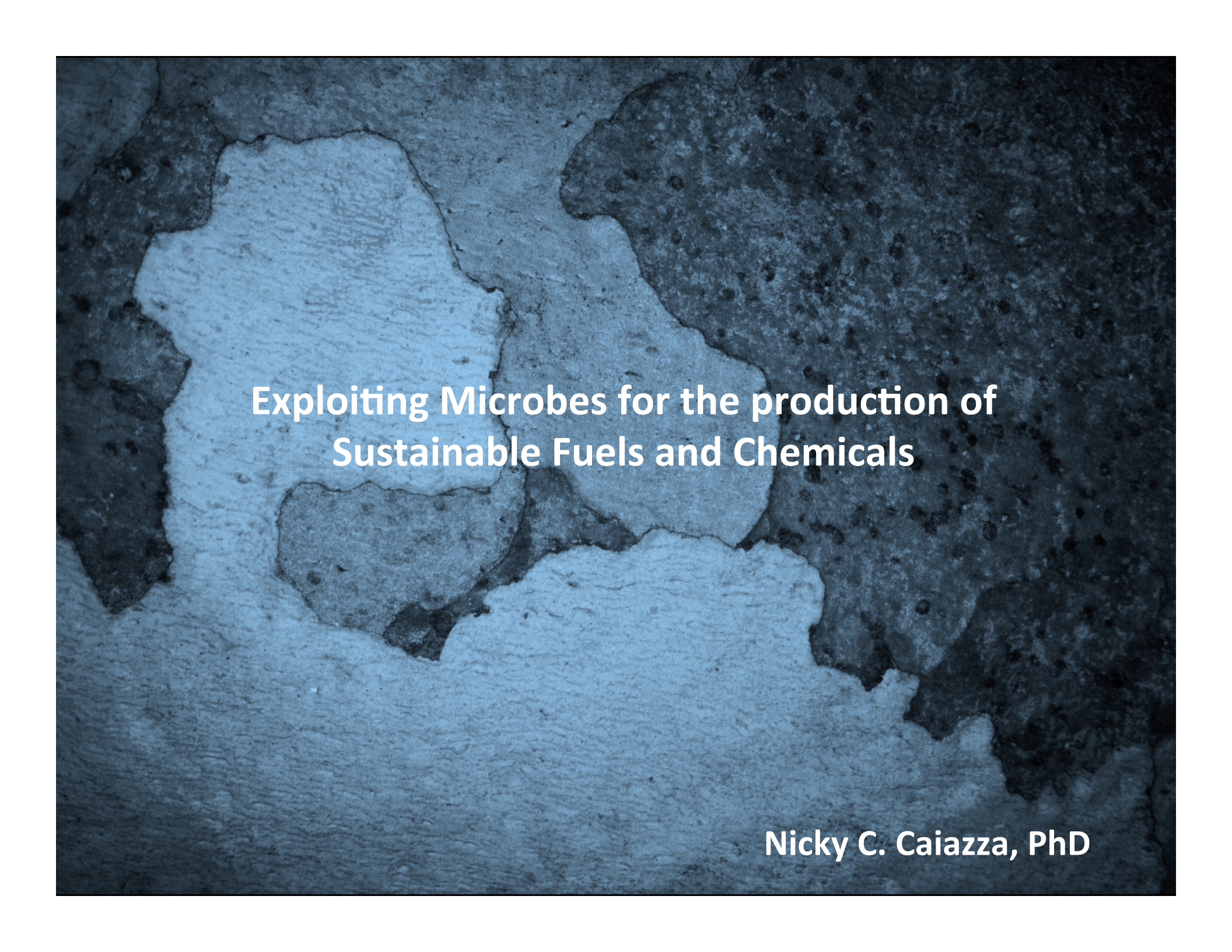


A microscopic image showing various microbial mats or colonies. The image has a blue tint and shows irregular, textured patches of different shades of blue and grey, representing different microbial communities or structures.

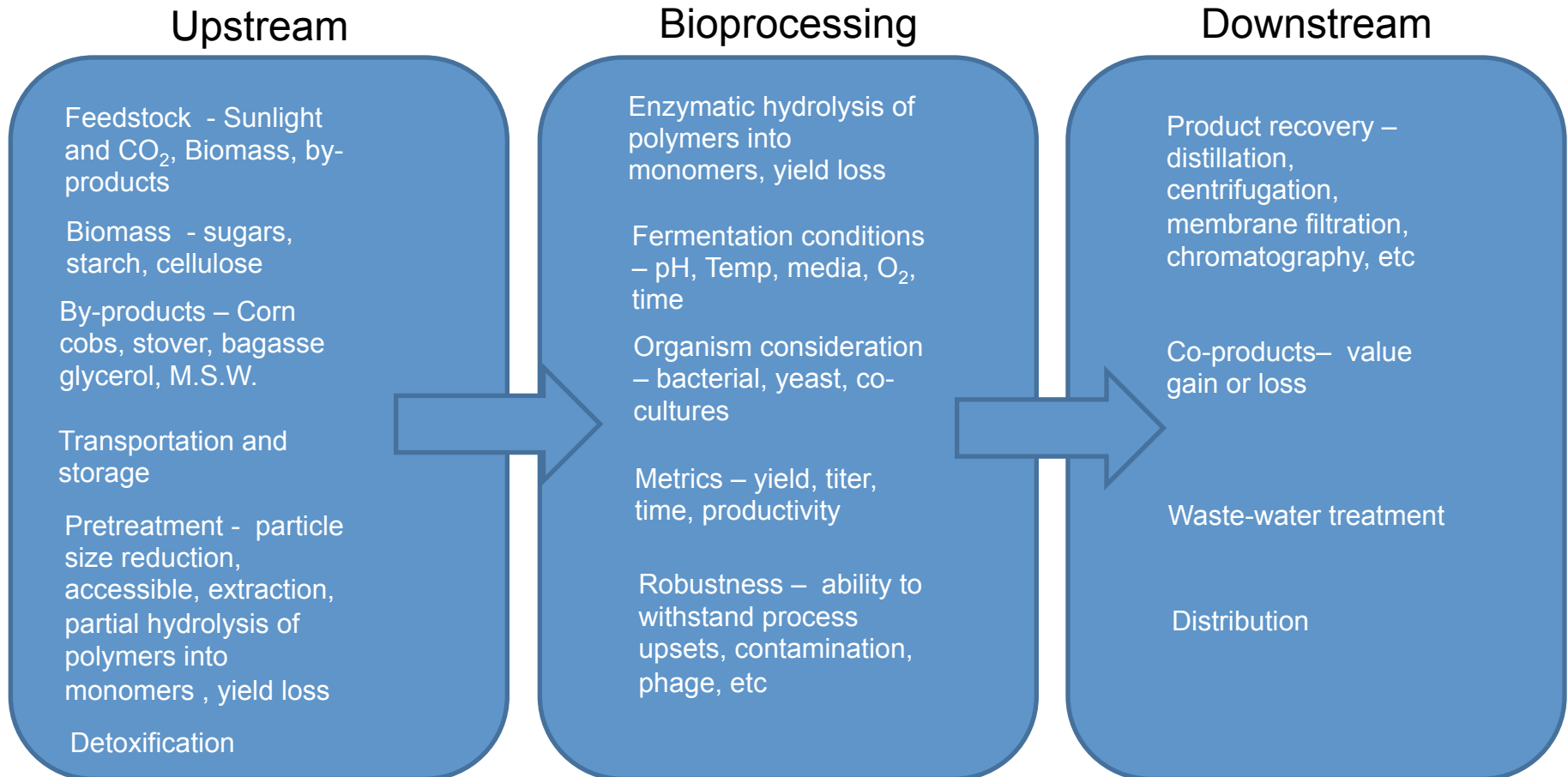
Exploiting Microbes for the production of Sustainable Fuels and Chemicals

Nicky C. Caiazza, PhD

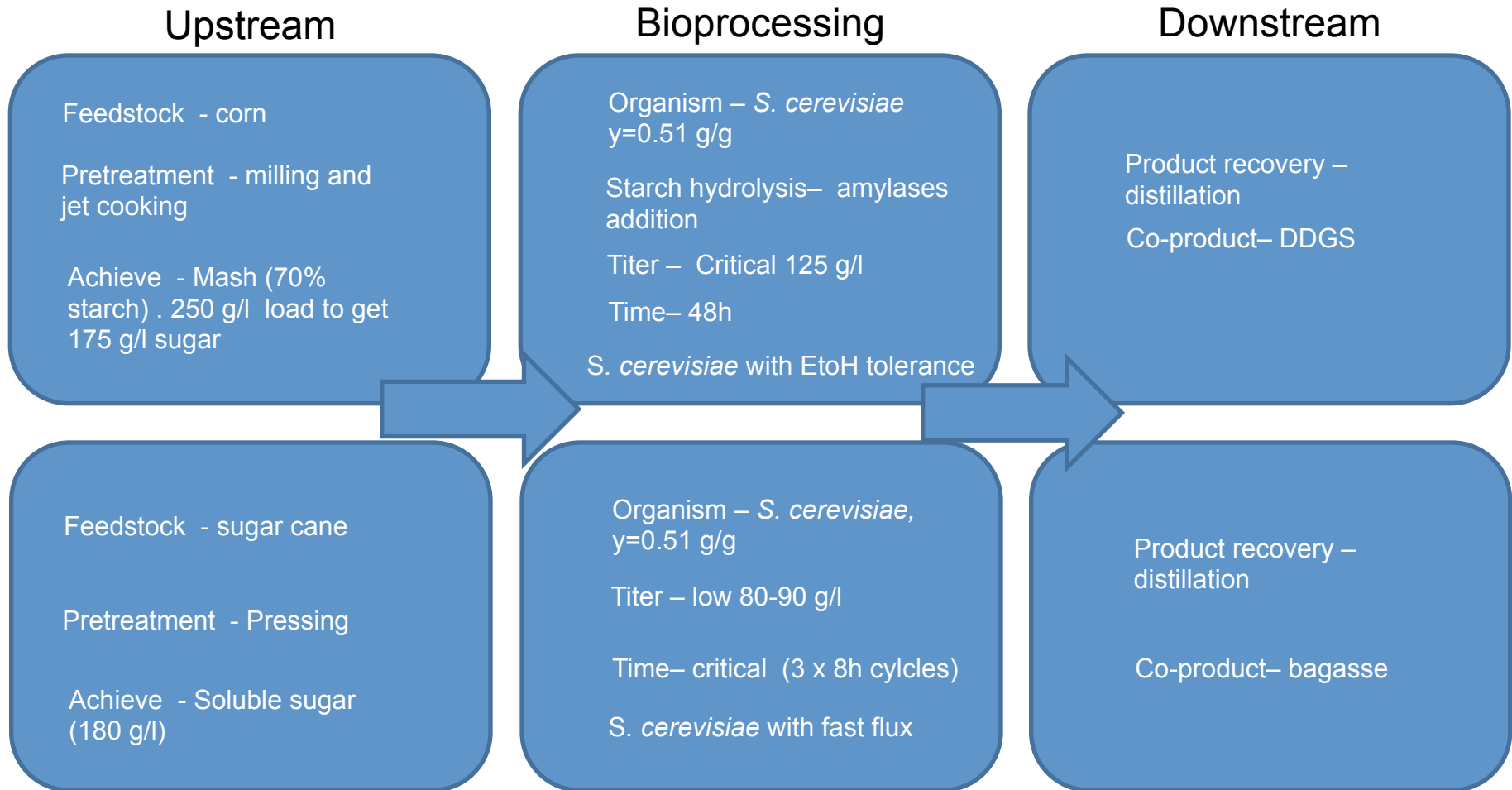
Main Components to Sustainable Fuels and Chemicals

