An overview of recent advances in lignocellulose to ethanol conversion technology

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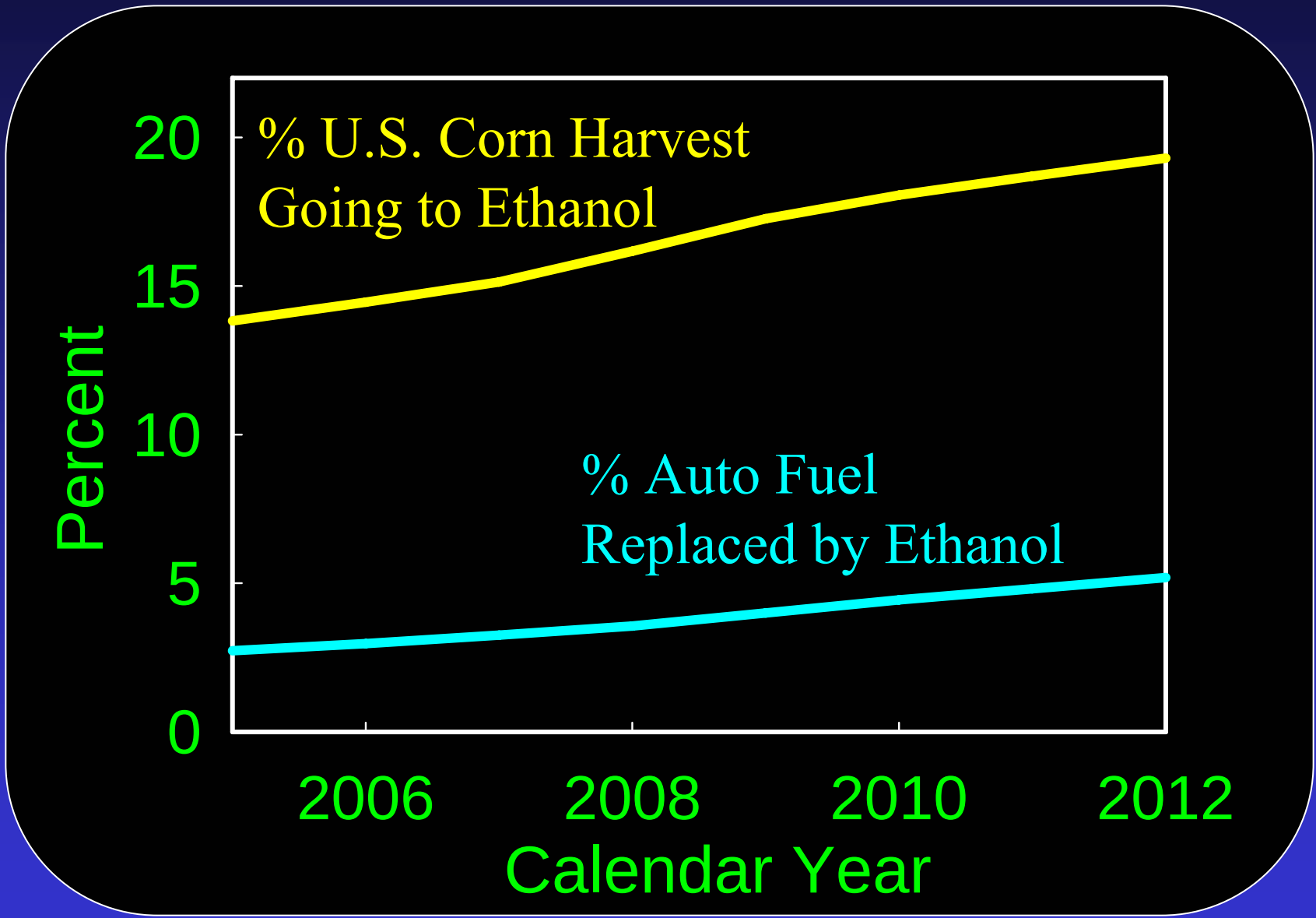
Potential of corn to replace oil for U.S. market



(RFA & NCGA, 2006)

Potential of lignocellulosic biomass to replace oil for U.S. market

Feedstocks	Million dry ton per yr	Billion gal of ethanol per yr
Agricultural Land (selected)		
Corn Stover	75	4.50
Wheat Straw	11	0.66
CRP Biomass	18	1.08
Perennial Crops	156	9.36
Forestlands (selected)		
Logging & Processing Residues	134	8.04
Total:	4,894	23.6

This is 17% of our total oil needs.

