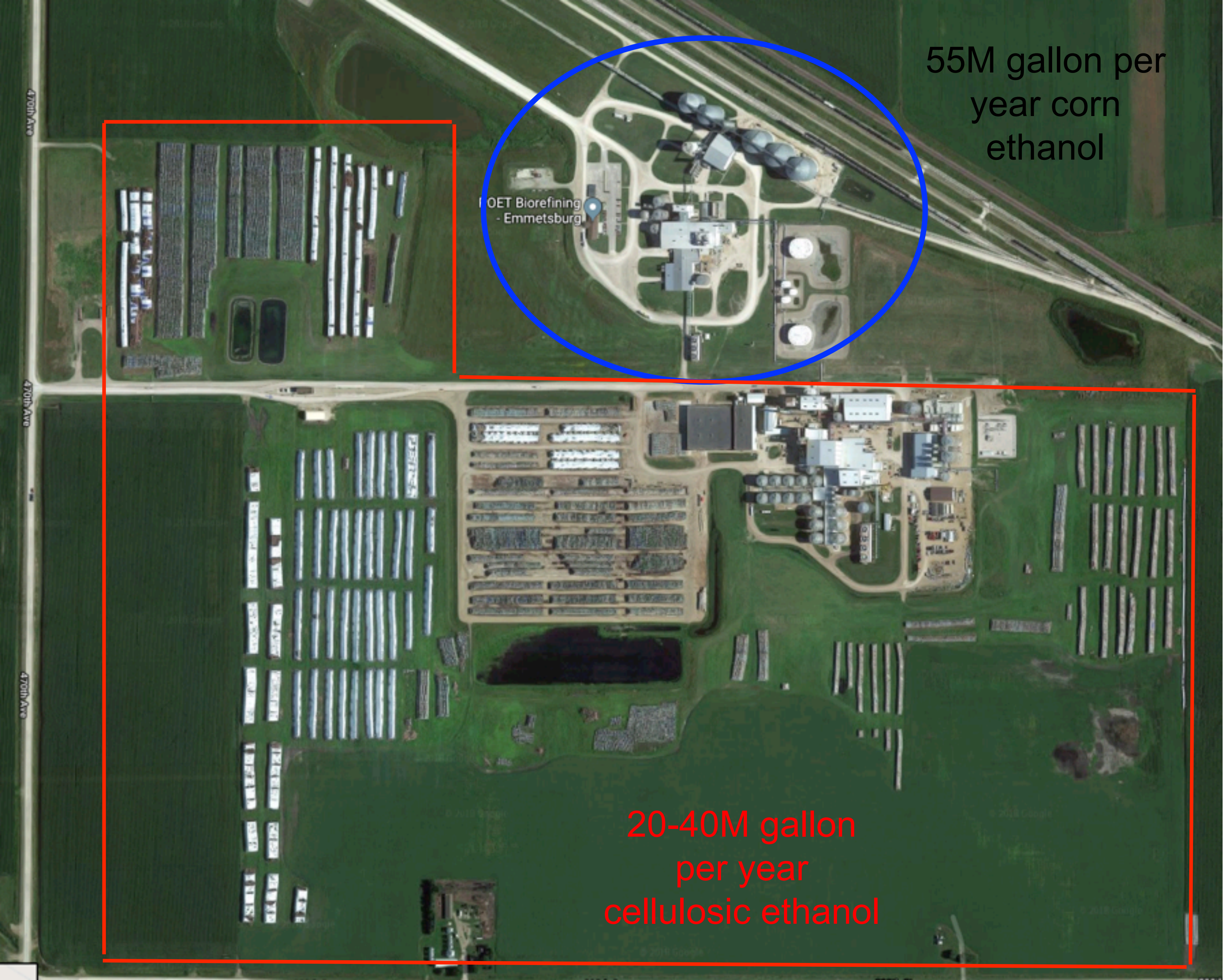
Hydrolysis
Convert cellulose to sugar with enzymes

Sugar

Fermentation - Conventional technology

Status

Poet operating at partial capacity
Abengoa and DuPont sold prior to startup



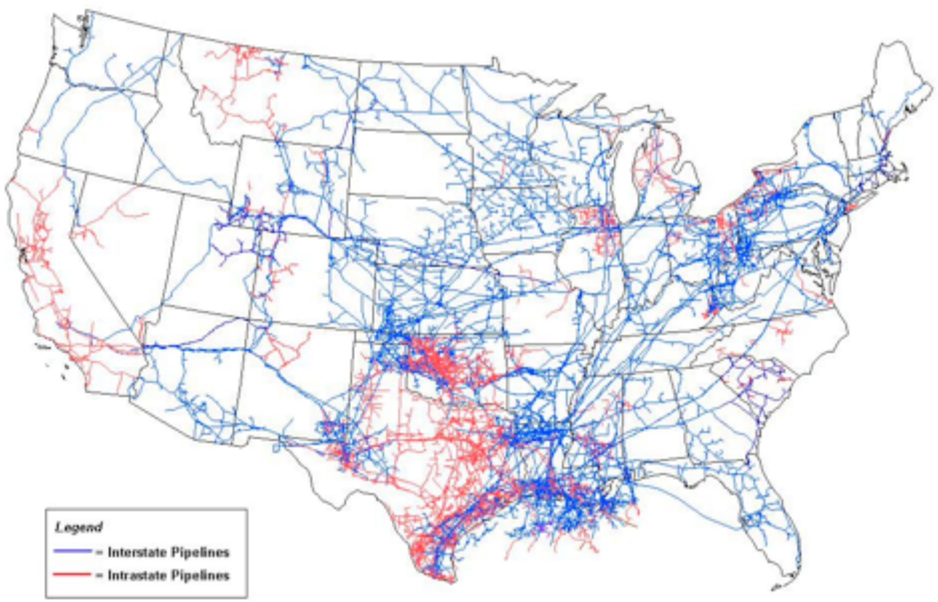
Corn ethanol CapEx ~$1.75/gallon

Cellulosic ethanol CapEx >$10/ gallon

Cost largely associated with biomass destruction, i.e., sugar production

<u>Cellulosic sugars are more expensive or valuable than commonly perceived</u>

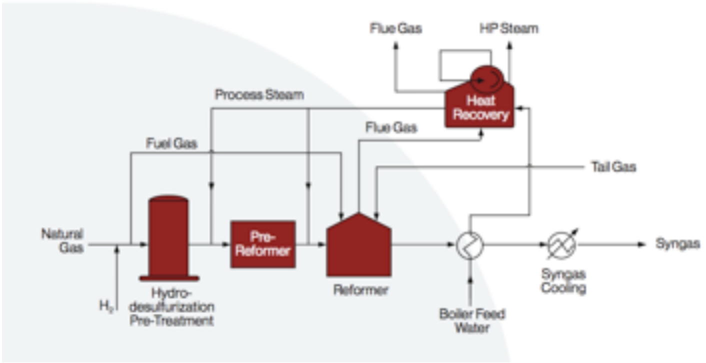
Industrial Gas Motivation for Mixotrophy

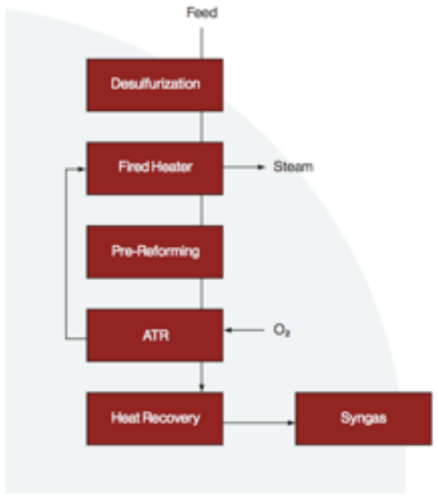


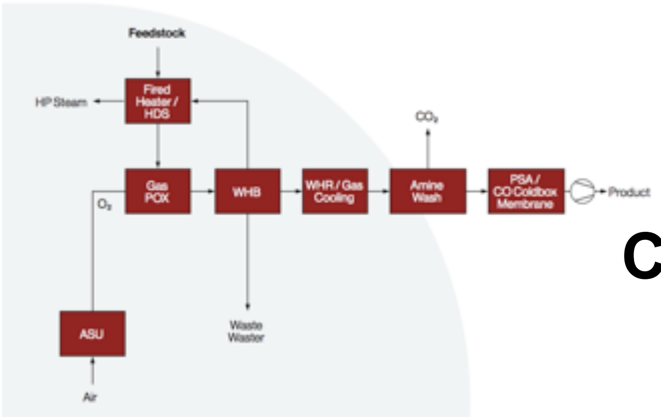
Source: Energy Information Administration, Office of Oil & Gas, Natural Gas Division, Gas Transportation Information System