- Sustainability – motivation
- Economics - driver
- Technology – solution
 - Upstream Process
feedstock, handling, pre-treatment, etc
 - Biological Process
fermentation, biotransformation, etc
 - Downstream Process
product recovery, co-products, etc

Main Components to Sustainable Fuels and Chemicals



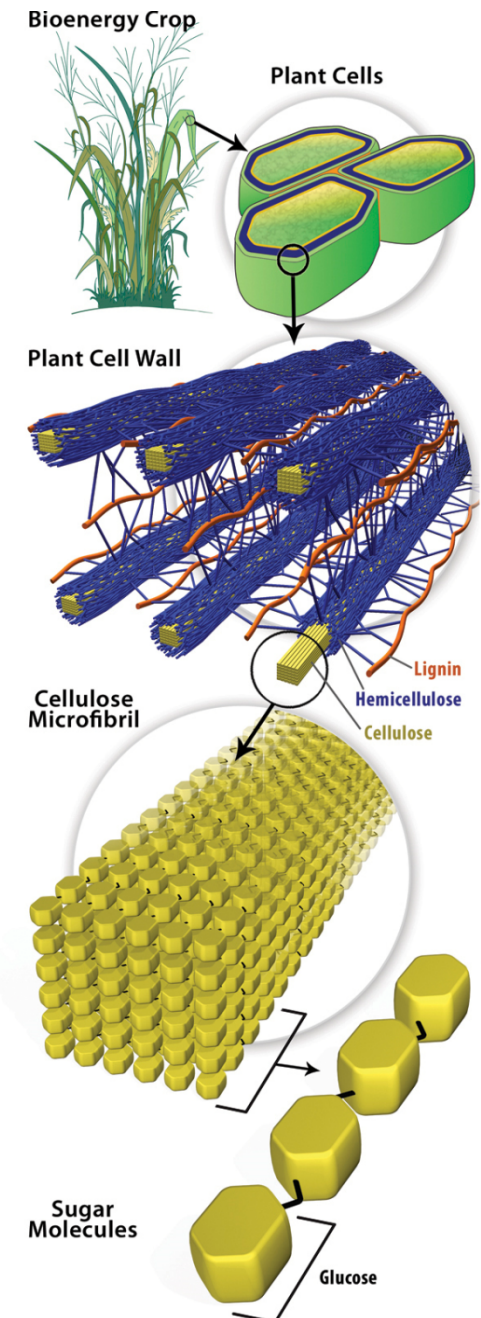
Main Components to Sustainable Fuels and Chemicals – ex corn and cane ethanol



Lignocellulosic Biomass

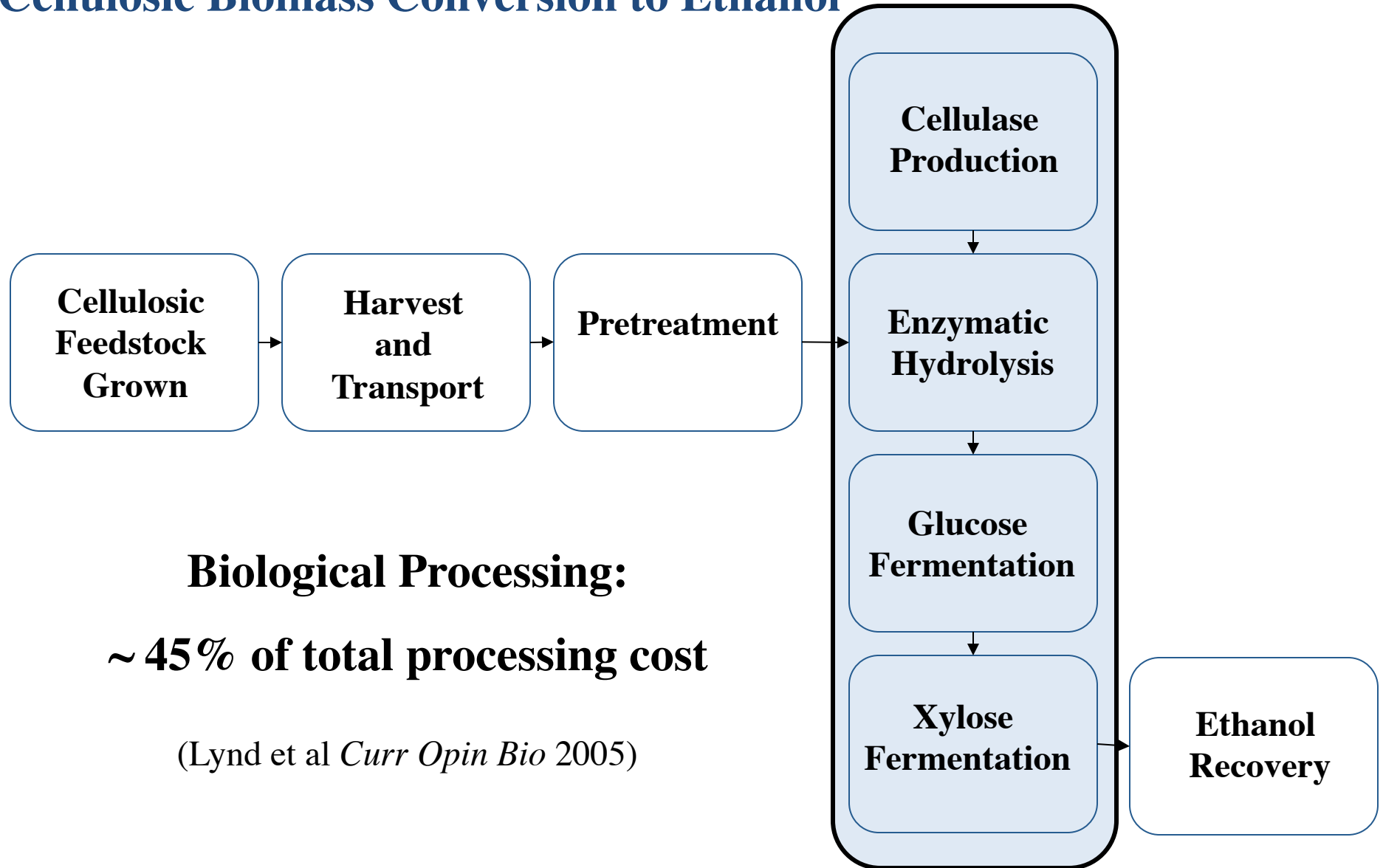
Biomass sugars are very challenging to access economically

- Plants evolved to resist degradation
- Engineering and biotechnology approaches to low cost sugar and biofuel production

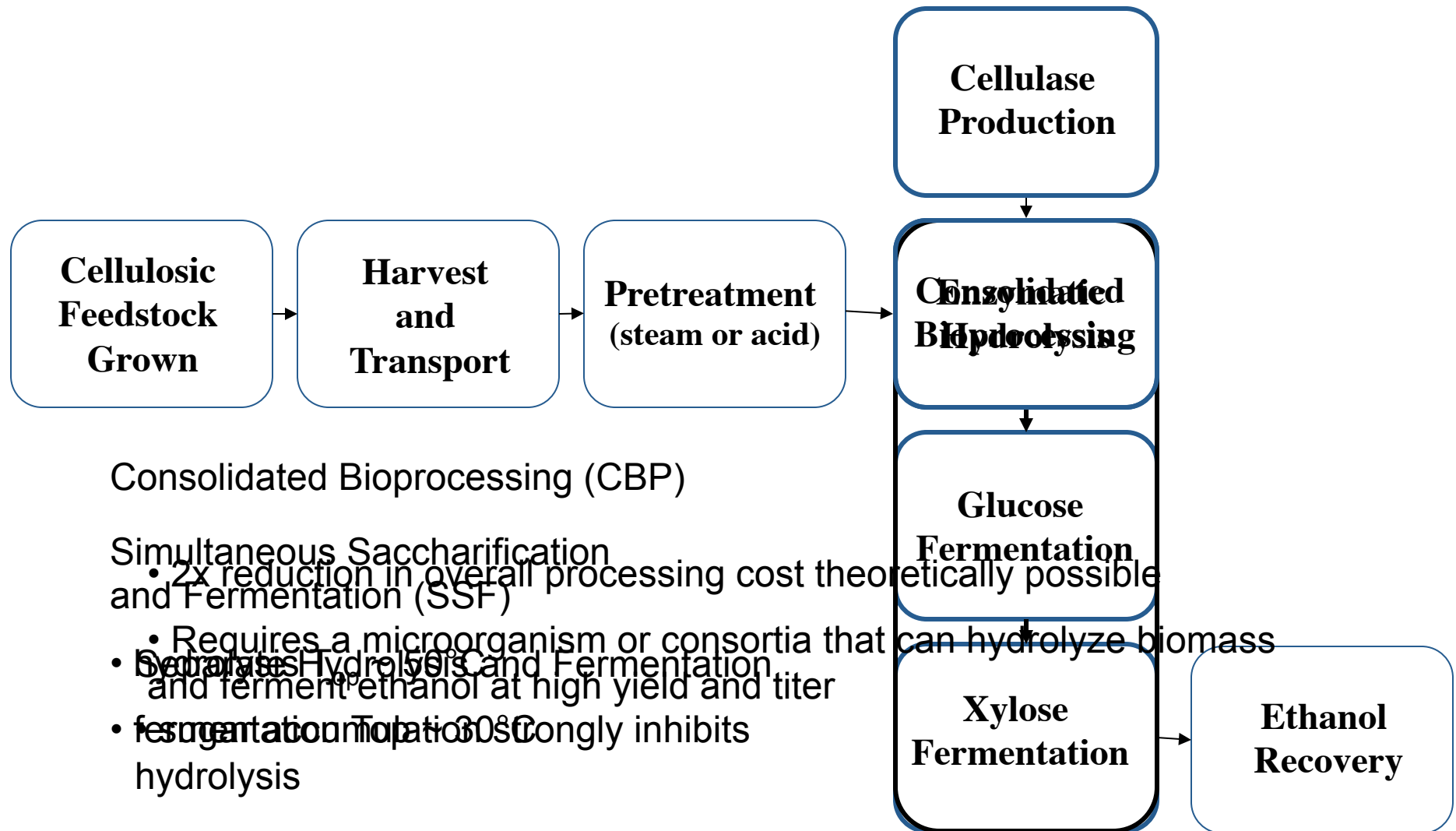


Bioenergy Research Centers: An Overview of the Science, U.S. Department of Energy, July 2009. DOE/SC-0116.