Notes: (1) 60 gal/ton ethanol yield; (2) source: http://feedstockreview.ornl.gov/pdf/billion_ton_vision.pdf

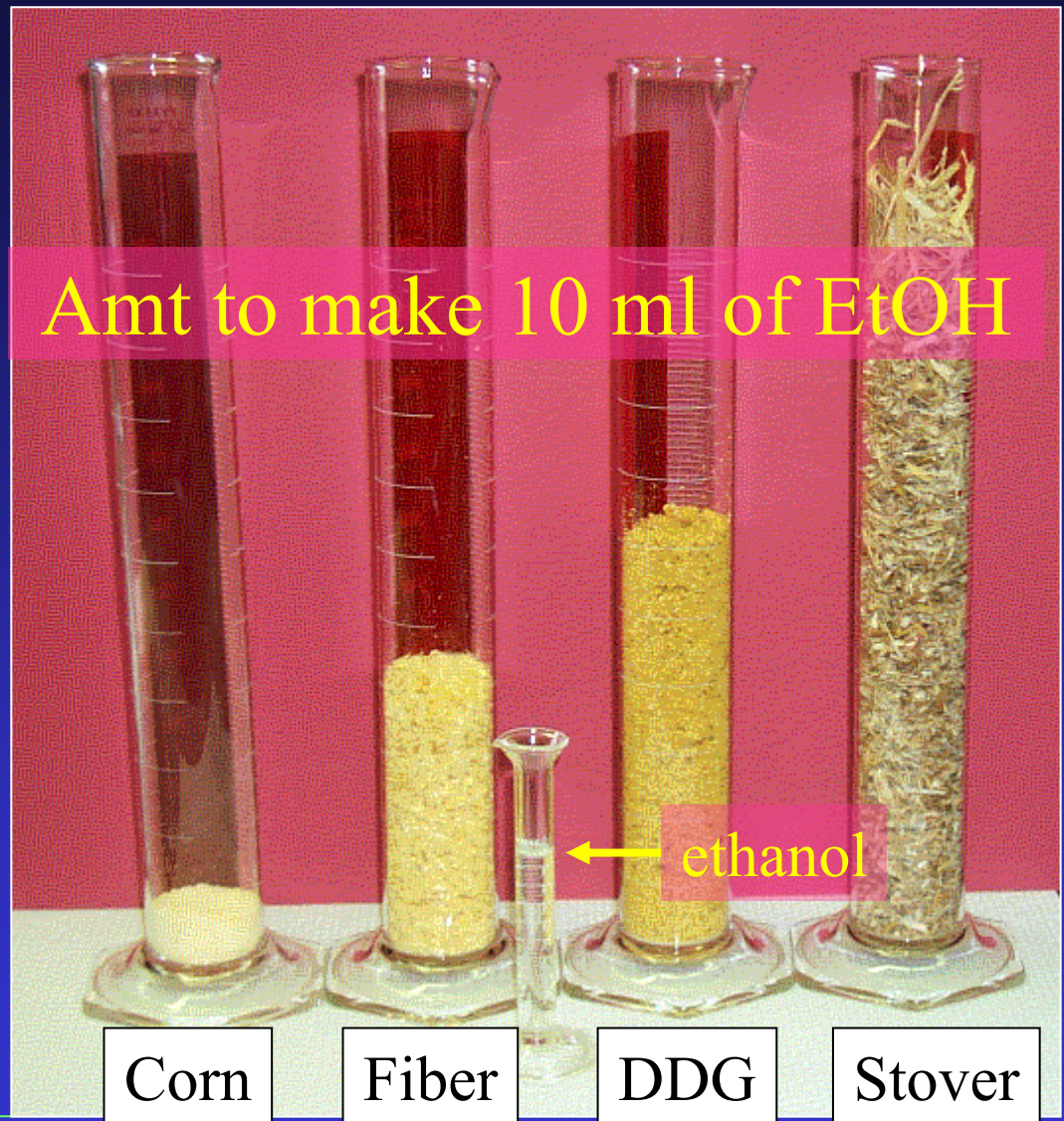
Chemical composition of biomass

Comp.	Corn Kernel	Corn Stover	Switch-grass	Poplar Hybrid
Ether Ext.	4.6	4.6	1.0	4.2
Protein	9.1	4.0	3.2	1.2
Starch	78.0	0.0	3.9	0.0
Cellulose	2.0	36.0	28.3	42.4
Hemi-cellulose	3.6	23.4	24.5	19.0
Klason lignin	trace	18.6	15.4	25.7
Ash	1.5	12.5	5.4	1.8