Pipeline

$1 – 1.2/kg H_2







Co-located Production

<$1/kg H_2

~$1.1M/ 1,000$Nm^3$/hr

~$1,000$Nm^3$/hr H_2 / 1M gal EtOH

Industrial Gas Motivation for Mixotrophy - SCALE

1M gallons of annual ethanol production requires 1,000Nm^3/hr of H_2

Today
18 Billion Gallons Ethanol

6.5 billion bushels of corn

Potential
18 Billion Gallons Ethanol

4.3 billion bushels of corn
+
120 steam methane reformers

Industrial Gas Motivation for Mixotrophy - SCALE

2nd Generation Opportunity

20 Billion Gallons Ethanol

20B gallons * $10/gallon EtOH = <u>$200B in capital</u>

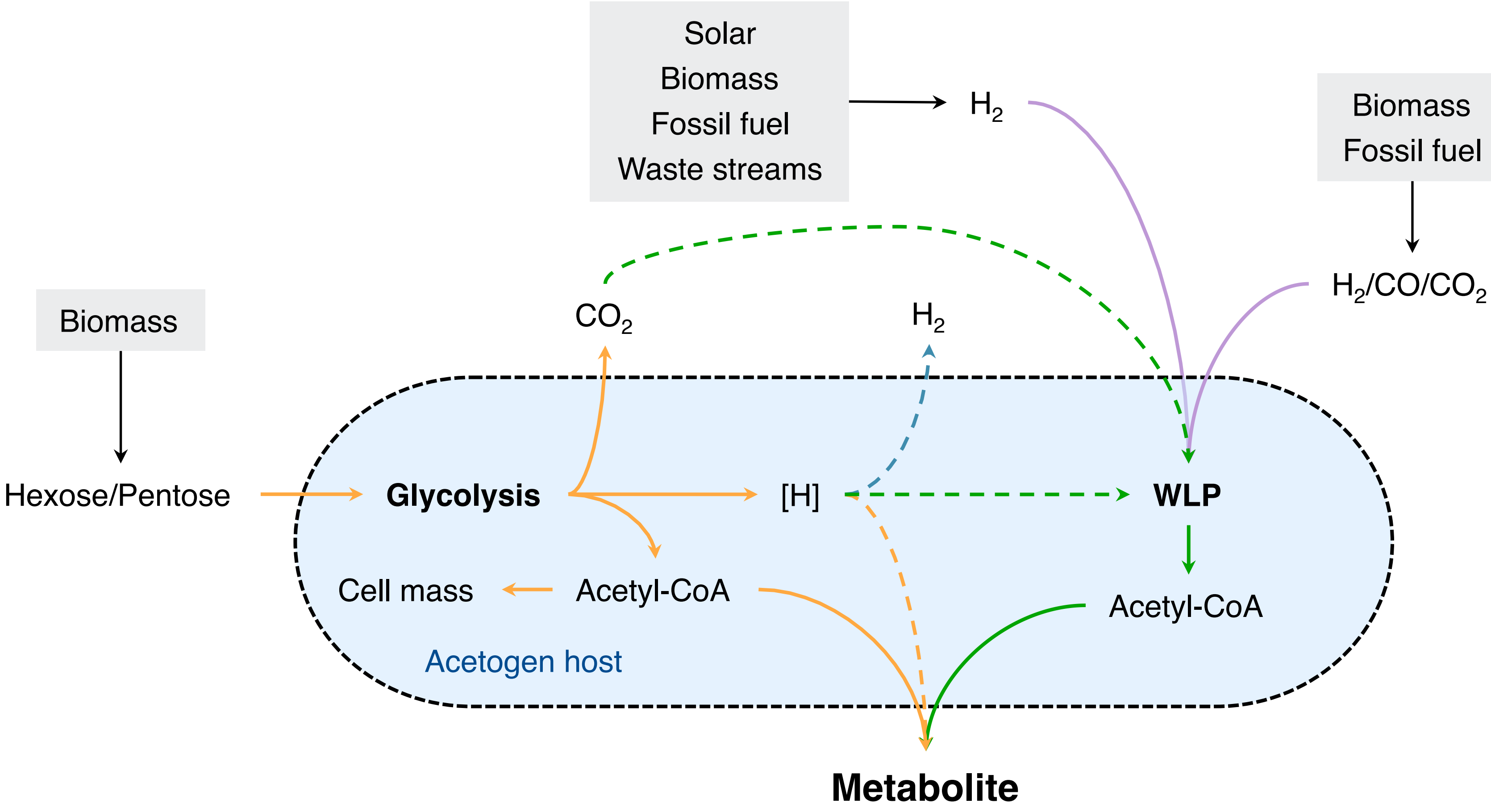
13.2B gallons * $10/gallon + 6.8B gallons * $1.1B/B gallon EtOH = <u>$139B in capital</u>

>30% reduction in capital required

&

Normalized for energy, H_2 comparable cost to glucose is $0.054/lb → recall, I claim starch can be $0.08/lb from corn

MixoFerm™ at its core (cellular level)



<u>MixoFerm™ with gas addition</u>

Gases can provide more reductant

Reducing equivalents [H] are used to fix CO_2 through the Wood-Ljungdahl Pathway (WLP)

Fast, AG, Schmidt, ED, Jones, SW, & Tracy, BT. 2015. *Curr Opin Biotechnol* 33:60-72.

Jones, SW, *et al.* 2016. *Nat Comm* 7:12800.

Energetic Requirements Comparison

Table 1

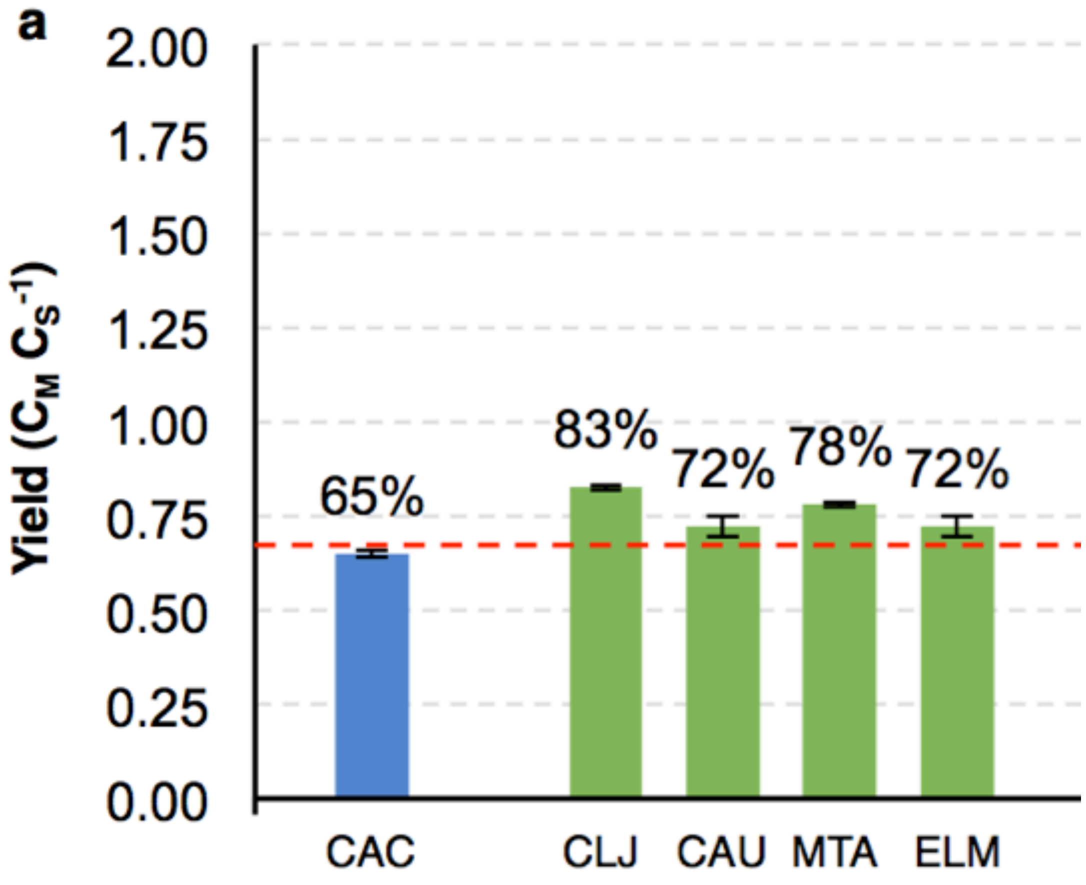
Energetic requirements of carbon fixation for acetyl-CoA production

Pathway	Energetic and electron requirements to fix one mole of acetyl-CoA			
	Mol NAD(P)H equivalent	Mol ATP	Total Mol H_2 (for reducing equivalents and ATP)	Number of enzymes
Reductive pentose phosphate	4	7	7.5	10
WL pathway	4	<1[a]	>4[b]	8
3HP/4HB cycle	4	6	7	13
Reductive TCA cycle	4	2	5	8

[a] Though one molecule of ATP is consumed in the WL pathway, some energy is conserved in the form of membrane gradients for ATP production. Thus, not quite one mole of ATP is consumed for every mole of acetyl-CoA that is produced.

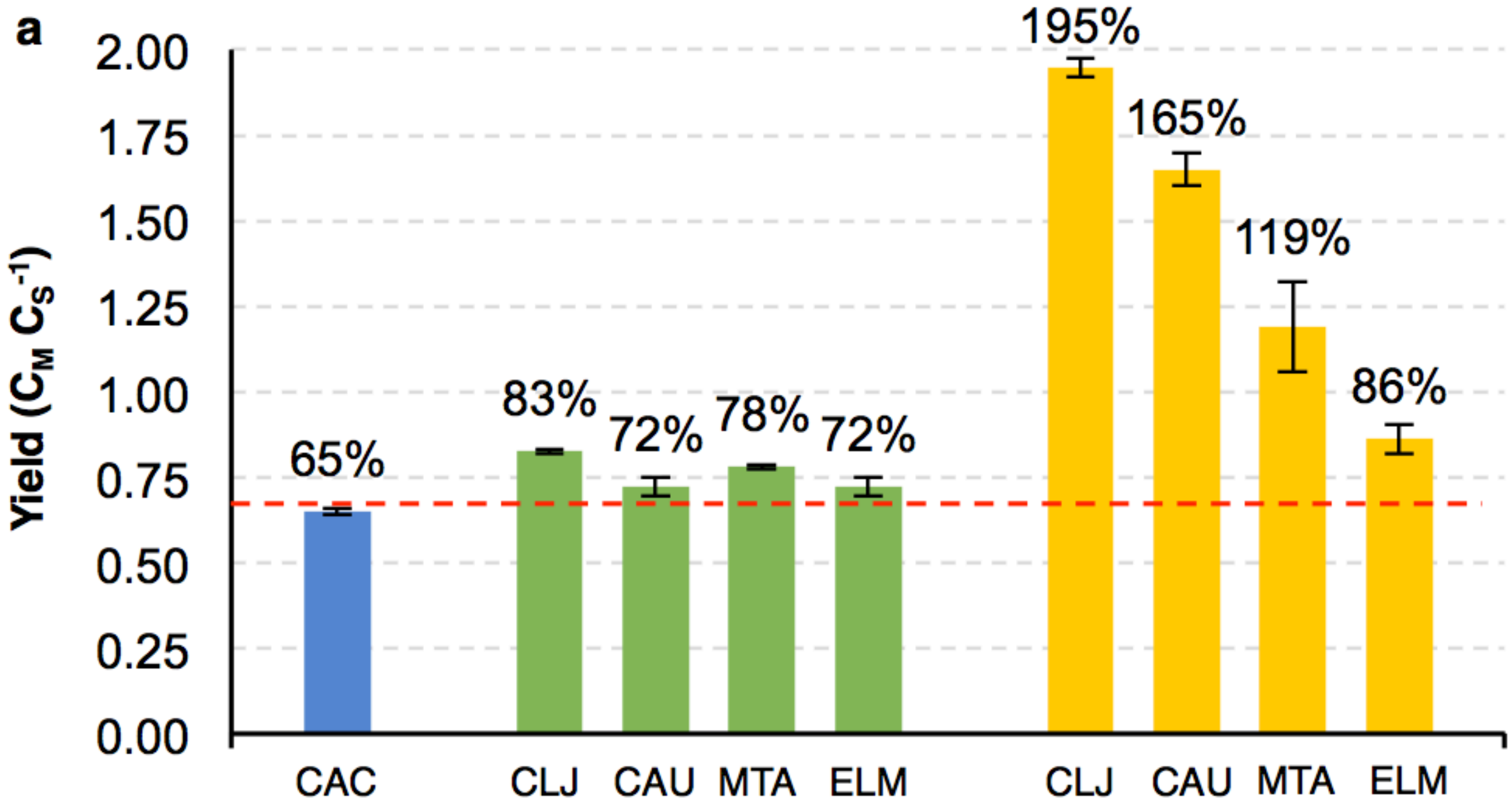
[b] In organisms with the WL pathway, H_2 cannot be directly oxidized to form additional ATP as shown in Eq. (2). Instead, a fermentation product (i.e. acetate or ethanol) is needed to recover the ATP deficit from acetyl-CoA production.