Cellulosic Biomass Conversion to Ethanol



Cellulosic Biomass Conversion to Ethanol



Conversion of Lignocellulosic Biomass with Thermophilic Bacteria

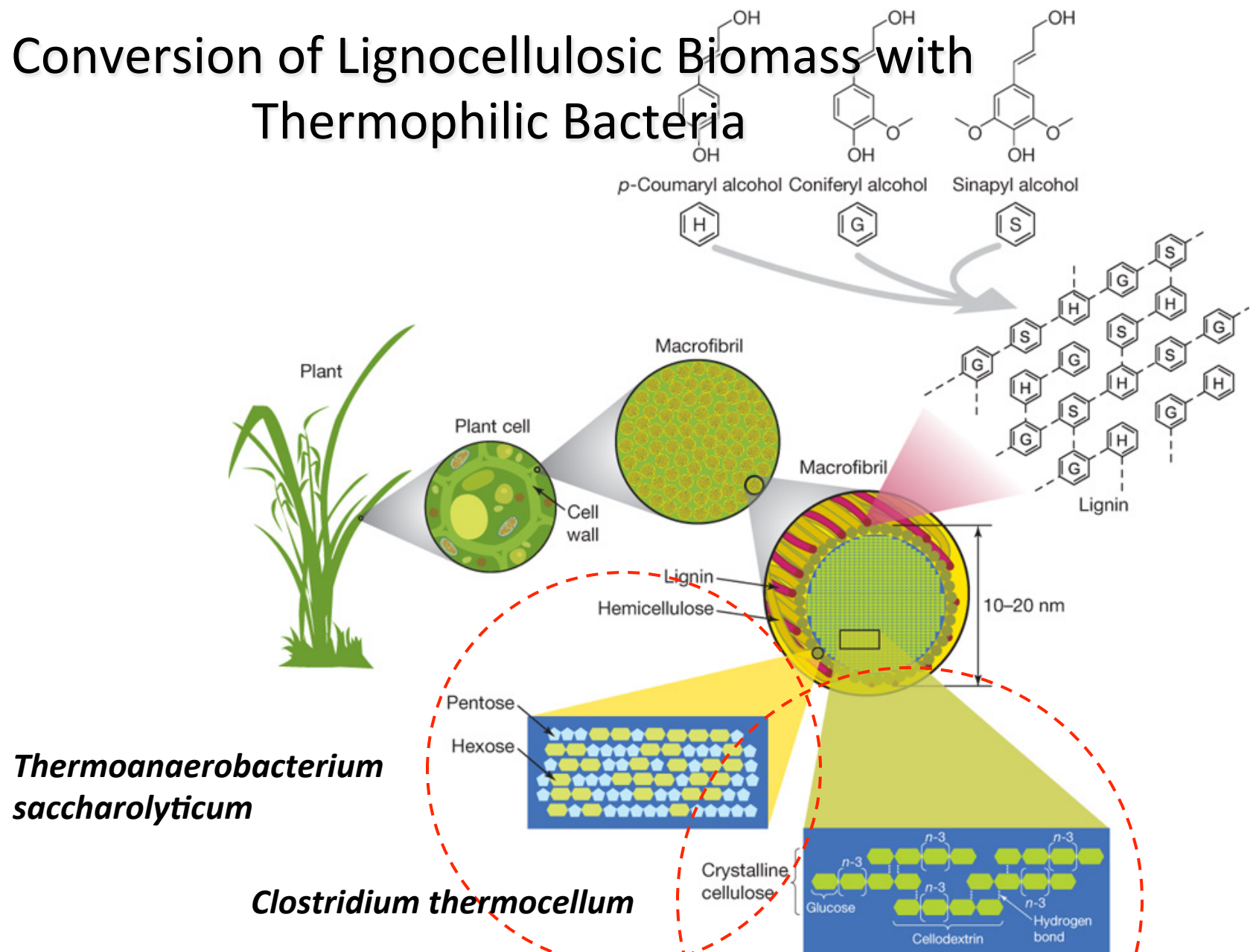


Figure from EM Rubin *Nature* **454**, 841-845 (2008)

T. saccharolyticum

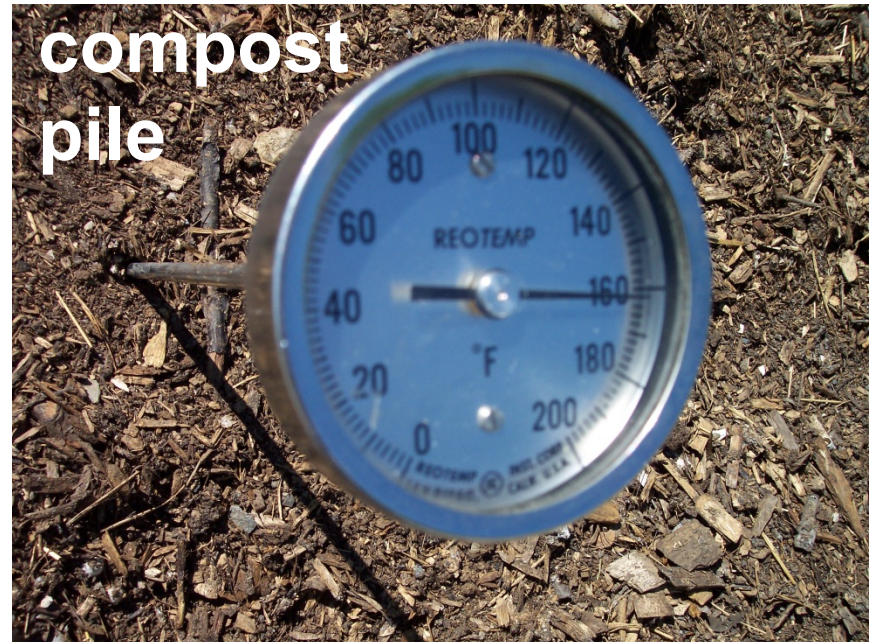


Temp: 50- 60°C pH: 5.0 – 7.0

Low G+C Gram positive, strict anaerobe

Generation time ~ 1 hr

C. thermocellum



Temp: 50- 60°C pH: 6.0 – 8.0

Low G+C Gram positive, strict anaerobe

Generation time ~ 1 hr on cellobiose
2-3 hr on cellulose

***T. saccharolyticum* JW/SL YS485**

Broad Substrate Utilization

Carbohydrates Utilized

glucose	cellobiose
xylose	sucrose
mannose	maltose
arabinose	starch
galactose	xylan
fructose	mannan
rhamnose	amorphous cellulose*
lactose	carboxymethyl cellulose*
trehalose	* partial utilization

C. thermocellum

Cellulose specialist

Carbohydrates Utilized

Cellulose
Cellodextrins
Glucose*
Fructose*
* poor utilization

Products

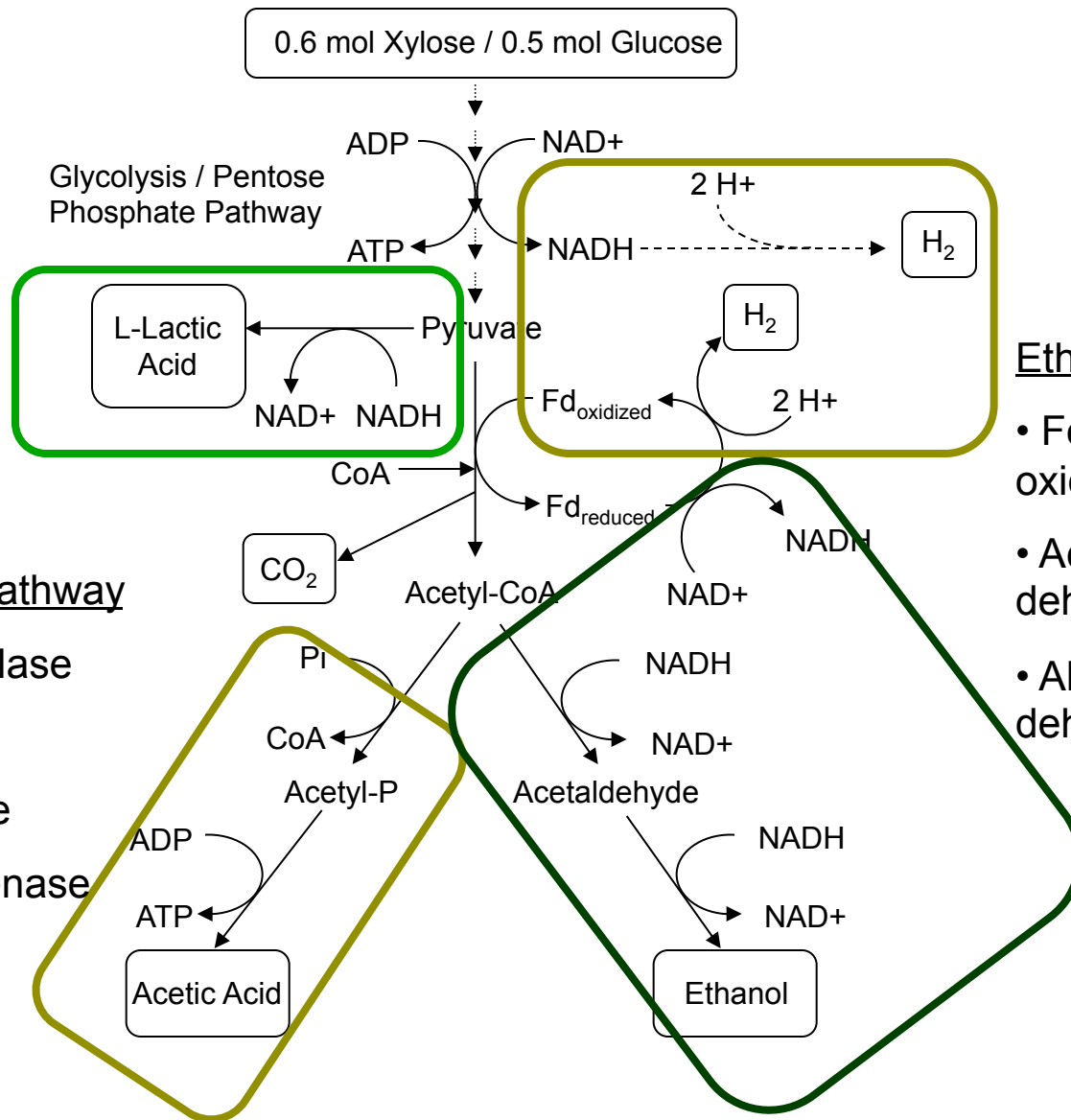
Ethanol, Acetic Acid, Lactic Acid, CO₂, H₂