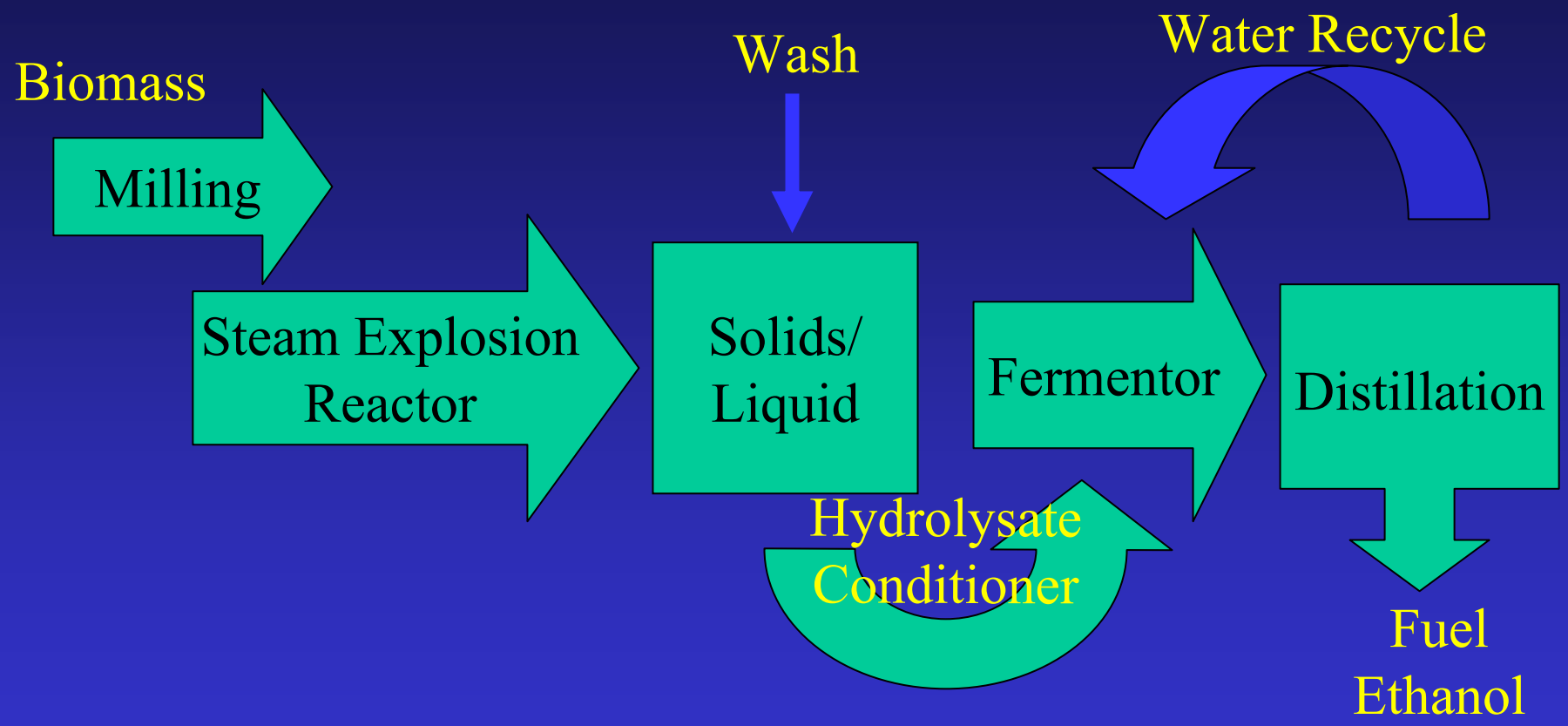
Sources: DOE Biomass Database & Dien

Challenges to processing fibrous biomass compared to grains

- ❖High bulk mat'l (wood less so)
- ❖2-phase reactions (β-glucan insoluble for > 10 d.p.)
- ❖Complex cell wall structure & lignin (e.g. storage vs. structural CHO's)
- ❖Xylan related sugars not fermented by *Saccharomyces*