Demonstration of MixoFerm



Conventional **MixoFerm™**

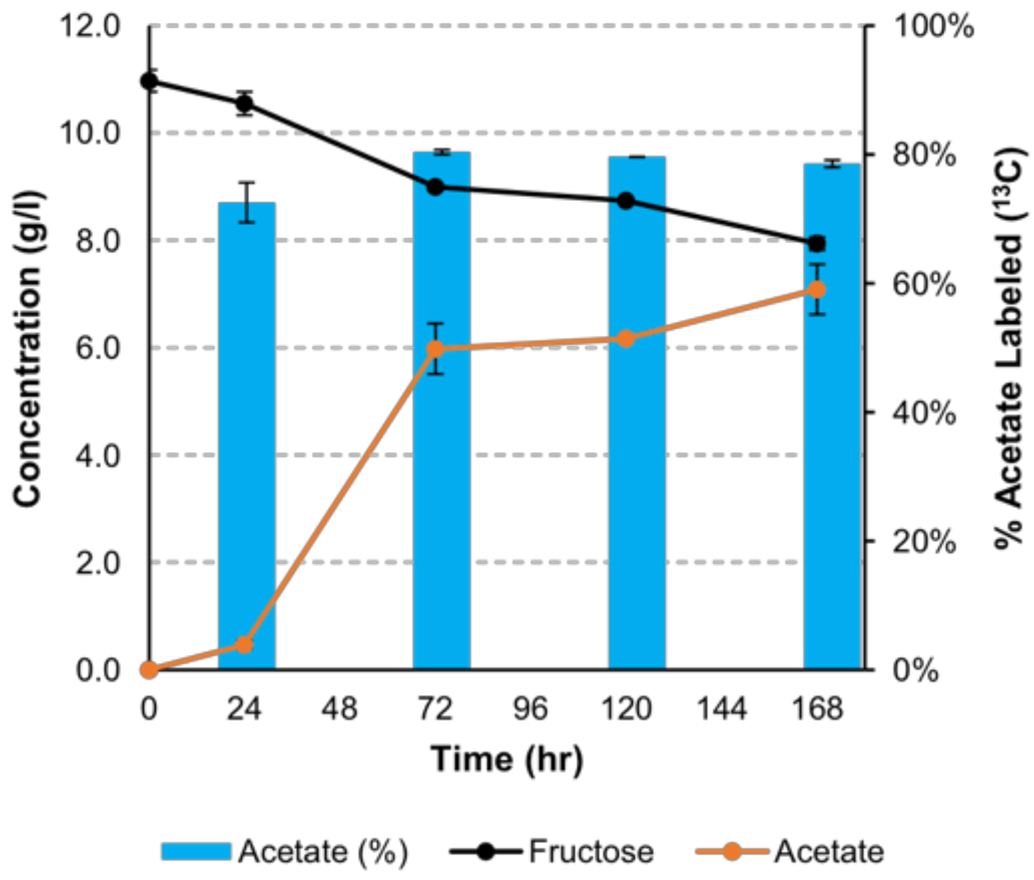
Demonstration of MixoFerm Plus



Conventional **MixoFerm™** **MixoFerm™ Plus Syngas**

Broad host range, which we have since curated >20 unique MixoFerm strains

MixoFerm – Catabolite Repression?



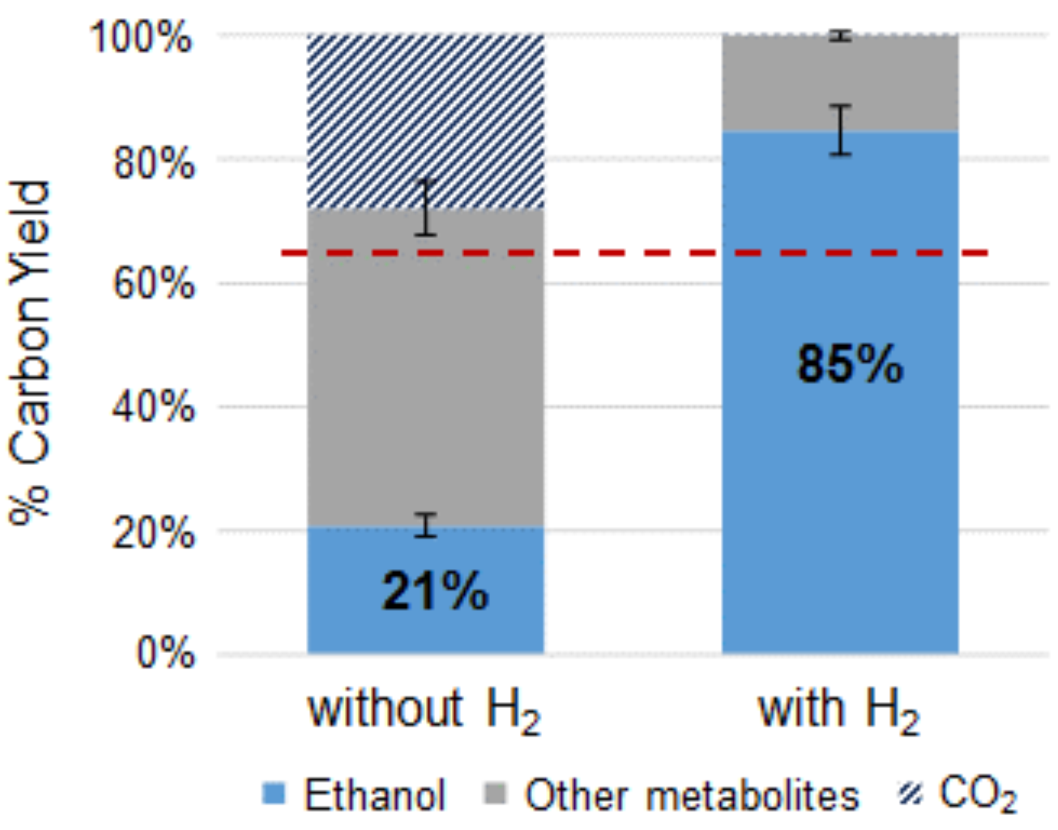
Grew *Clostridium ljungdahlii* on ^{12}C fructose and ^{13}C syngas mixture. Any ^{13}C-labeled metabolite had to come from syngas utilization.

Syngas is consumed simultaneously with fructose at high incorporation levels – actually preferred

Jones, SW, *et al*. 2016. *Nat Comm* 7:12800.

H_2 MixoFerm Plus for CO_2 Capture & EtOH

H_2 addition to *C. autoethanogenum*



- Effectively captured all CO_2
- Shifted metabolism to reduced product formation
- Achieved ~65wt% yield of EtOH on consumed fructose

Benefits of Mixotrophy

Key ratio to benefits of MixoFerm™ is:

NAD(P)H to Acetyl-CoA

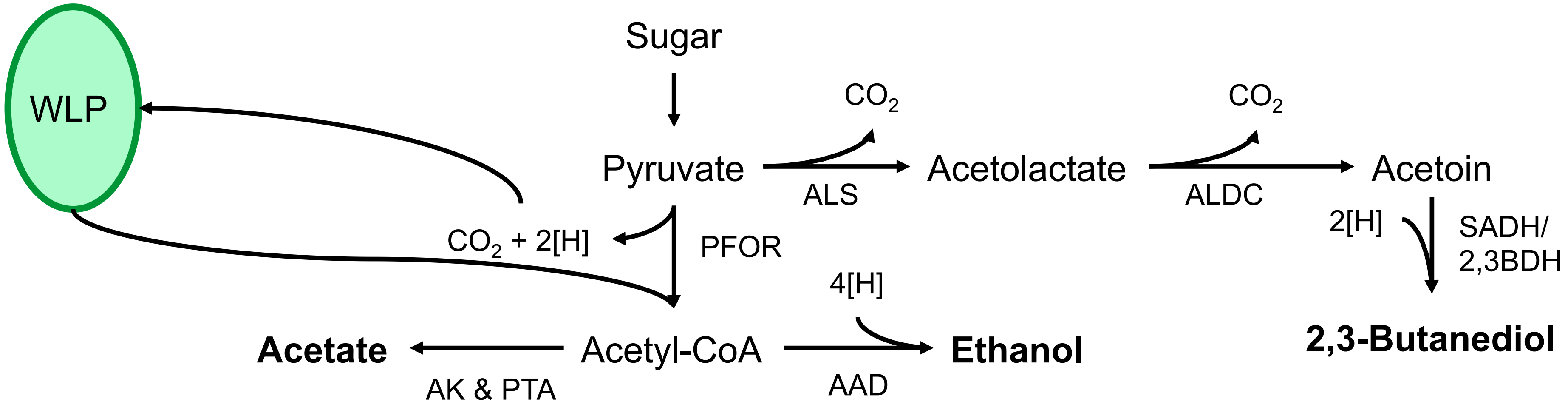
Focus of first demonstration of MixoFerm™

NAD(P)H/Acetyl-CoA ratio	Increase in yield of MixoFerm™ over standard fermentation	Example molecules
0	51%	Acetic acid or Acetone
0.5	35%	Isopropanol
1.0	22%	Butyric acid
1.5	11%	2,3-Butanediol
2	2%	Ethanol or n-Butanol
3	0%	Propionic acid