Lactic Acid Pathway

- L-lactate dehydrogenase

Acetic Acid and H₂ Pathway

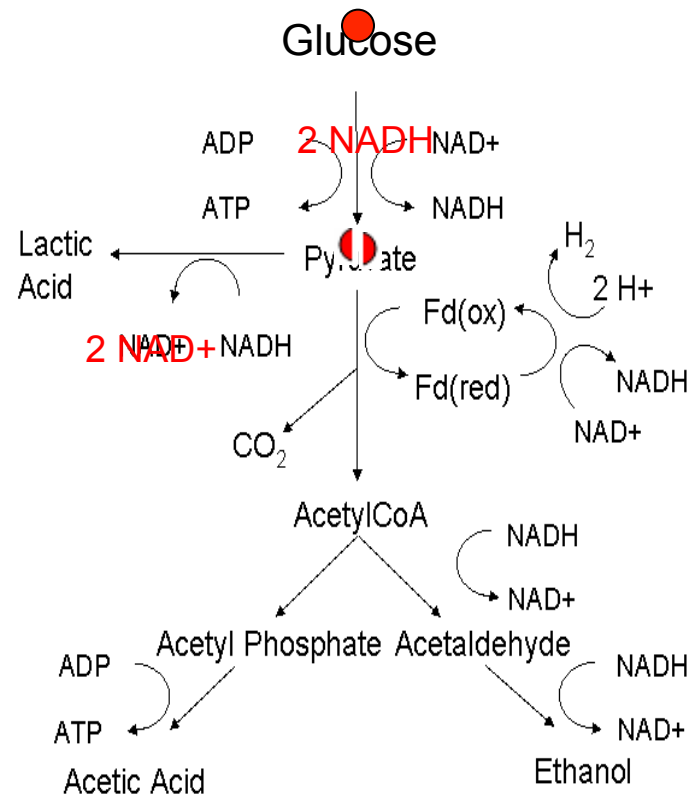
- Phosphotransacetylase
- Acetate kinase
- NADH hydrogenase
- Ferredoxin hydrogenase



Ethanol Pathway

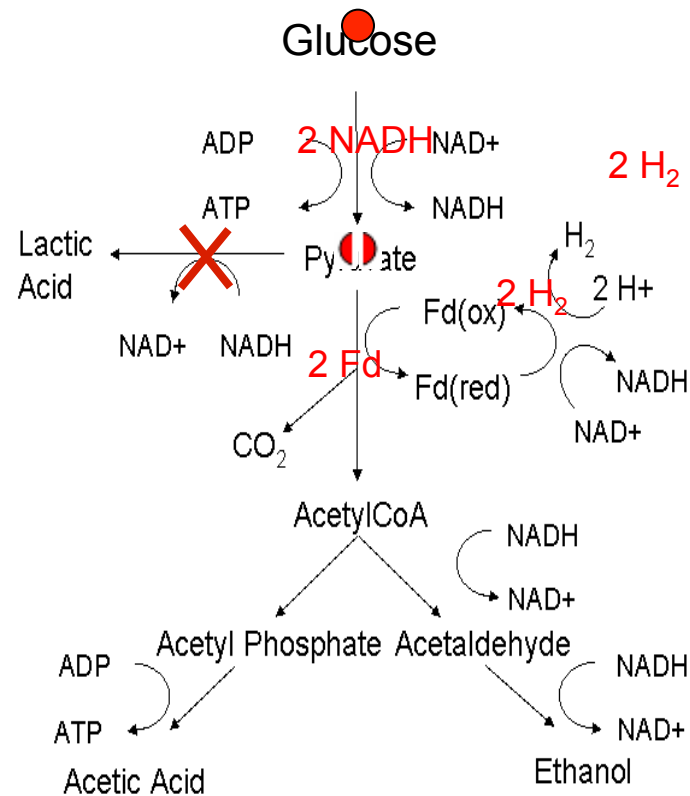
- Ferredoxin:NADH oxidoreductase
- Acetaldehyde dehydrogenase
- Alcohol dehydrogenase

Lactic Acid Production

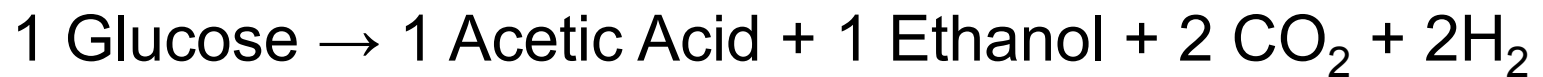
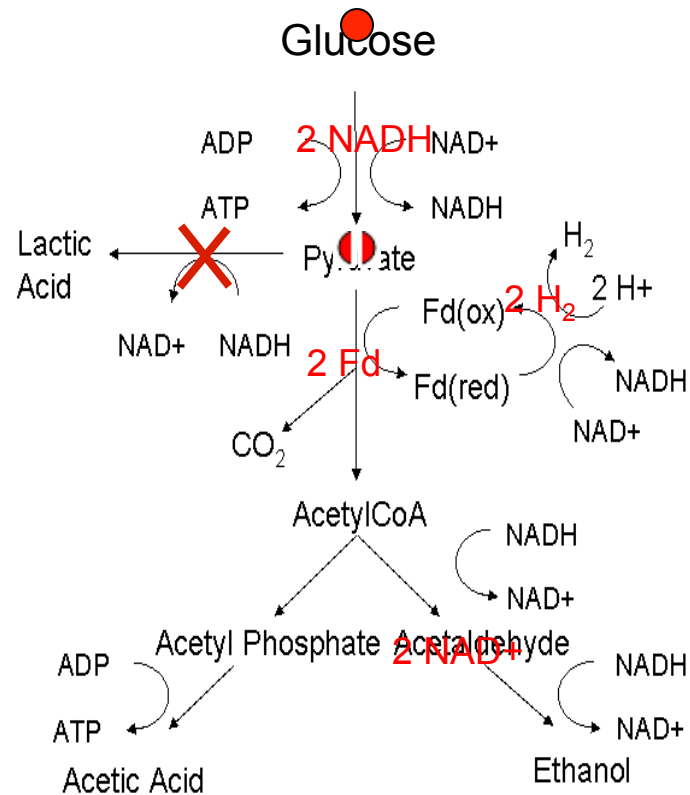


1 Glucose $\rightarrow$ 2 Lactic Acid

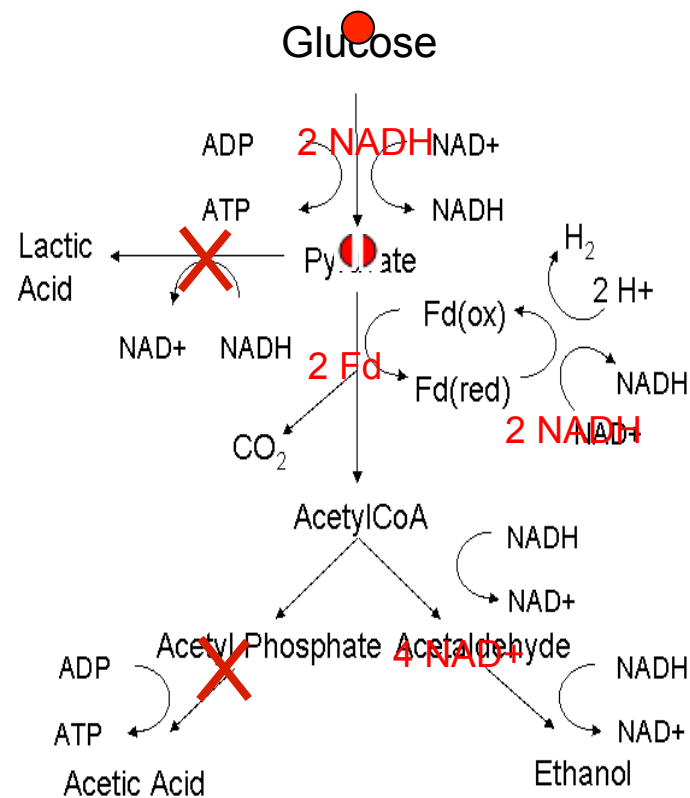
Acetic Acid Production At $\leq 1 \times 10^{-3}$ atm hydrogen (Inter-species hydrogen transfer)



Ethanol and Acetic Acid Production



High yield ethanol production



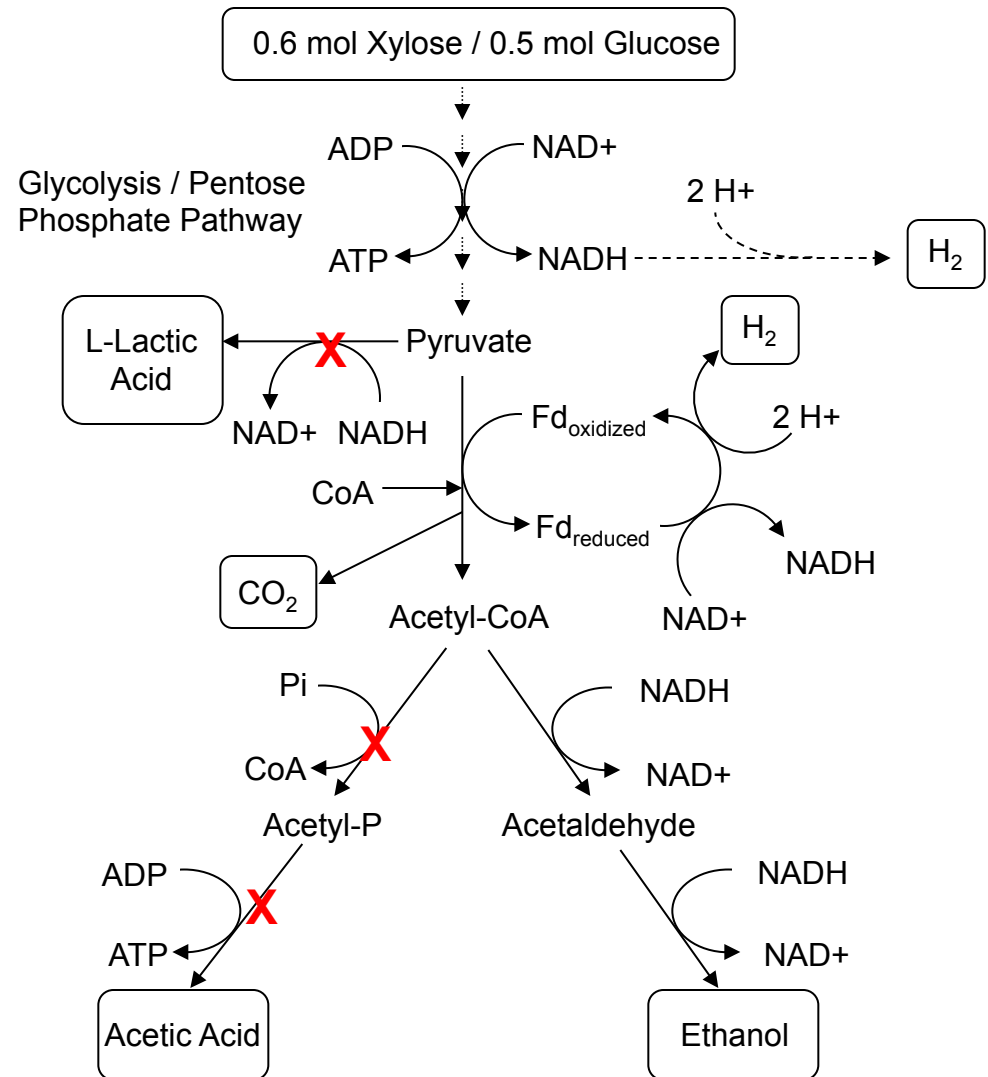
Metabolic Pathway Engineering- High Ethanol Yield