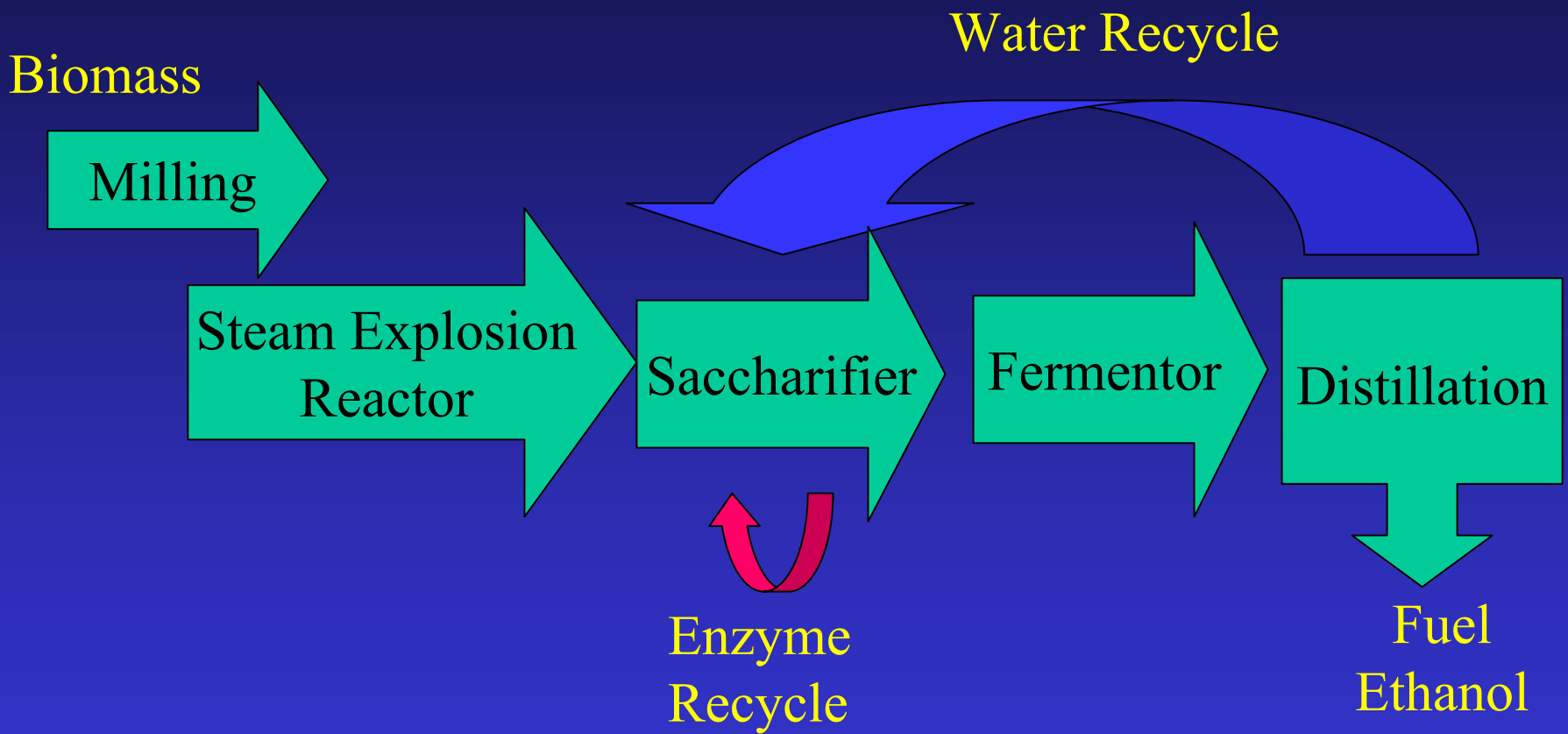


SSF process for converting biomass to bio-ethanol



Corn Stover:	80 gal/ton, DM (NREL, 2002)
Poplar Hybrid:	68 gal/ton, DM (NREL, 1999)
Dent Corn:	116 gal/ton, DM (2.7 gal/bu)