Acetone Production

No known acetogen naturally produces **acetone.**

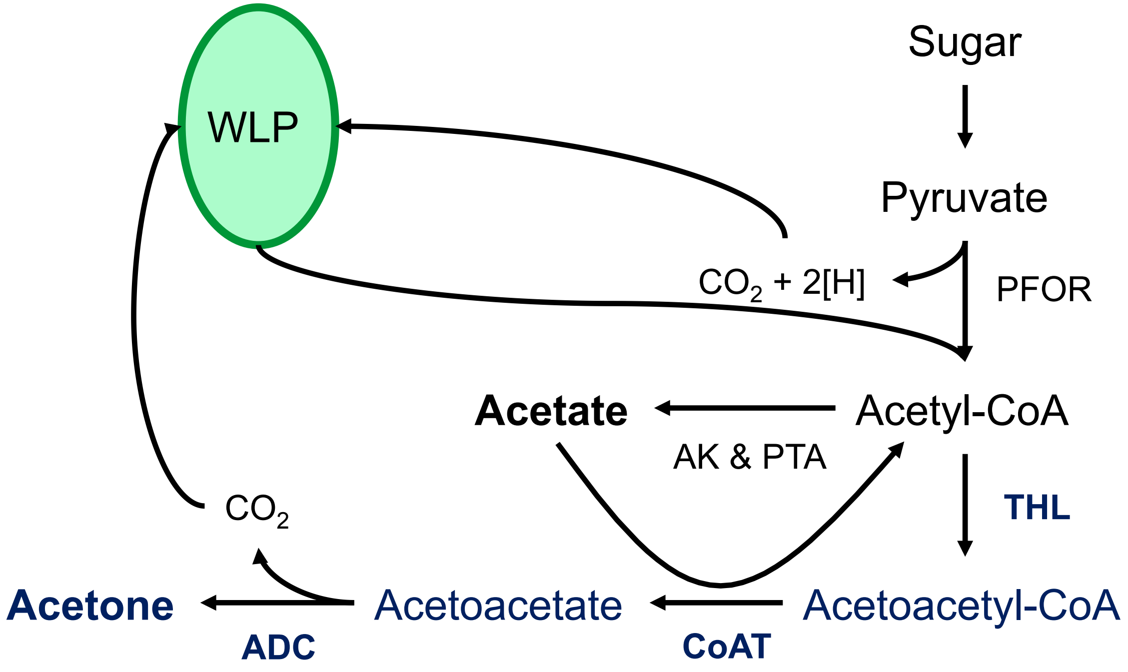
Acetogen host: *C. ljungdahlii*

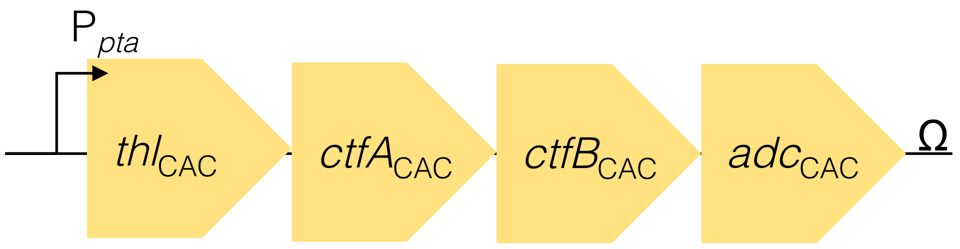


Köpke, M., et. al. 2014. *Appl Environ Microbiol* 80:3394-3403.