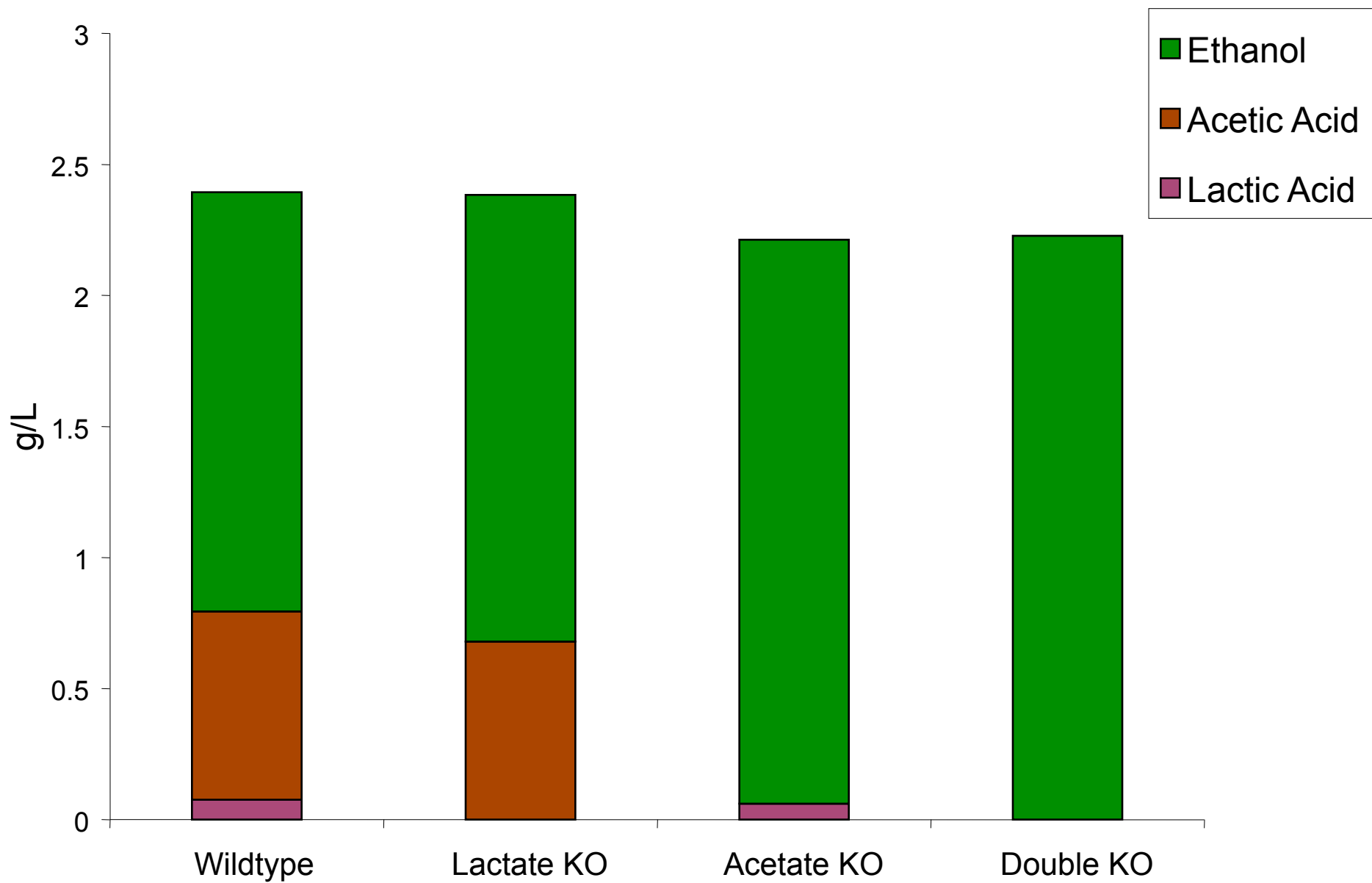
- Requires pathway knowledge
- Requires capability to make genetically modifications

Genetic tool development - *T. saccharolyticum* Platform

- **Natural competence**
- **Recombination using linear DNA**
- **Replicating plasmid isolated from strain *T. saccharolyticum* B6A-RI**
- **Shuttle vectors for *E. coli* and yeast**
- **Antibiotic resistance genes (Kan, Erm, Str, Cat)**
- **Constitutive and inducible promoters (fructose)**
- **Knockout and overexpression libraries**

Genetic Tools – natural competence and efficient homologous recombination facilitate the creation of marked mutations

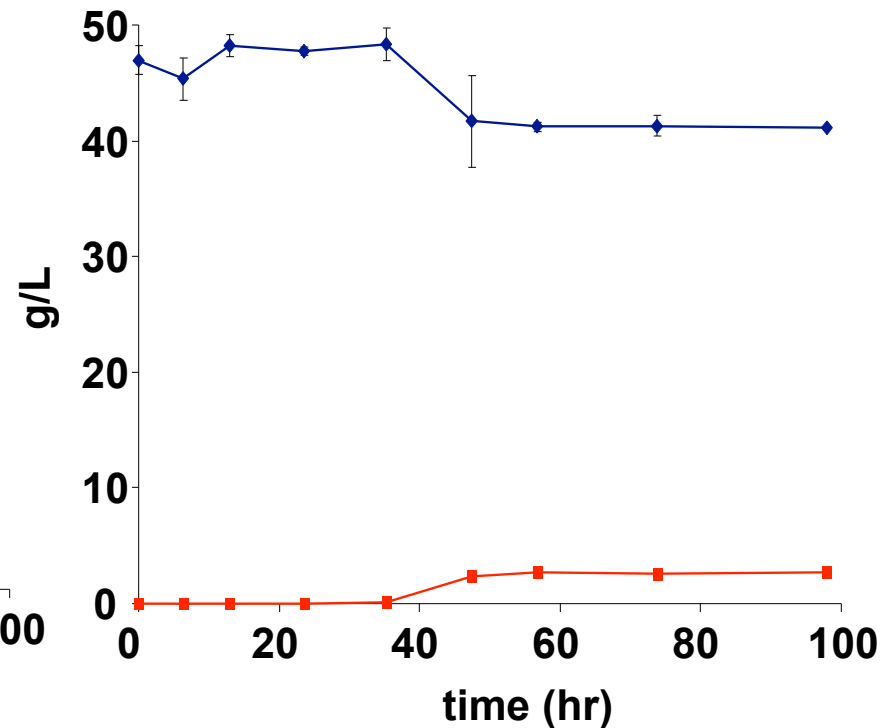
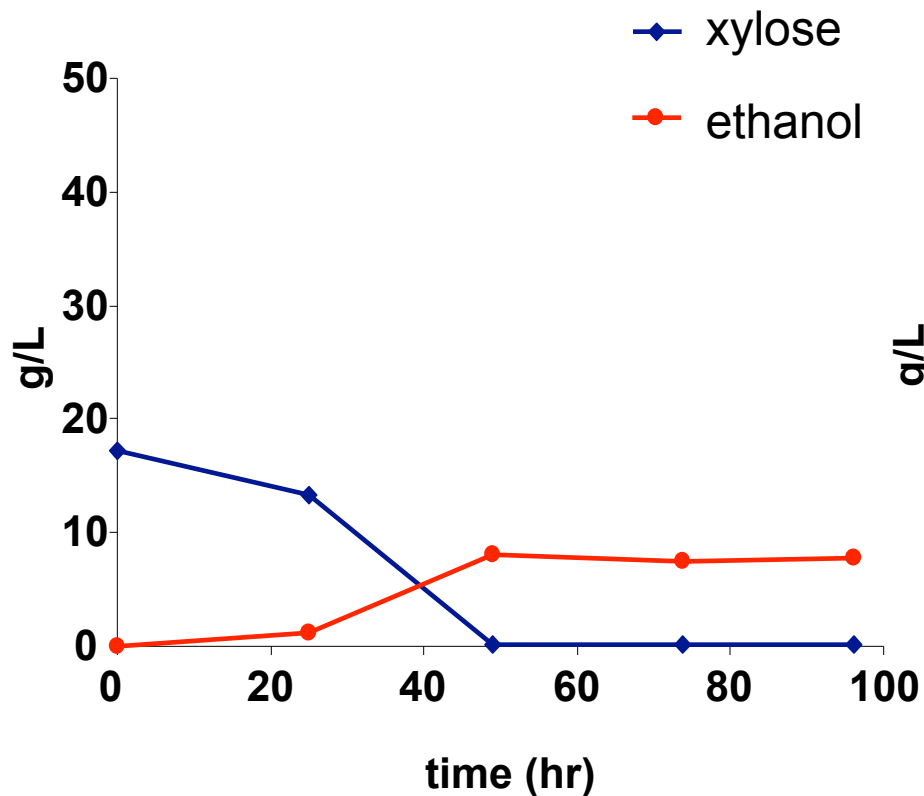




Initial 4.2 g/L Xylose, 2.5 g/L YE, 56°C

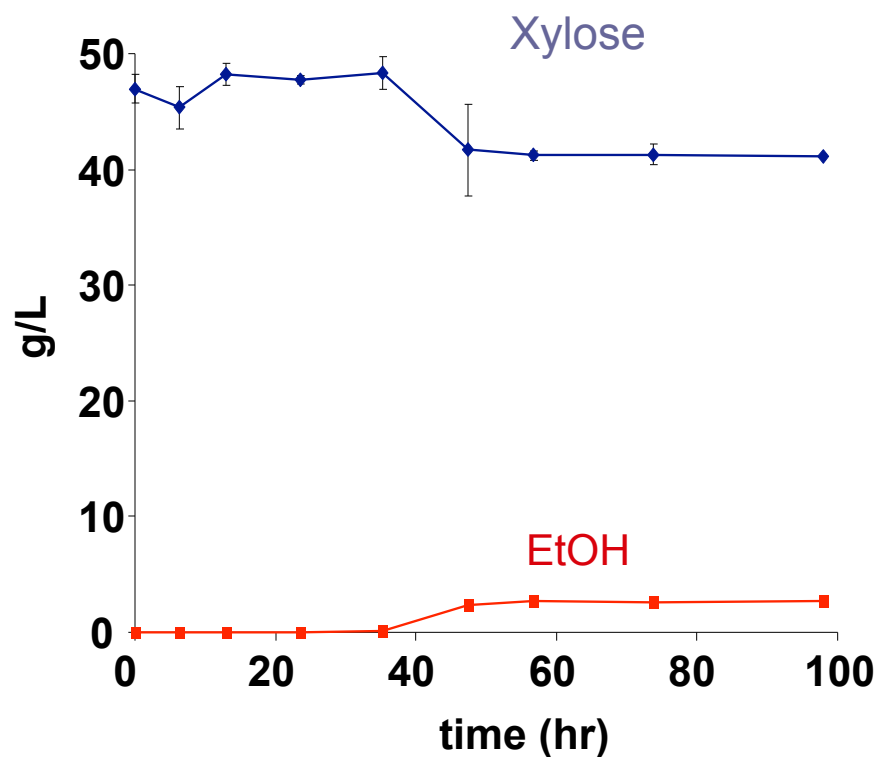
Improving Final Ethanol Concentration

1st generation strain (ALK1) had satisfactory ethanol yield, but could not produce ethanol at high substrate concentrations