Separate hydrolysis & fermentation (SHF) process

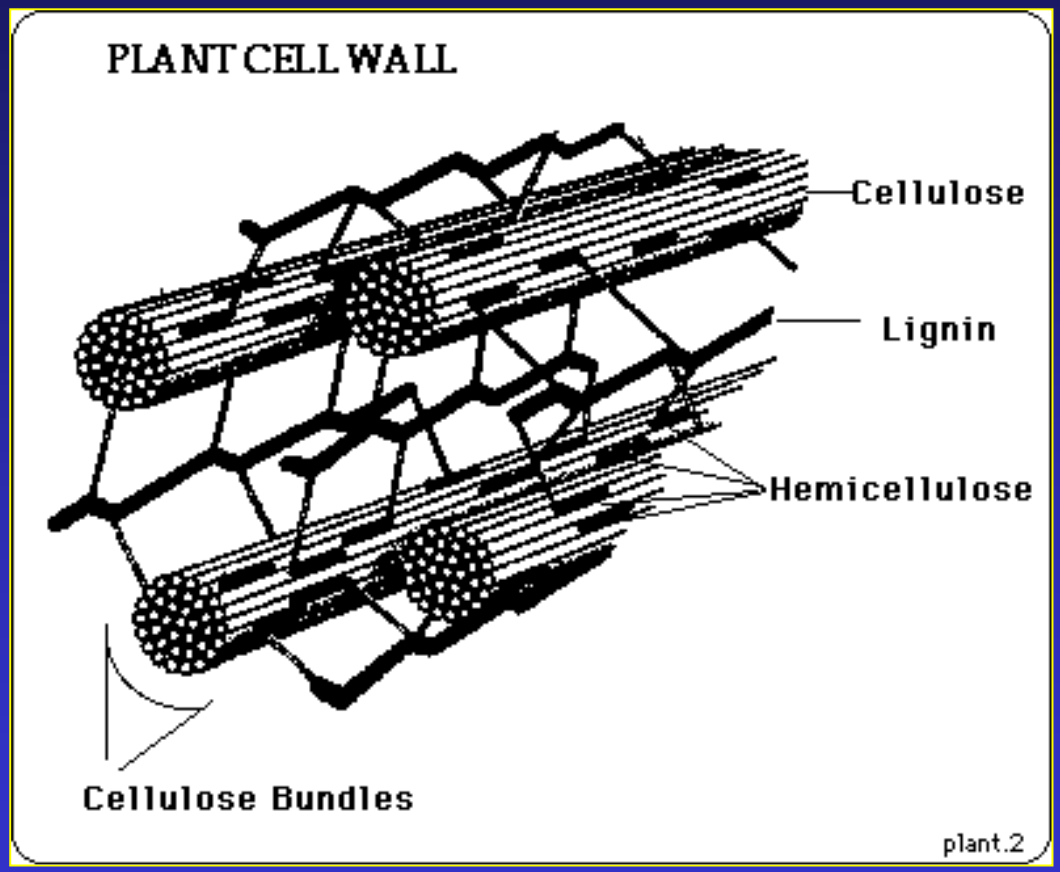


Advantages: fewer solids in reactor, easier to remove inhibitors, higher temp. enzyme rxn.