Acetone Production

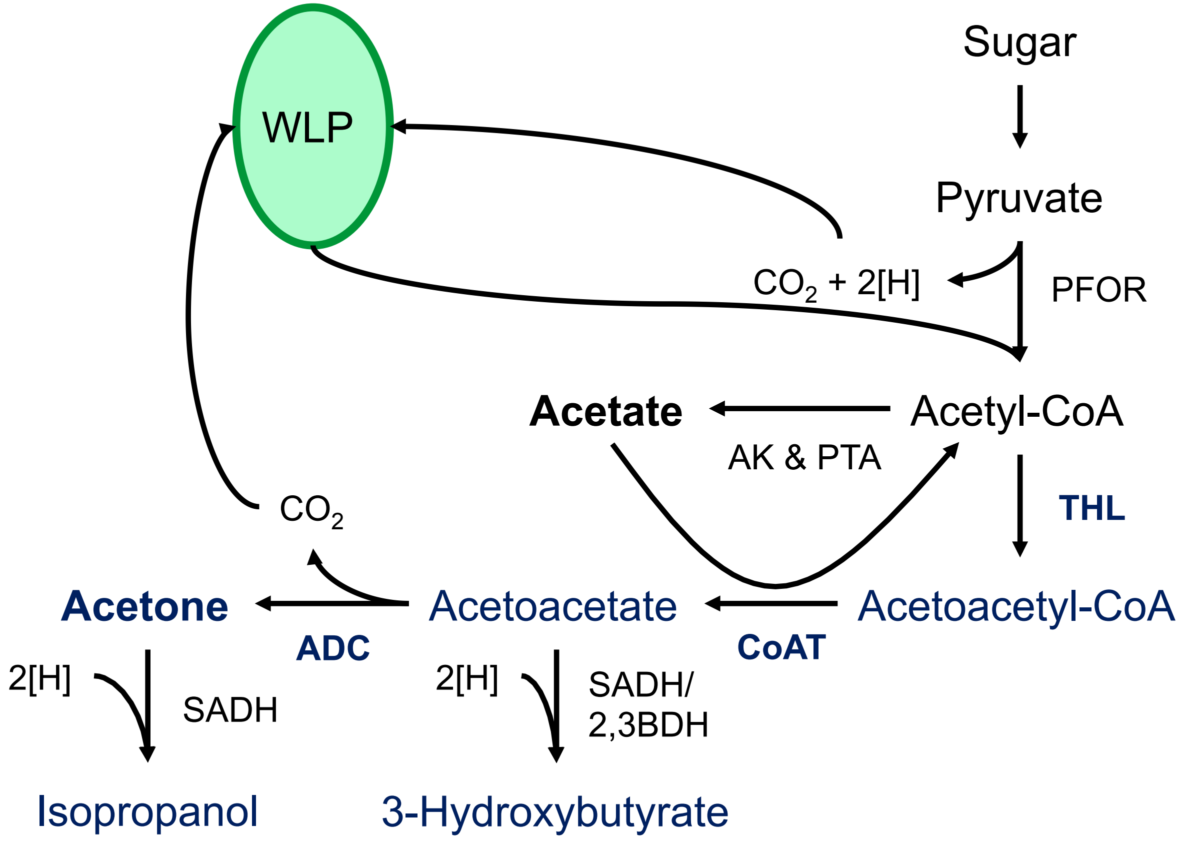
Acetogen host: *C. ljungdahlii*

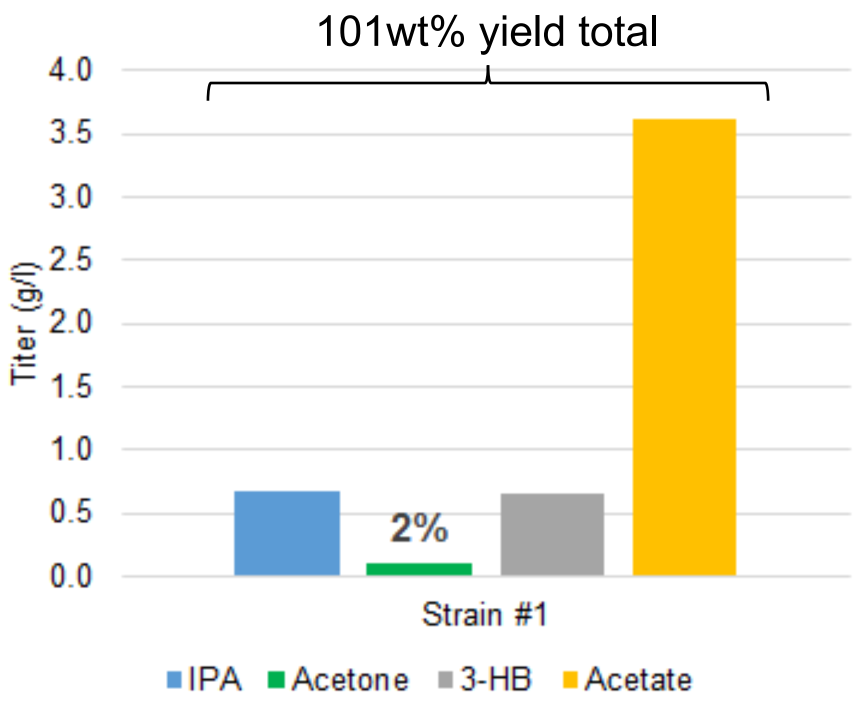




Acetone Production

Acetogen host: *C. ljungdahlii*

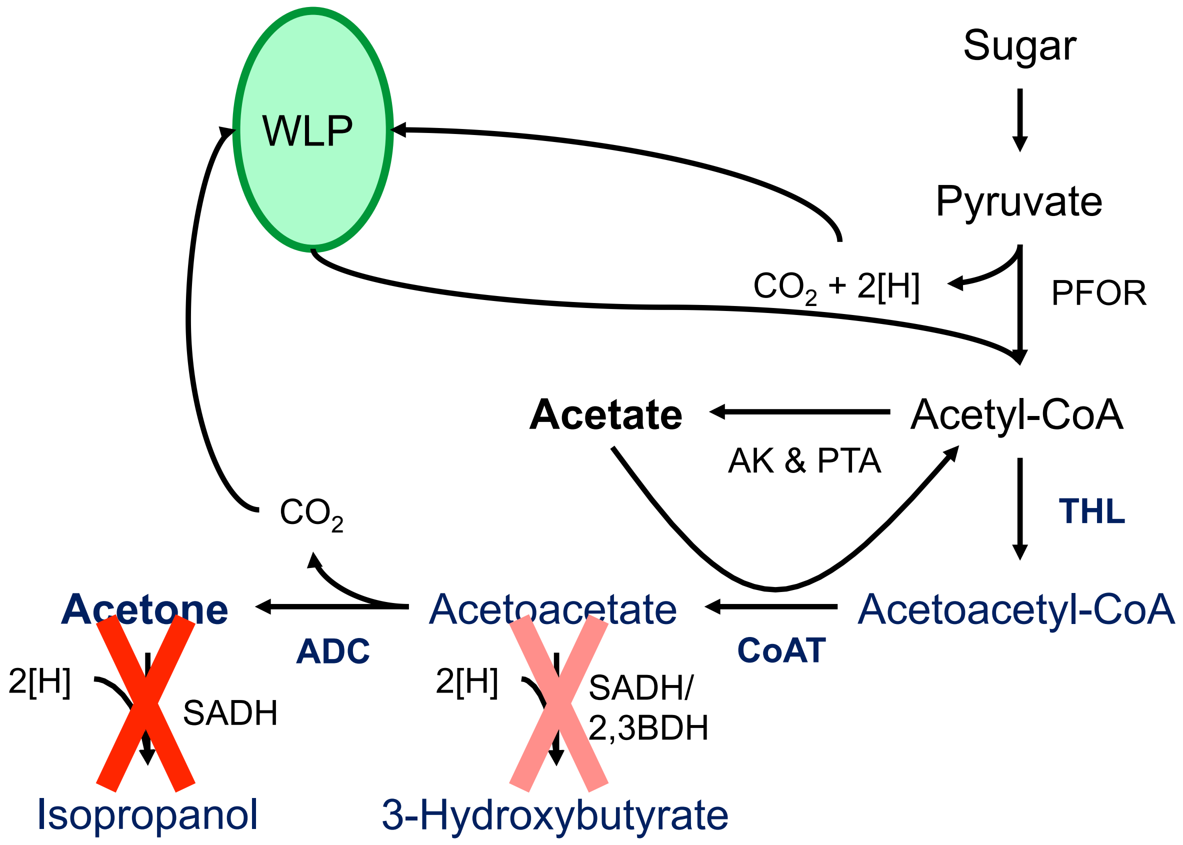


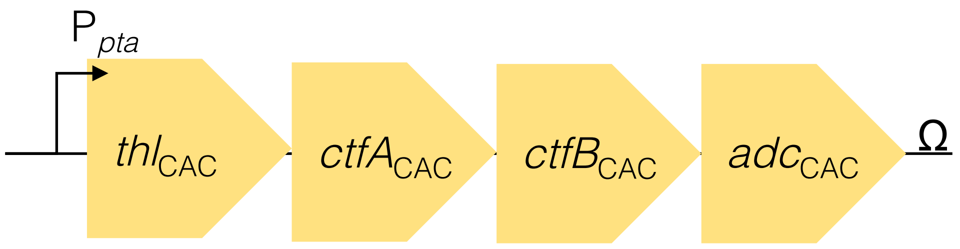


Strain #1 largely produced side-products

Acetone Production

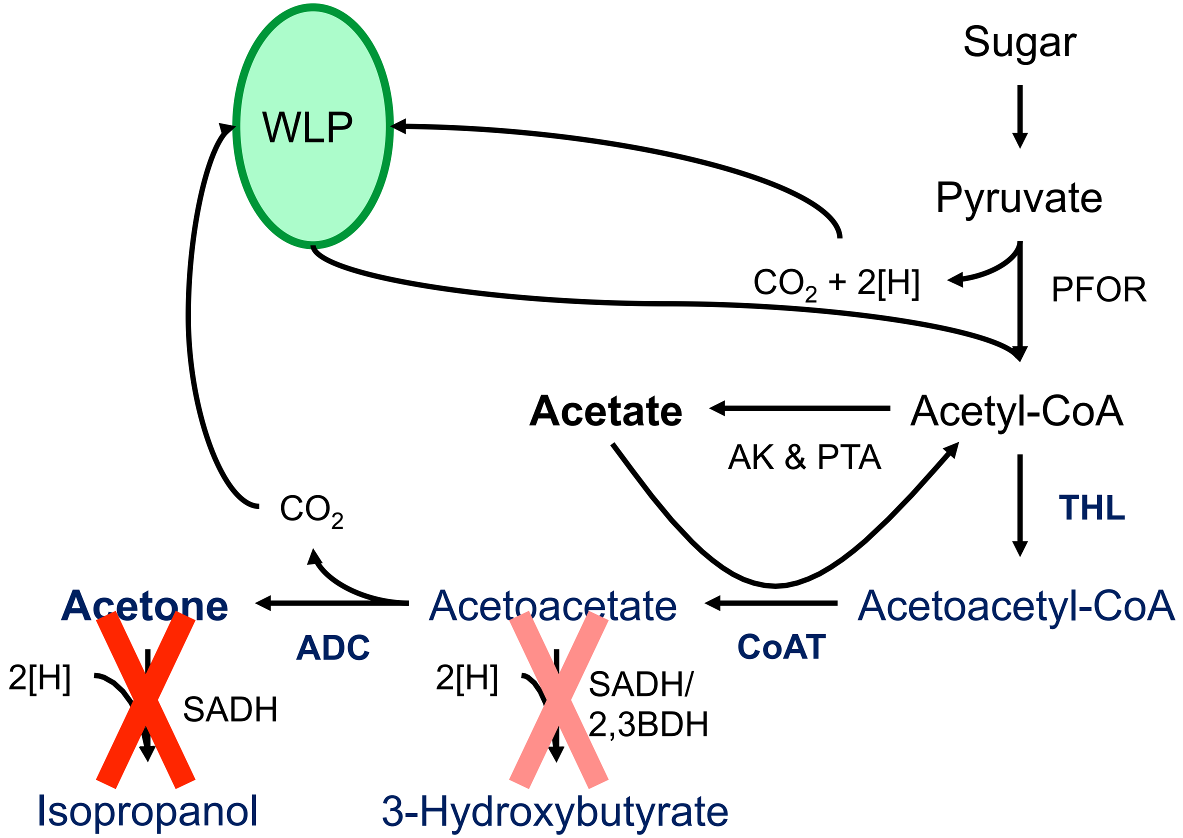
Acetogen host: *C. ljungdahlii*

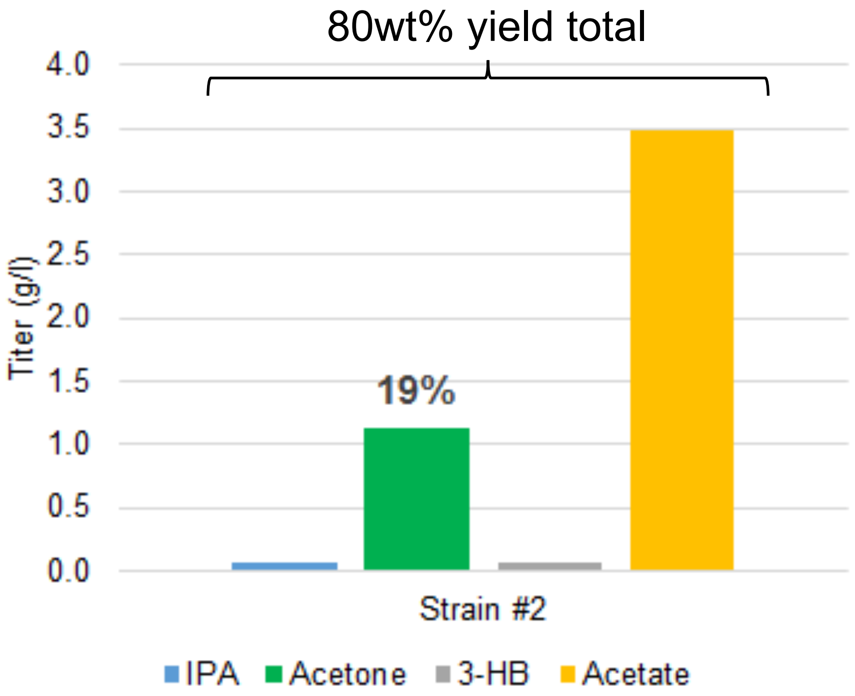




Acetone Production

Acetogen host: *C. ljungdahlii*