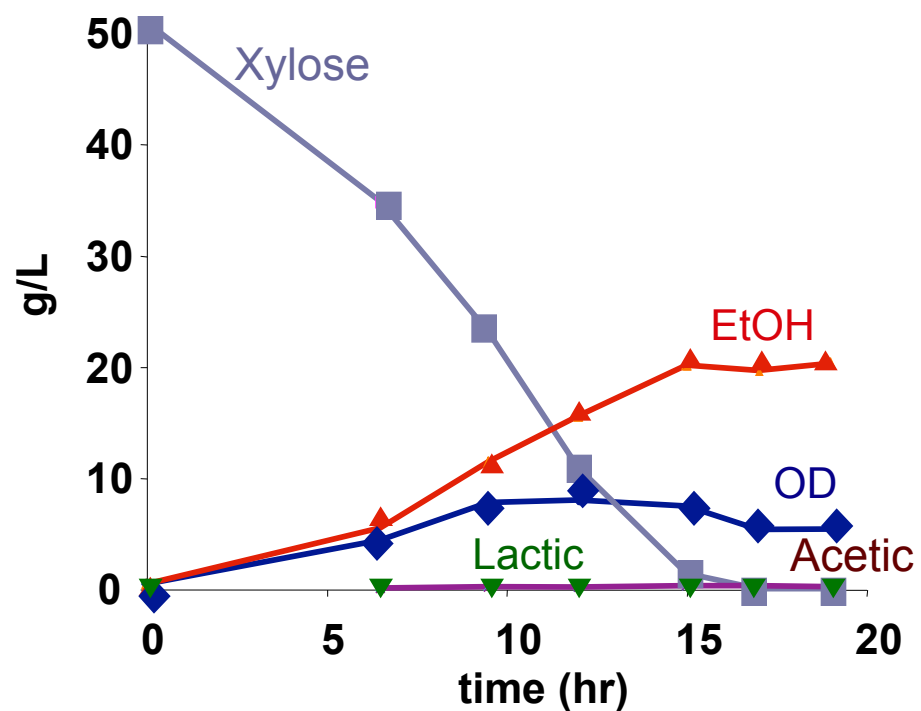


After ~ 3000 hours of continuous culture

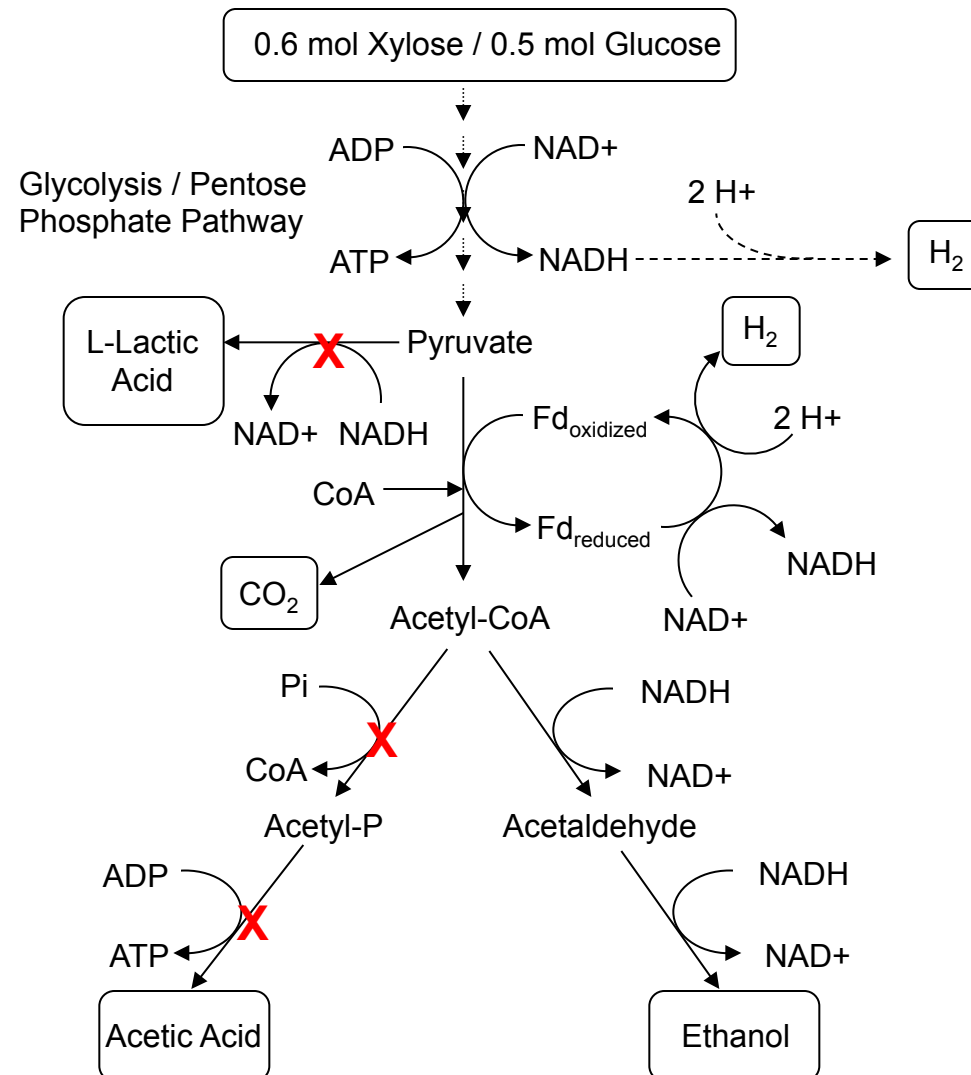
Unadapted – strain ALK1



Adapted – strain ALK2

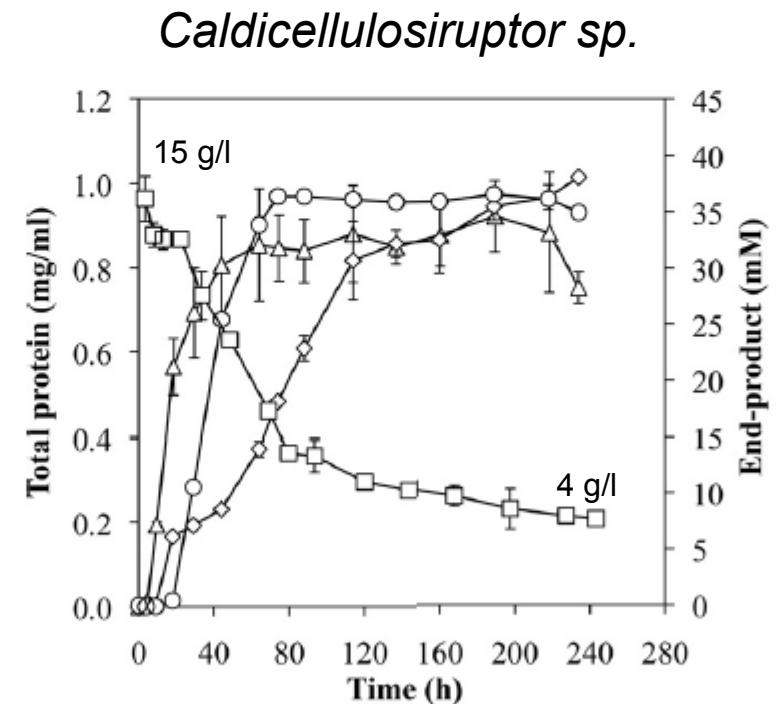
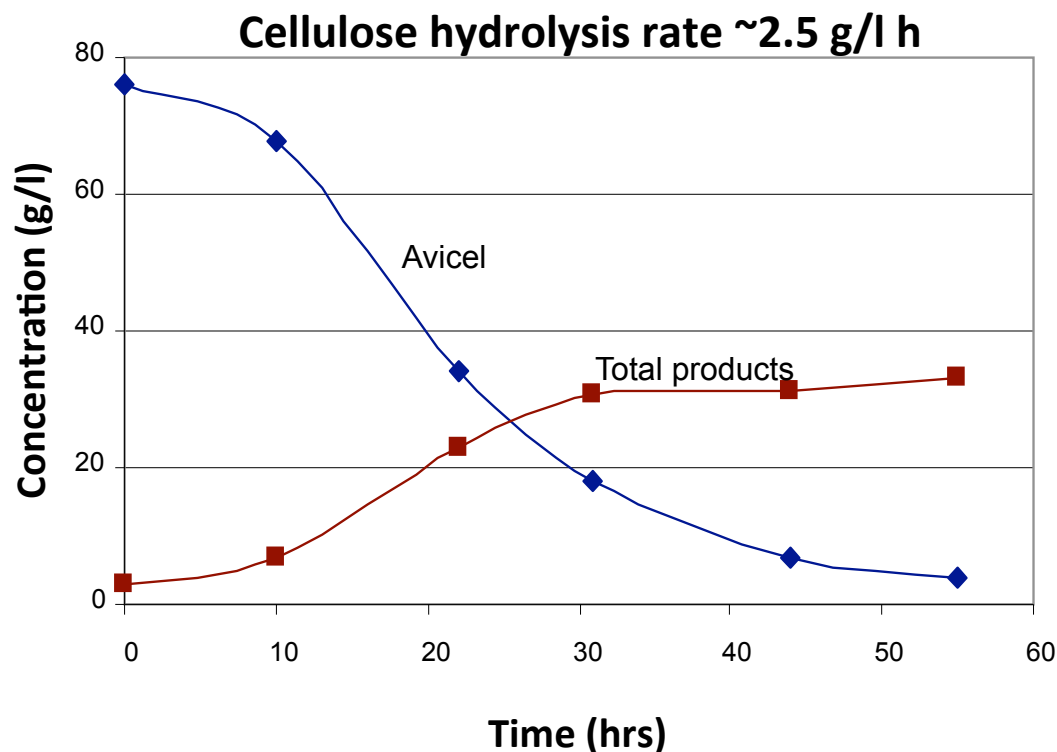