Pretreatment

What is expected of a pretreatment?

Allow cellulase access to cellulose polymers by disrupting cell wall structure



- ✓ Dissolve Hemicellulose
- ✓ Displace Lignin
- ✓ Swell Cellulose Bundles

Chemical mechanisms

Hemicellulose

Acid hydrolyzes, alkali dissolves, hot-water acts as weak acid

Lignin

Molecular oxygen, ozone, peroxide break lignin-ether bonds, Δ (140°C+) melts lignin, acid hydrolyzes, alkali saponifies ferulic/arabinose ester bonds

Cellulose

Ammonia disrupts H bonds, solvents & conc. acid dissolve cellulose polymer

High solids dilute acid pretreatment of corn stover

Rxn Solids Concentration	Xylose Yield	Cellulose Conversion[a]	Total Sugar Hydrolysate Conc.[b]
(wt%)	(%)	(%)	(g/l)
20	78	93	94
30	75	95	143

[a]cellulose digestibility measured by SSF testing
[b]total of glucose, xylose, arabinose, galactose, and mannose in the pretreated liquid stream

Reactor: Sund Defibrator vert. pulp digestor (1 t/d)
Conditions: 190°C, 1 min, 0.045 g H_2SO_4/ g dry corn stover

(D. Schell, R. Elander, and J. McMillan, NREL, 2003)