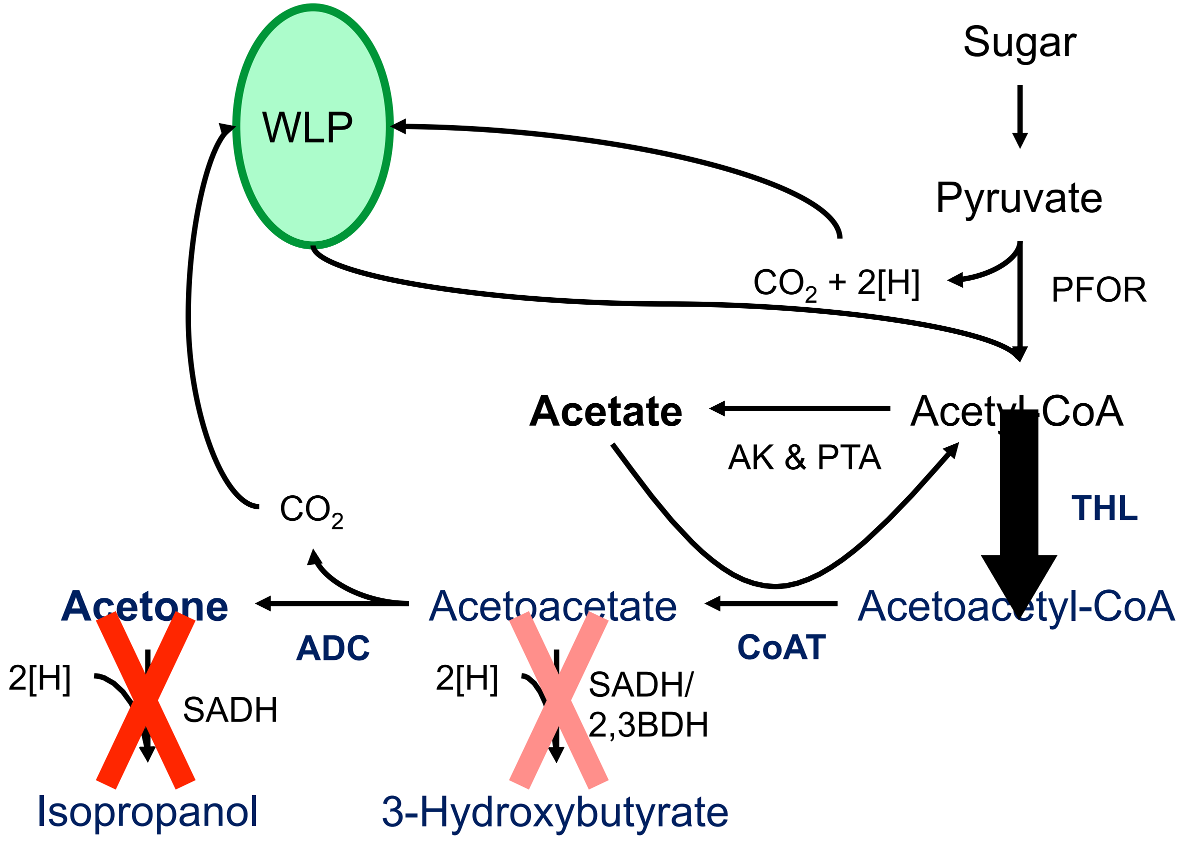


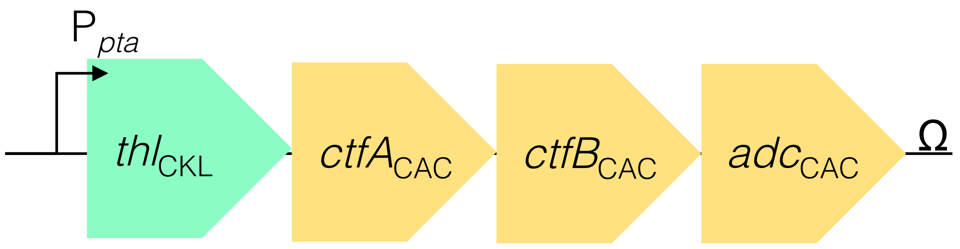


Strain #2 mostly eliminated side-products

Acetone Production

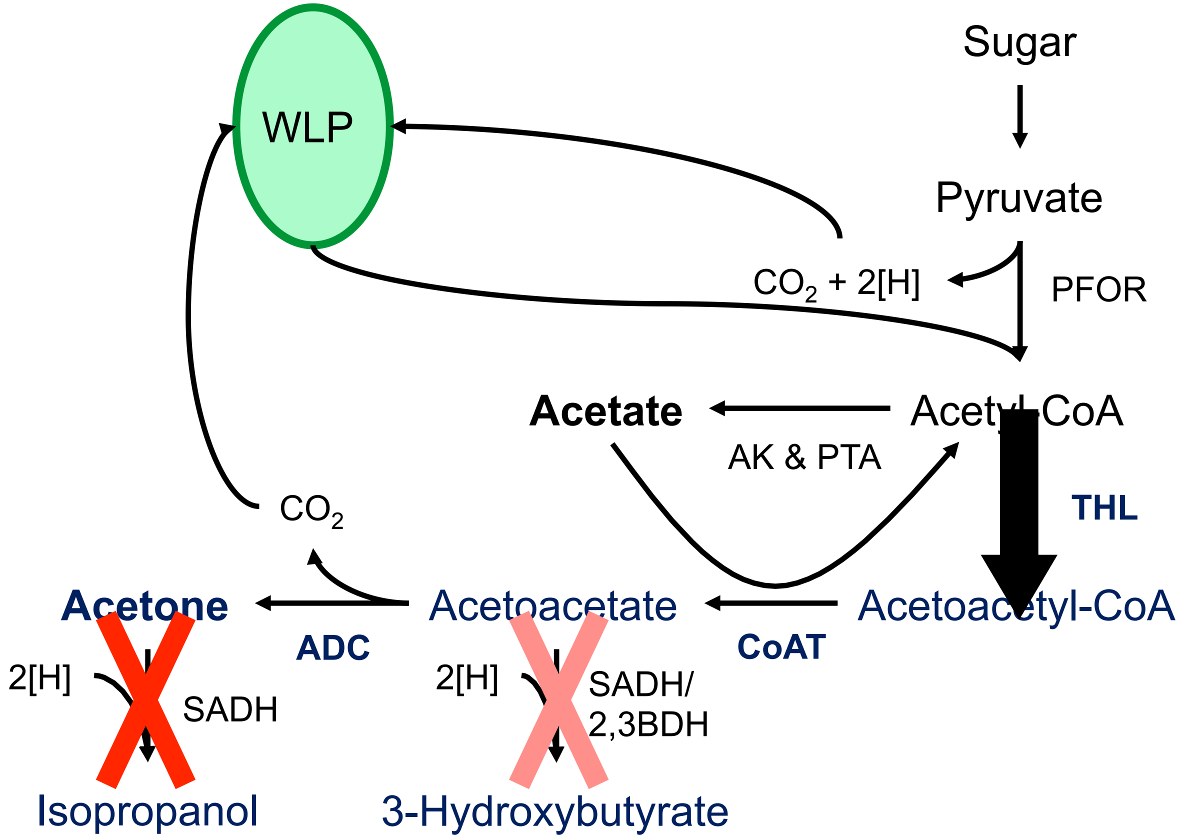
Acetogen host: *C. ljungdahlii*

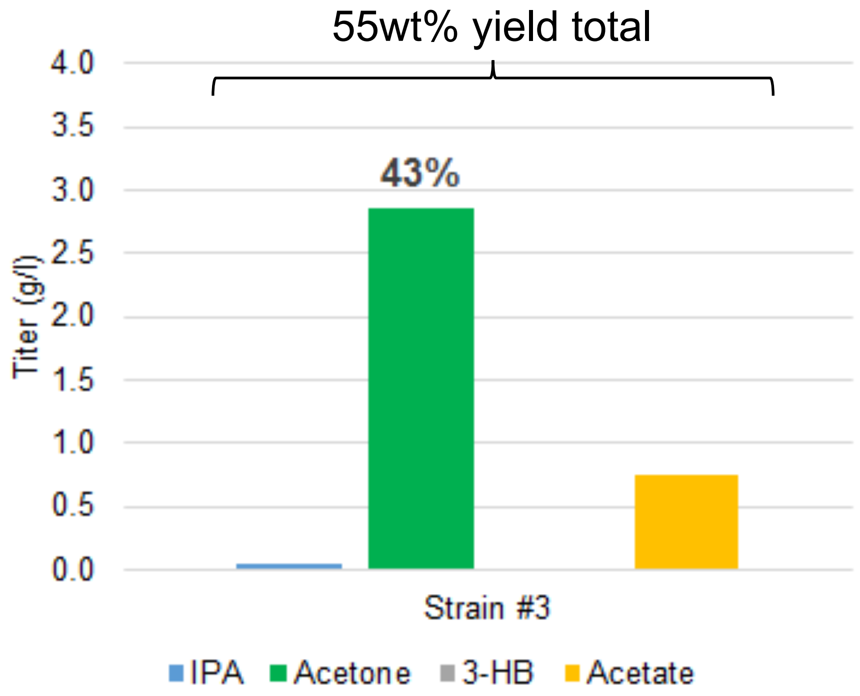




Acetone Production

Acetogen host: *C. ljungdahlii*





Strain #3 produced acetone >95% of theoretical maximum

Jones, SW, *et al*. 2016. *Nat Comm* 7:12800.

Batch Fermentation

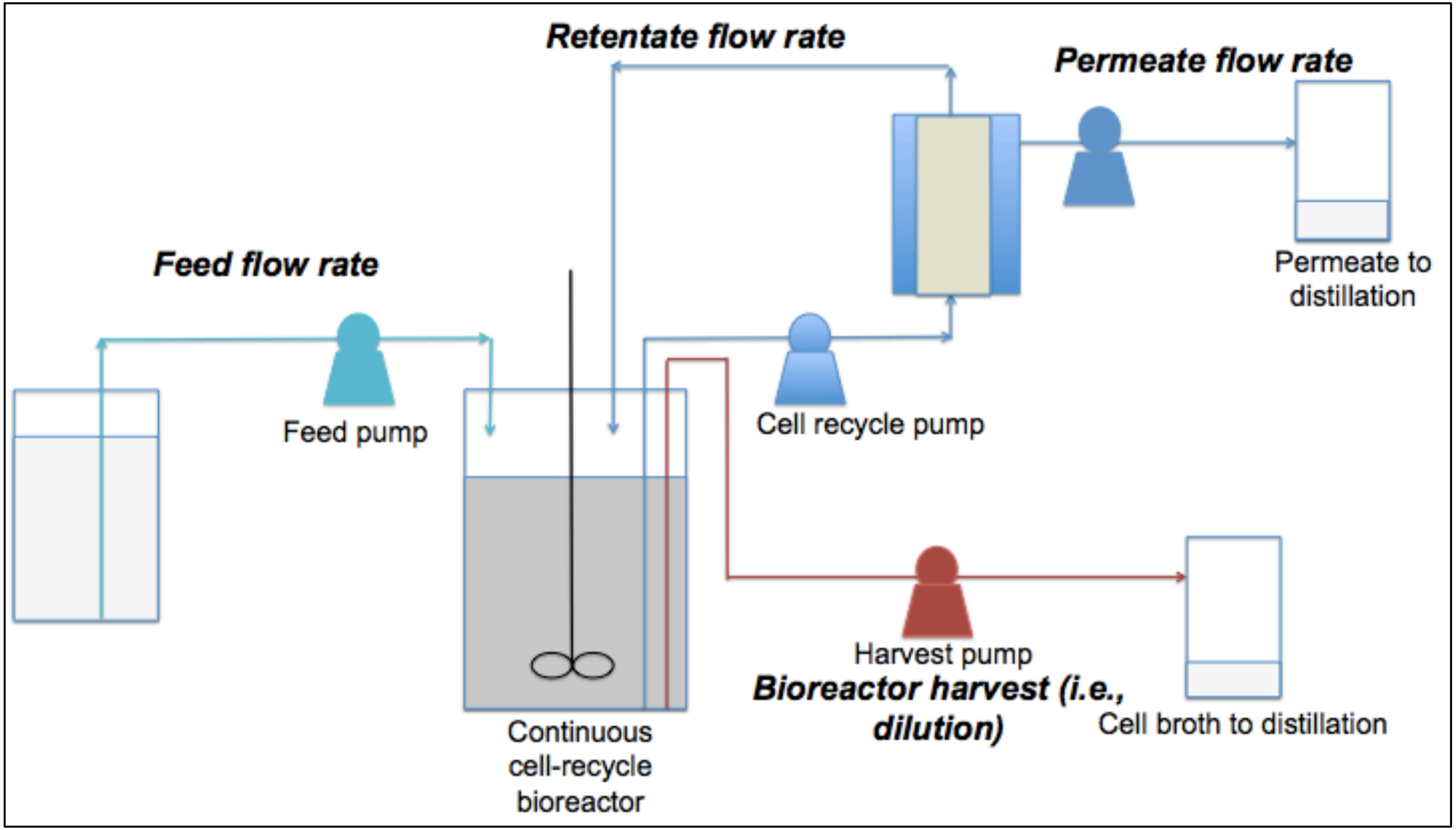
<u>Strain #3</u>
C. ljungdahlii ΔSADH (pTCtA-Ckl)