After ~ 3000 hours of continuous culture



***C. thermocellum* platform**

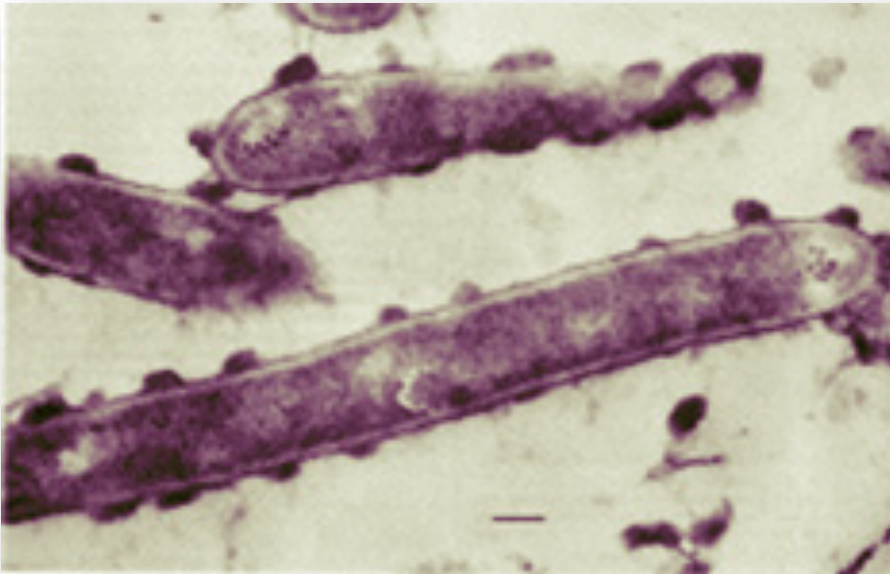
- **Strength** - the worlds best known cellulose degrading organism
 - Capable of hydrolyzing crystalline cellulose at some of the fastest recorded rates (~ 2.5 g/lh)
 - Cellulosome manufacturing organism – Organelle-like enzyme complex with varied composition and >60 enzymes



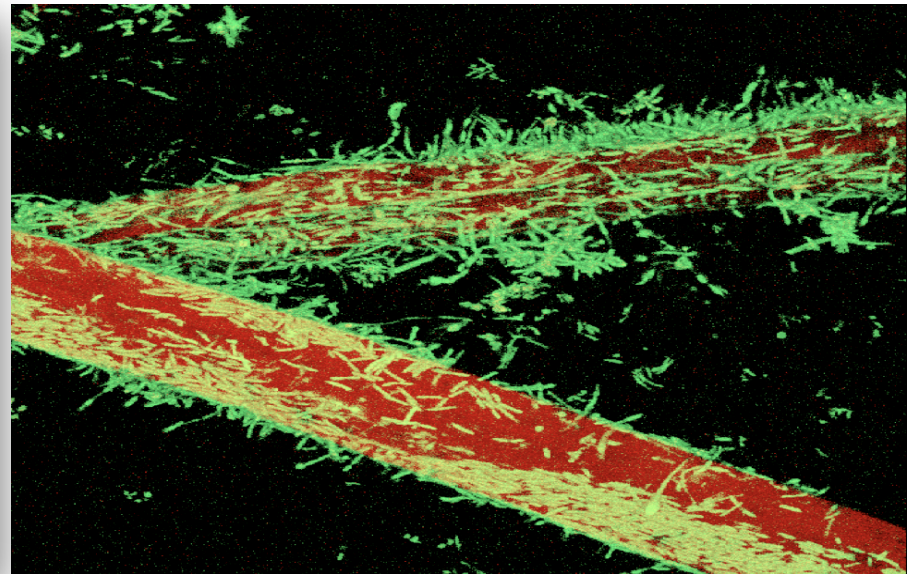
Hamilton-Brehm et al AEM 2010

***C. thermocellum* platform**

- **Strength** - the worlds best known cellulose degrading organism
 - Capable of hydrolyzing crystalline cellulose at some of the fastest recorded rates (~2.5 g/lh)
 - Cellulosome manufacturing organism – Organelle-like enzyme complex with varied composition and >60 enzymes



C. thermocellum, cellulosomes on cell surface



C. thermocellum growing on cellulose.

***C. thermocellum* is also well known for being genetically intractable**

“Cellulase, Clostridia, and Ethanol”

Demain et al, *MMBR Reviews*. 2005

“G
eth
info
the

“Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential?”

Alper and Stephanopoulos, *Nat Rev Microbiol*. 2009

“the innate capacity and potential of these cells which may be able to use recalcitrant substrates or withstand toxic products is immense.”

However

“strains typ
molecular
scientific
modifiab

“The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass”

Maki et al, *In. J. Biol. Sci*. 2009

“an additional limiting factor to the exploration of *C. thermocellum* for CBP for biomass conversion has been its recalcitrance to genetic modification”

***C. thermocellum* genetic tools**

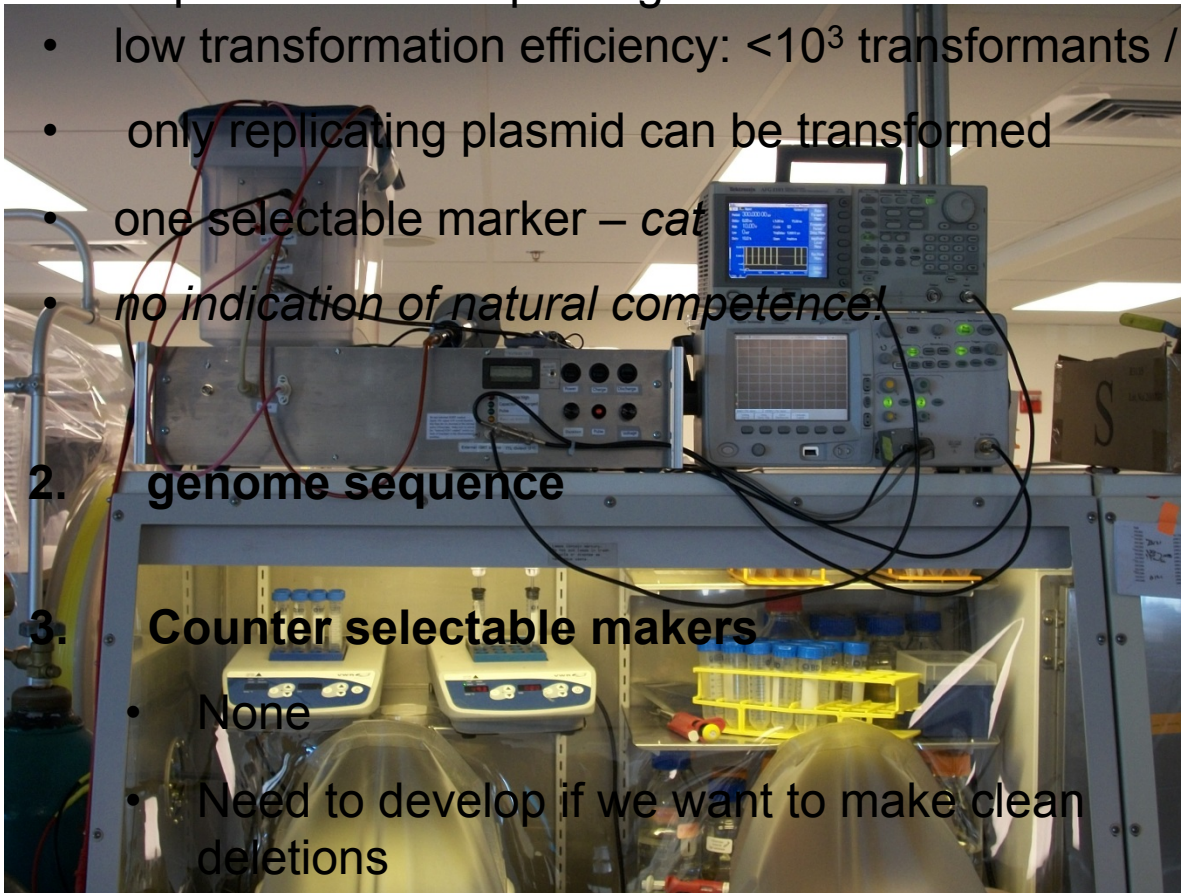
1. electrotransformation

- requires a custom pulse generator
- low transformation efficiency: $<10^3$ transformants / μg DNA
- only replicating plasmid can be transformed
- one selectable marker – *cat*
- *no indication of natural competence!*