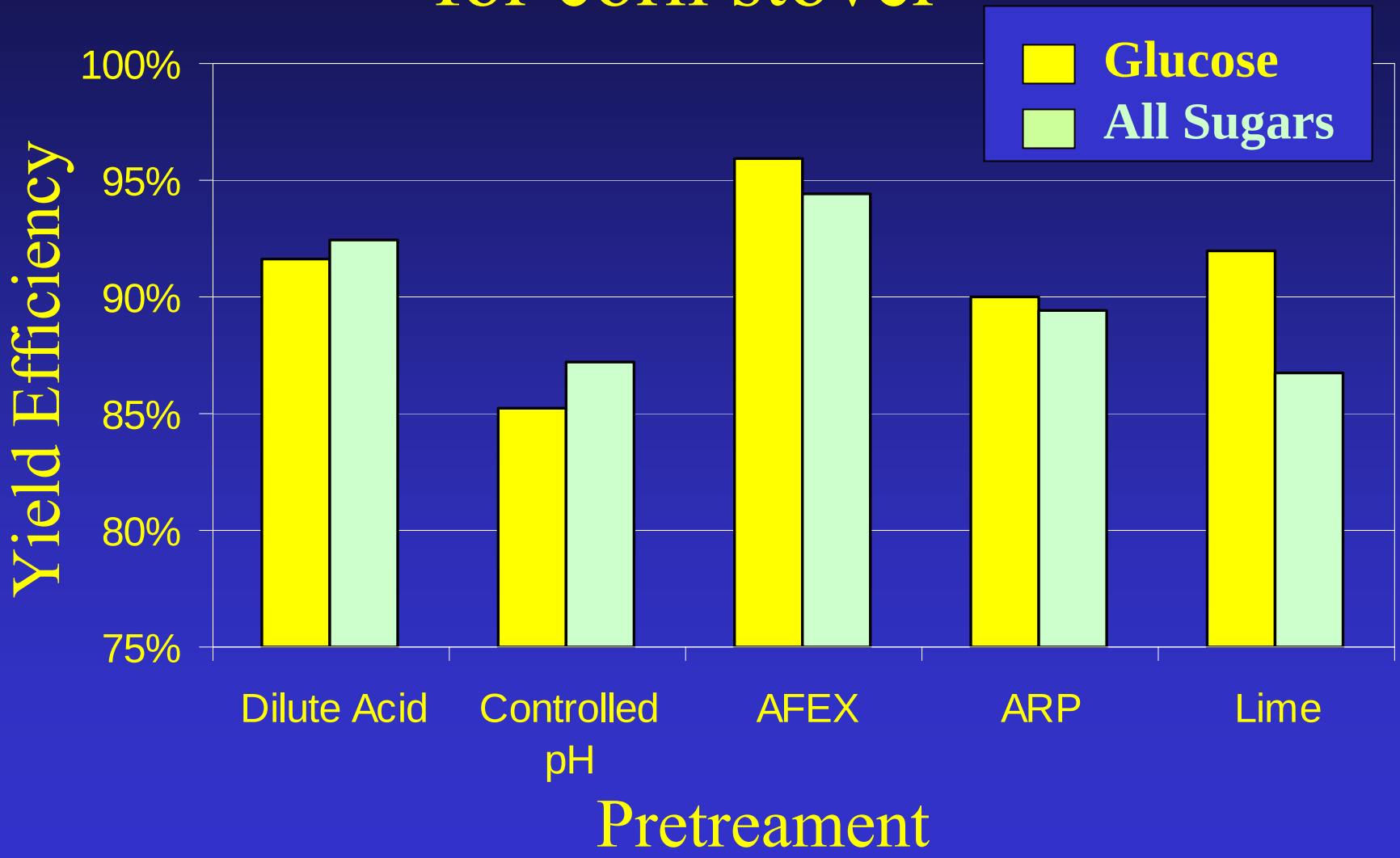
CAFI results for corn stover

Preteatments Evaluated (Major PI)

- Ammonia Fiber Explosion (AFEX) (B. Dale)
- Ammonia Recycle Percolation (Y.Y. Lee)
- Controlled pH Hot-Water (M. Ladisch & N. Mosier)
- Concentrated Lime (M. Holtzapple)
- Dilute Acid (C. Wyman)