Batch fermentation on fructose

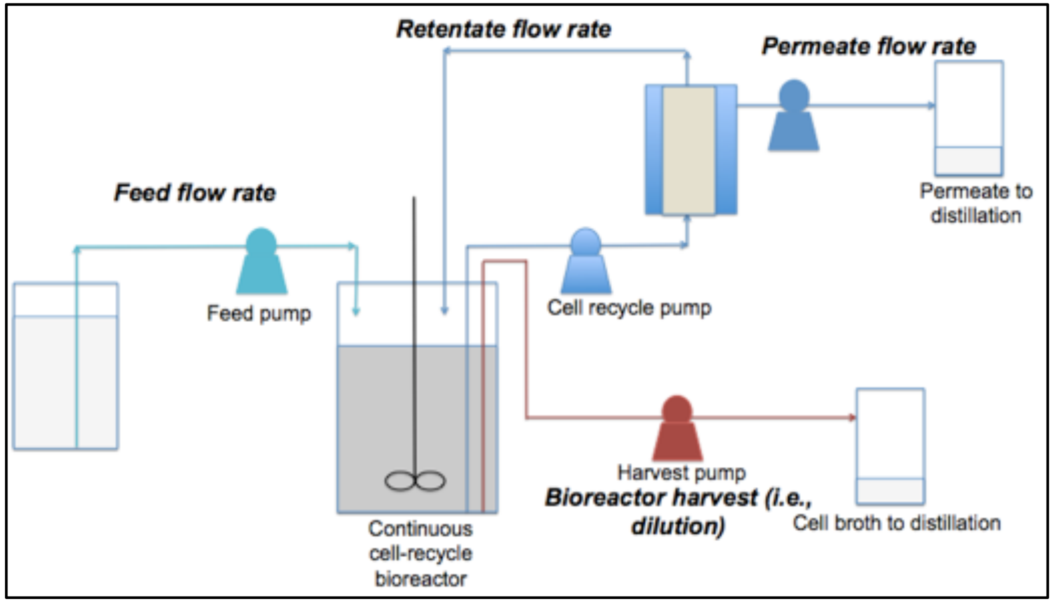
Parameter	Batch fermentation
Yield (wt%)	43
Productivity (g/L/hr)	0.05
Titer (g/L)	6

Productivity too low for commercial production

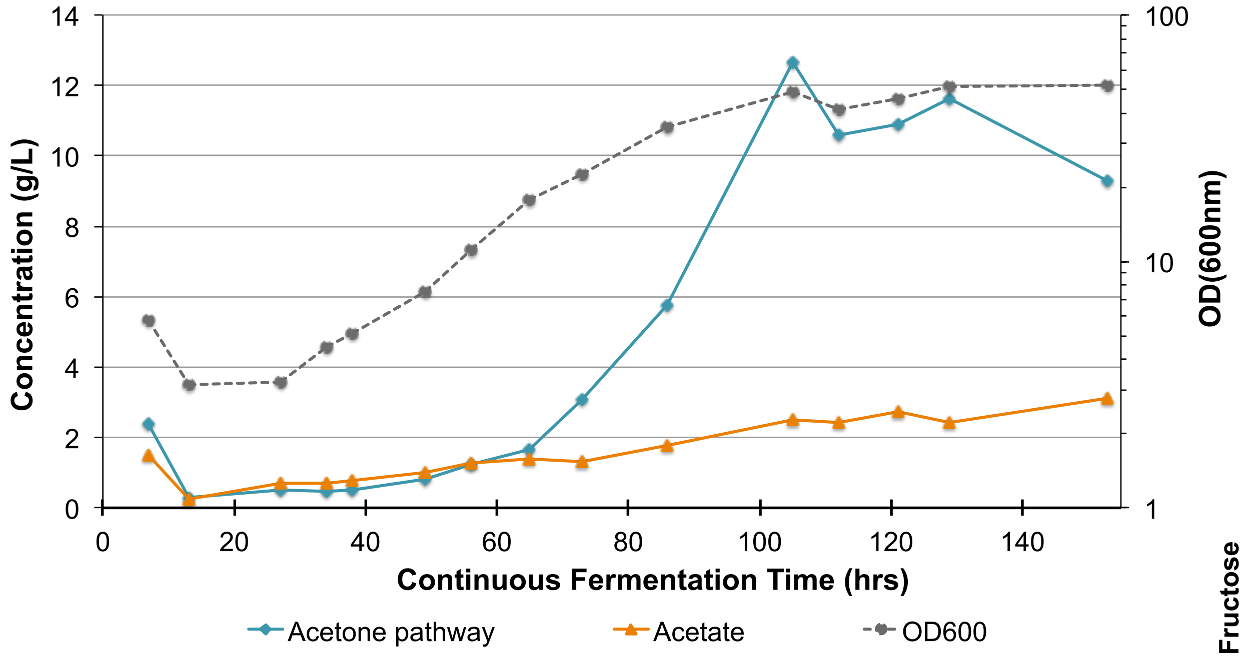
Continuous Fermentation



Continuous Fermentation



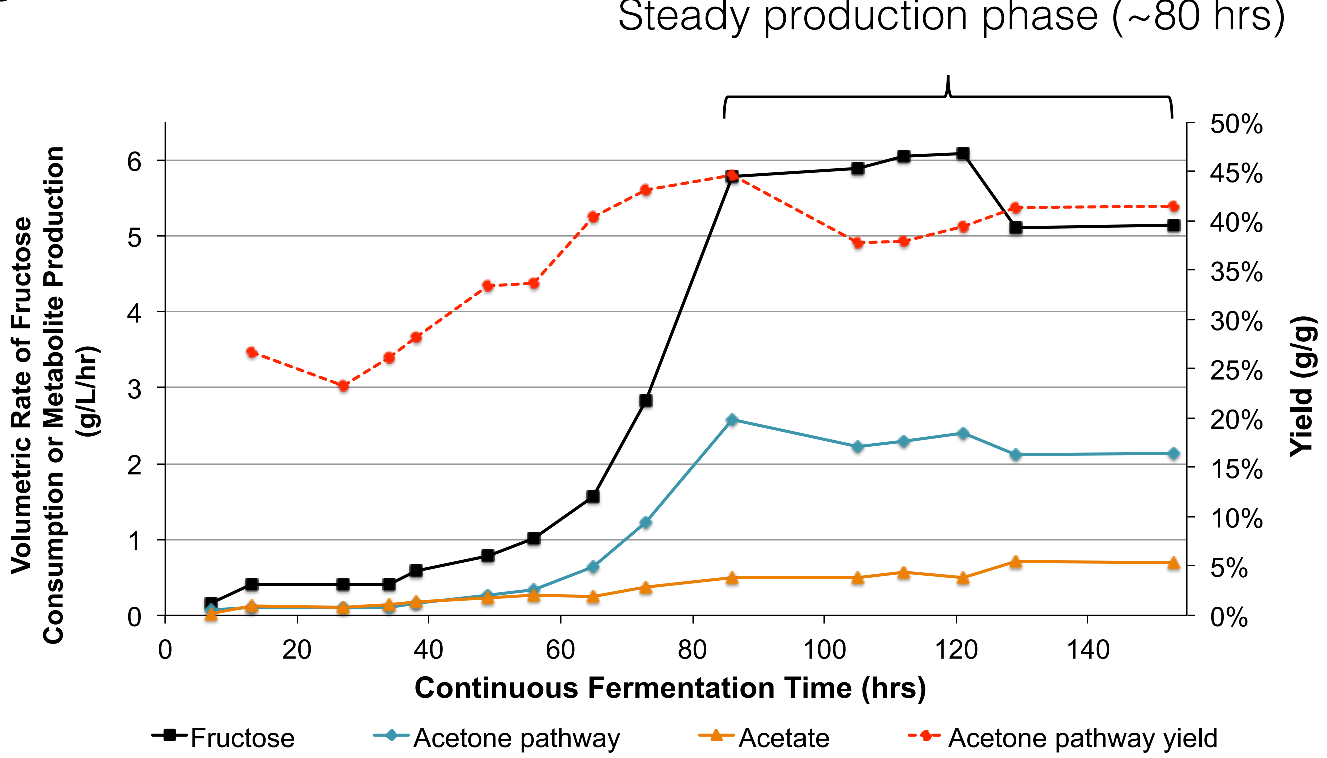
Continuous Fermentation



Substrate was **Fructose**

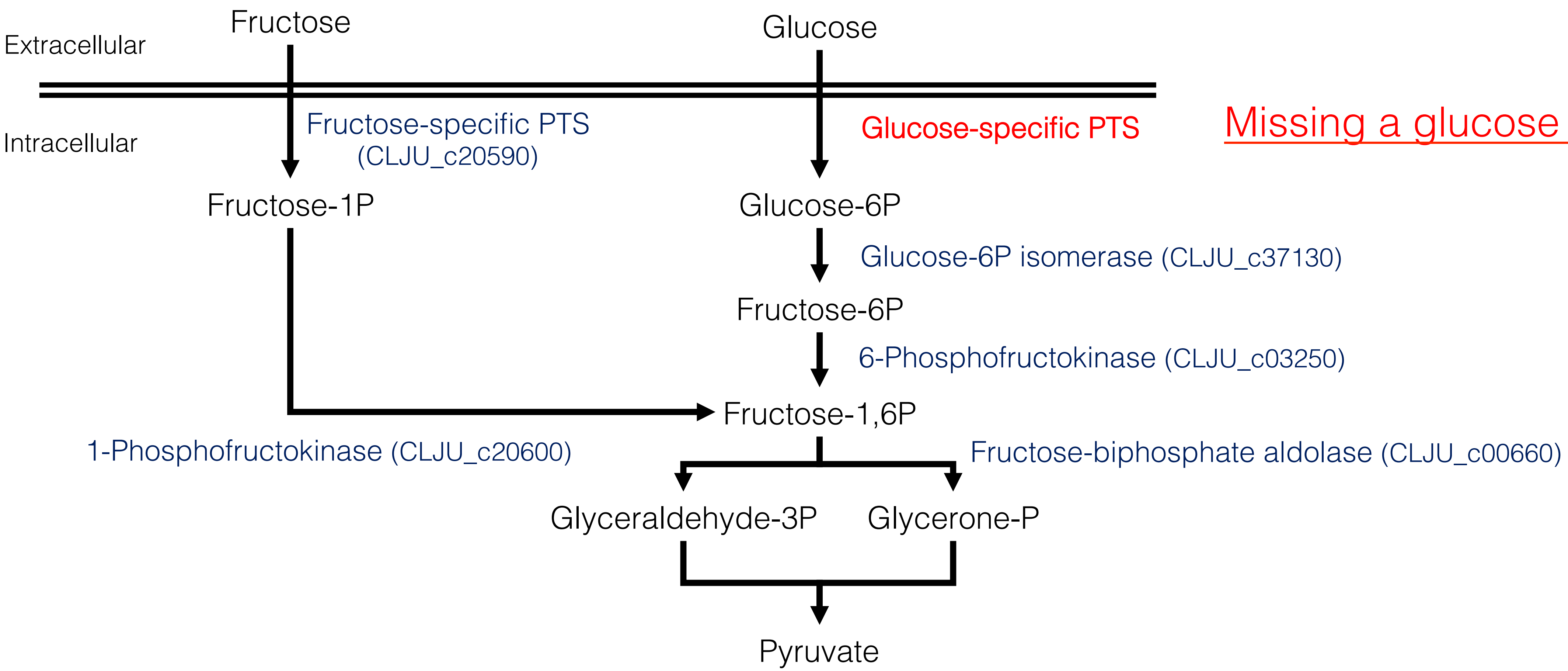
Acetone pathway products:

- Acetone – 11 g/L
- IPA – 1 g/L
- 3-HB – 1 g/L



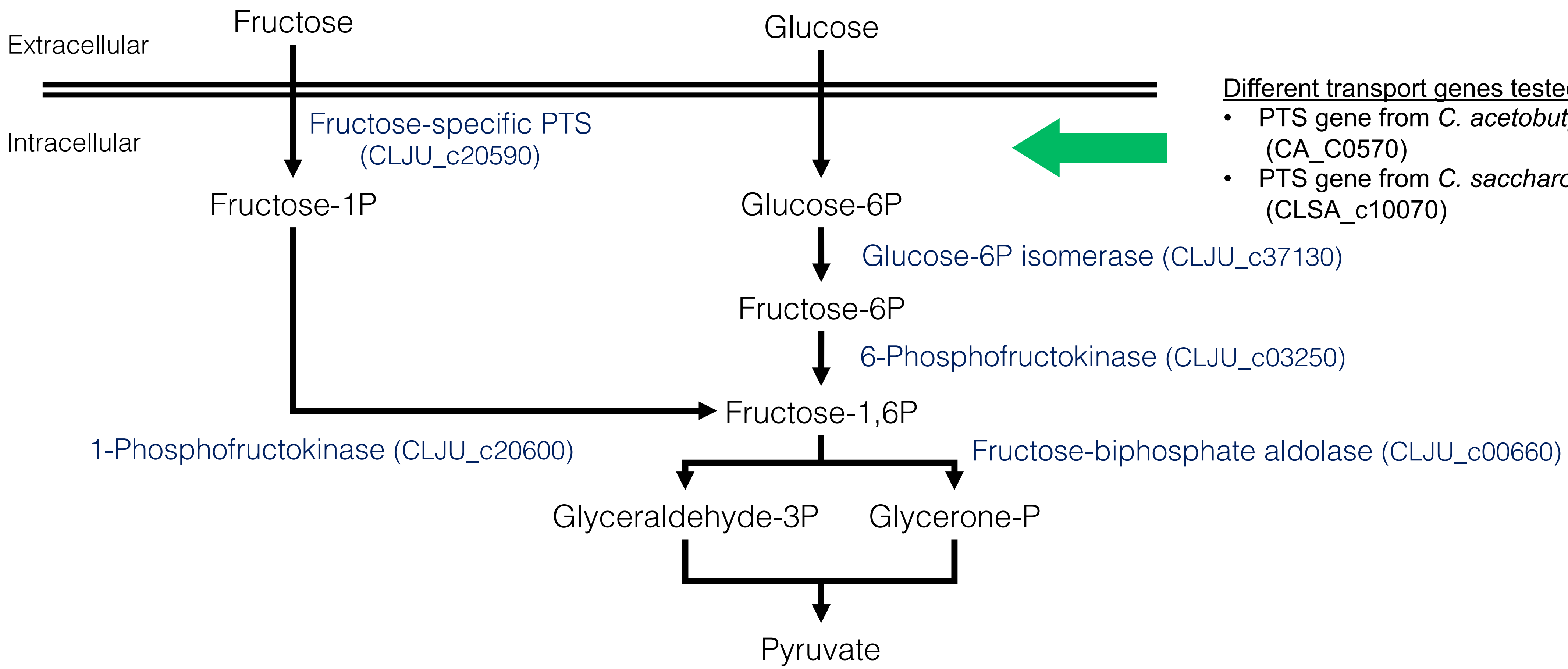
Jones, SW, *et al*. 2016. *Nat Comm* 7:12800.

Glucose Utilization



Missing a glucose transport system

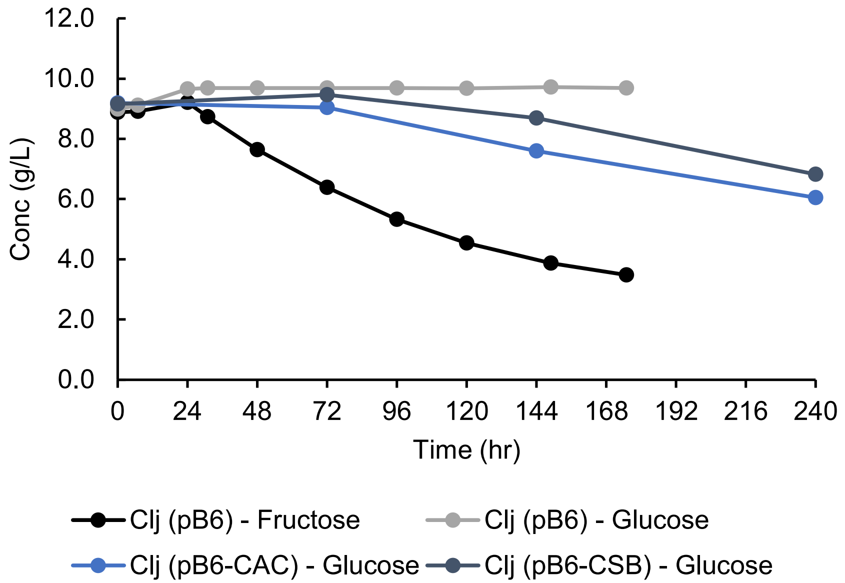
Glucose Utilization



<u>Different transport genes tested:</u>

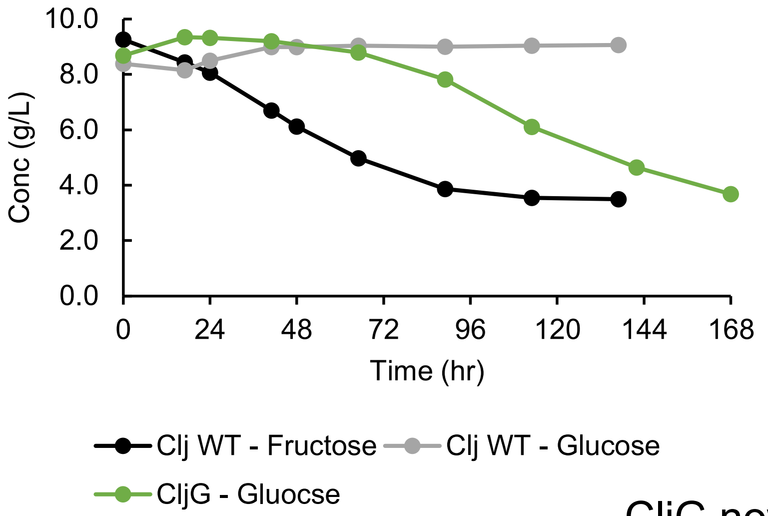
- PTS gene from *C. acetobutylicum* (CA_C0570)
- PTS gene from *C. saccharobutylicum* (CLSA_c10070)

Glucose Utilization



Plasmid expression enabled glucose utilization, though at a significantly slower rate.

Gene from *C. saccharobutylicum* was integrated into the genome and the strain was adaptively evolved in a chemostat



WT fructose consumption rate: 0.074 g/L/hr

CljG glucose consumption rate: 0.056 g/L/hr

CljG now consumes **glucose at a rate 75%** of fructose rate.

Enabled glucose utilization through engineering and evolution

Other Substrate Utilization

		Carbohydrate										
	Strain	Glucose	Fructose	Mannose	Galactose	Xylose	Arabinose	Ribose	Sucrose	Cellobiose	Maltodextrin	Glycerol
Mesophilic	*Acetobacterium carbinolicum*	+[a]	++[b]	-[c]	+	−	+	+	+	−	+	−
	Blautia producta	+	+	+	+	+	+	+	+	+	−	+
	Clostridium autoethanogenum	−	++	−	−	++	++	+	−	−	−	−
	Clostridium drakei	++	++	+	−	++	++	−	++	++	−	++
	Clostridium ljungdahlii	−	++	−	−	++	++	+	−	−	−	−
	Clostridium magnum	+	+	+	+	+	+	++	+	+	−	+
	Clostridium scatologenes	++	++	+	+	++	++	−	++	−	−	++
	Eubacterium aggregans	+	+	−	−	−	−	−	+	−	+	−
	Eubacterium limosum	++	++	−	+	+	−	−	++	−	+	++
	Sporomusa ovata	−	+	−	−	−	+	−	−	+	−	−
	Sporomusa termitida	+	+	+	+	−	−	+	++	+	+	++
	Treponema azotonutricium	+	+	+	+	+	+	+	+	+	+	+
	Terrisporobacter glycolicus	+	+	−	+	+	−	−	−	−	+	+
Thermophilic	*Moorella thermoacetica*	+	+	−	−	+	−	−	+	−	−	−
	Moorella thermoautotrophica	+	+	−	−	+	−	+	−	−	−	−
	Moorella glycerini	+	+	+	+	−	−	−	−	−	−	+
	Thermoanaerobacter kivui	+	+	+	−	−	−	−	−	−	−	−

Various Gas Substrate Utilization

	Gas compositions and pressures							
	$CO_2:H_2:N_2$ (30:50:20)		$CO:H_2:N_2$ (30:50:20)		$CO:N_2$ (40:60)		$CO:H_2:CO_2:N_2$ (55:20:10:15)	
Strain	10 psig	30 psig	10 psig	30 psig	10 psig	30 psig	10 psig	30 psig
Blautia producta	++[a]	++	+[b]	+	+	+	++	+
Clostridium autoethanogenum	–[c]	+	+	+	+	++	+	+
Clostridium drakei	++	++	++	++	+	+	++	+
Clostridium ljungdahlii	+	++	–	++	+	–	–	++
Clostridium scatologenes	+	++	+	+	++	+	++	++
Eubacterium aggregans	–	++	–	–	–	–	–	–
Eubacterium limosum	+	+	+	++	++	++	++	+
Terrisporobacter glycolicus	+	+	–	–	–	–	–	–
Thermoanaerobacter kivui	+	+	–	–	+	+	+	+

Summary

- Clostridial mixotrophy works – evolution did us a favor
- Robust opportunity to couple known gas production technology from fossil sources with known fermentation technology
- New industries are motivated and will serve an important, strategic role in development, scale-up and commercial deployment

CHALLENGES!

- Gas delivery in MixoFerm Plus – plug and play with Lanzatech experience?
- Optimal gas composition based on economics and preference of organisms
- Capital efficient solutions for renewably source H_2 or syngas mixtures
- Improved –omics and organism engineering tools

Acknowledgements

Entire WDL team

University of Delaware collaborators:
- Ellinor Carlson
- Alan Fast
- E. Terry Papoutsakis
- Jennifer Au
- Maciek Antoniewicz

Air Liquide collaborators:
- Ilana Aldor
- M Sundar