2. genome sequence

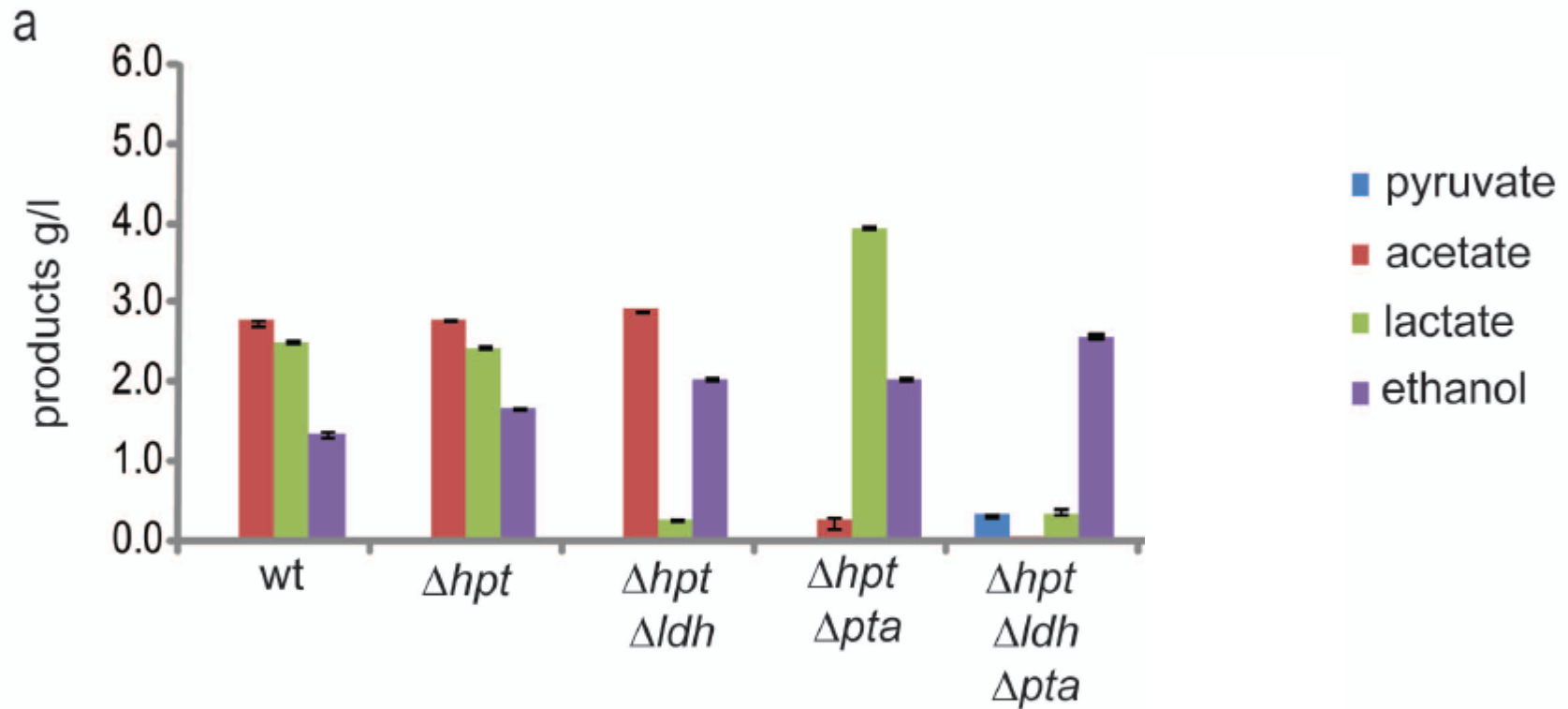
3. Counter selectable makers

- None
- Need to develop if we want to make clean deletions

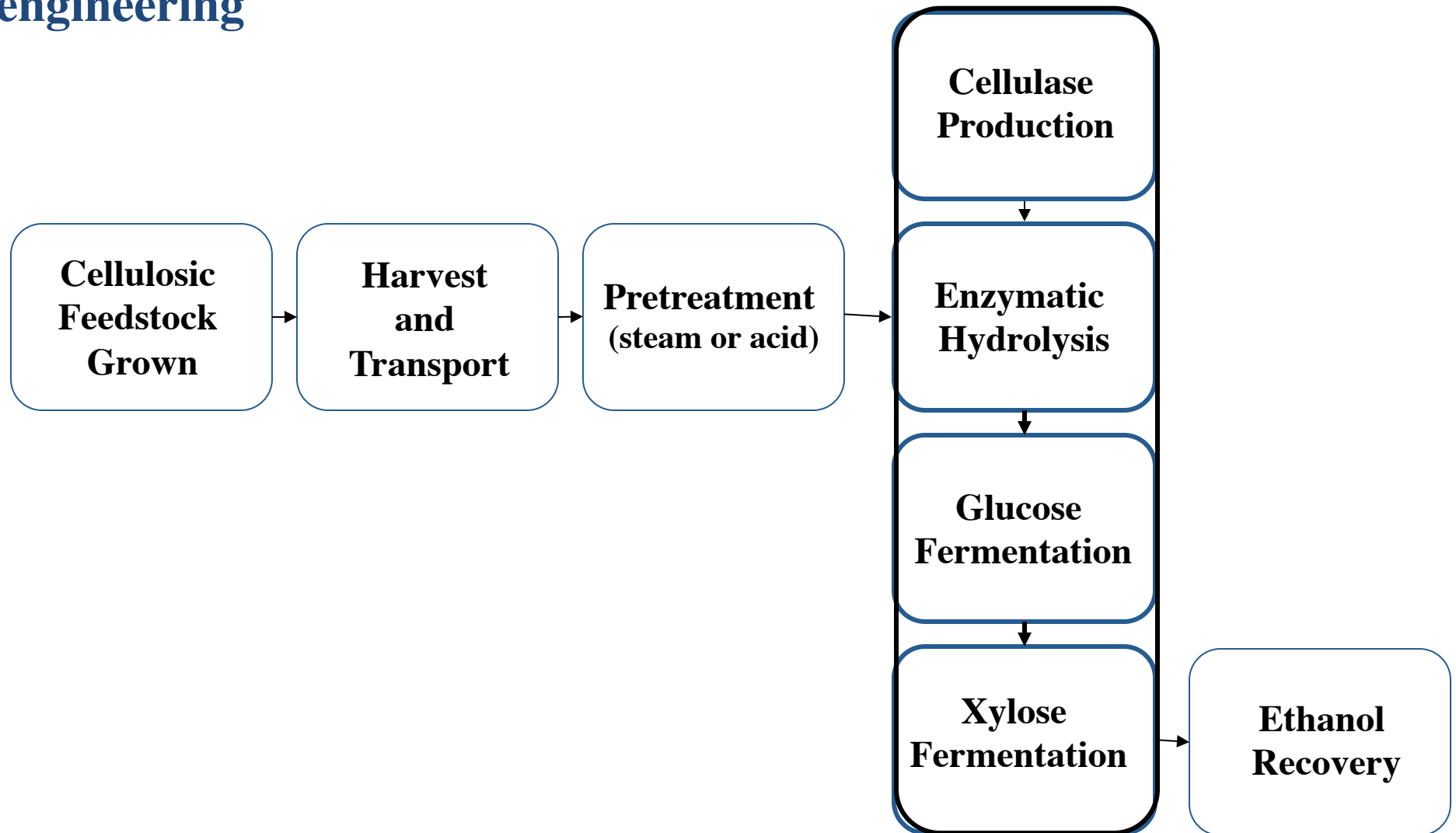


C. thermocellum genetic system for making clean deletions

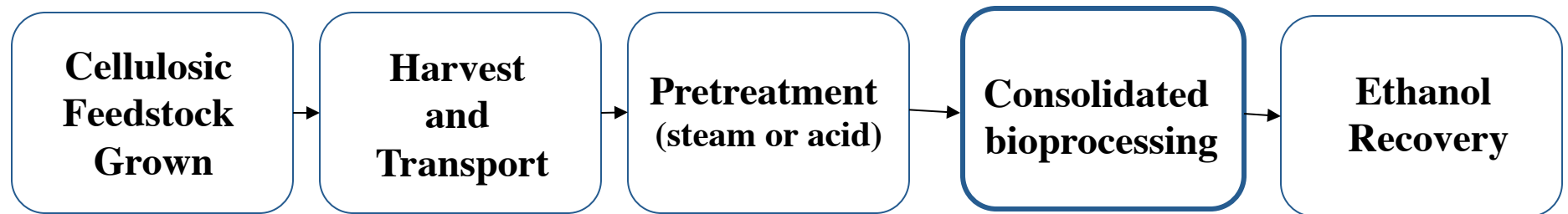
Creation of Combined Lactic, Acetic Acid KO's



Cellulosic Biomass Conversion to Ethanol – benefits of metabolic engineering



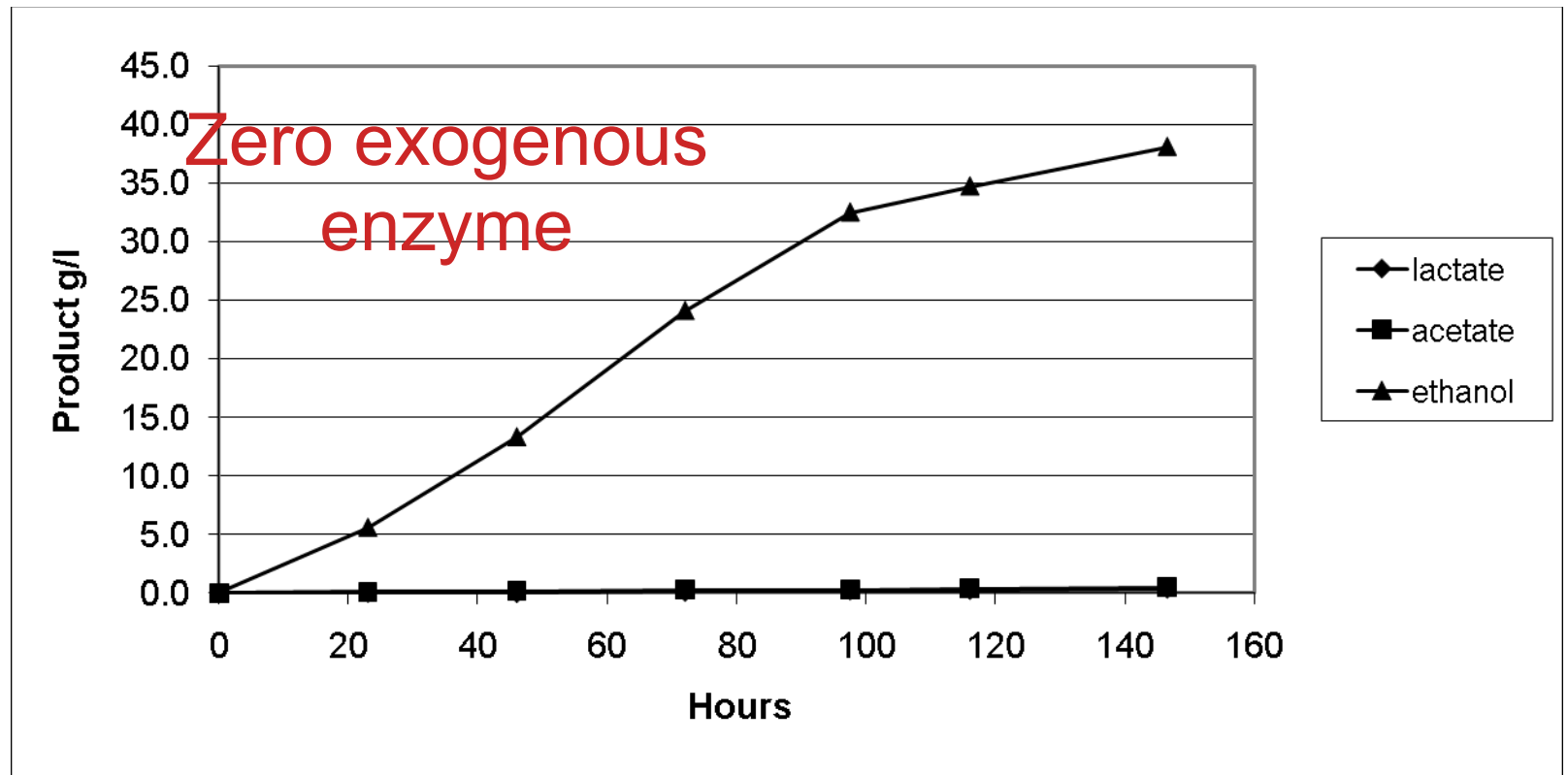
Cellulosic Biomass Conversion to Ethanol – benefits of metabolic engineering



Consolidated bioprocessing

Performance of an Engineered Co-Culture

Ethanol Production via Consolidated BioProcessing



Co-culture of *C. thermocellum* Ev Δ hpt Δ ldh Δ pta and *T. saccharolyticum* ALK2 on 9% Avicel PH-105.

Thermophilic Bacteria Technology Summary

- **Leverages highly evolved biological systems for biomass utilization**
- **New genetic tools have opened doors to strain development**
- **Product losses due to mixed fermentations have been eliminated**
- **High product yields, titers, and rates appear to be achievable**