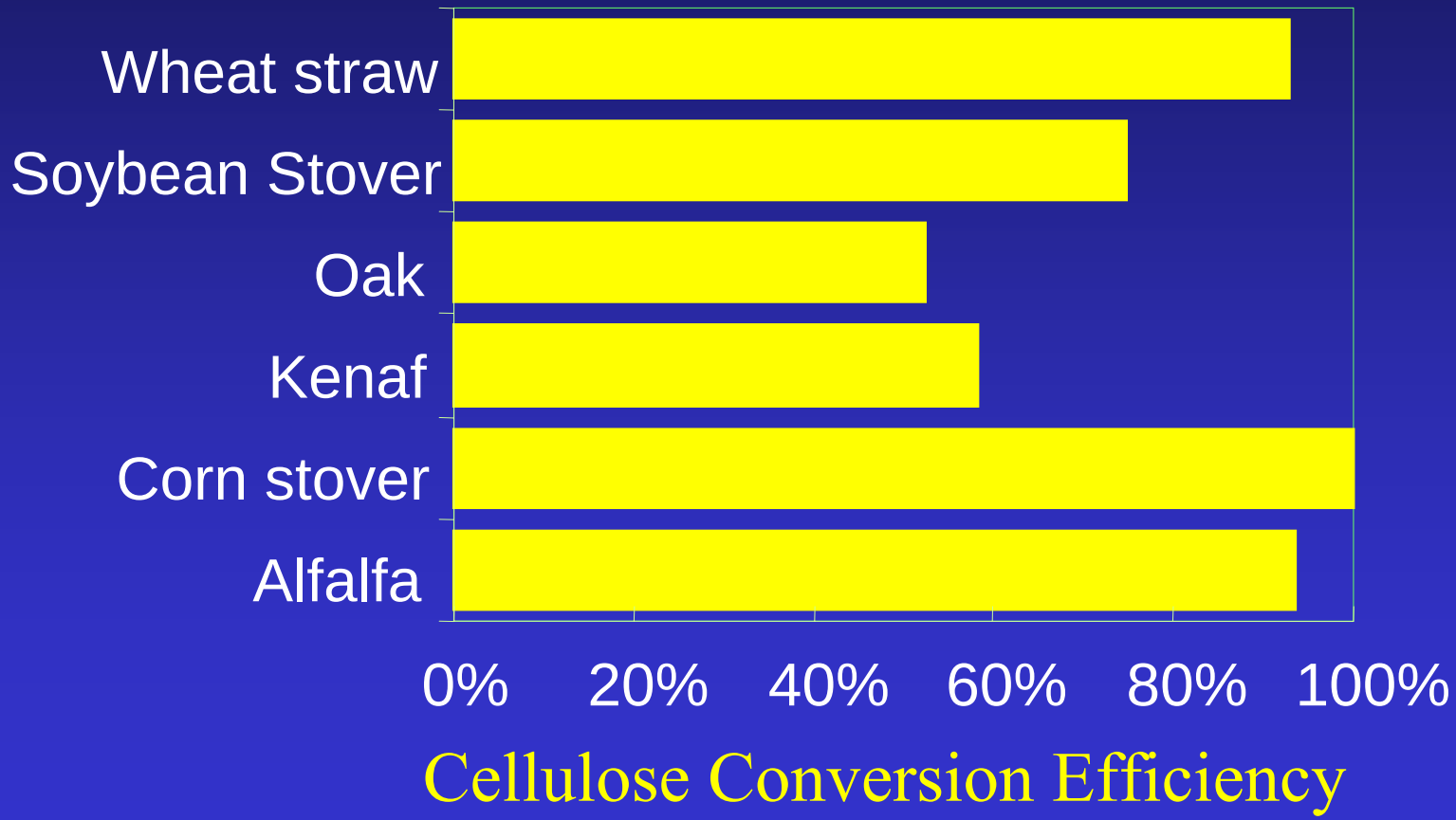
Funded by USDA

Comparison of yields for corn stover



Source of biomass matters!

Alkaline Peroxide Pretreated Biomass

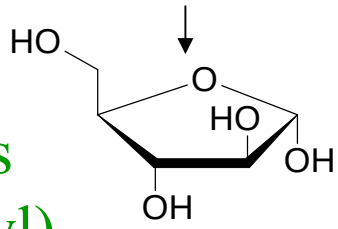


(Gould, 1984)

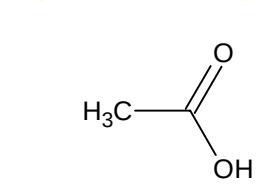
Inhibitor Removal

Inhibitors formed during hydrolysis

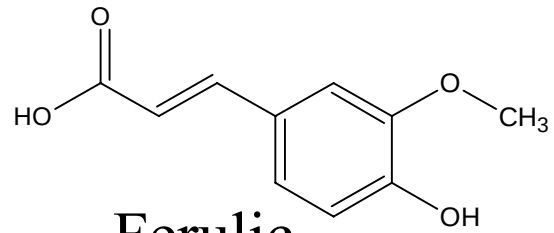
Hemicellulose



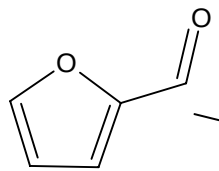
Pentoses (Ara, Xyl)



Acetic

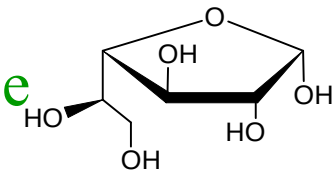


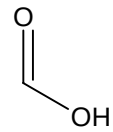
Ferulic



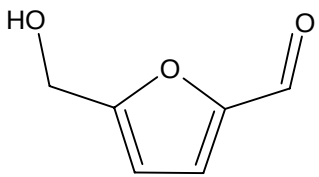
Furfural

Galactose





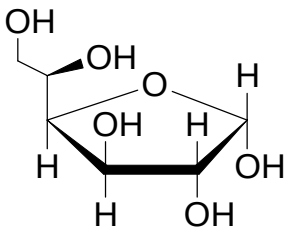
Formic

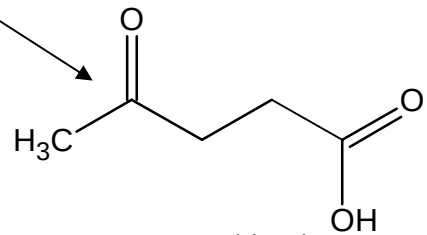


HMF

Cellulose

Glucose





Levulinic

Lignin

Phenolics

Conditioning hydrolysates

- **"Over-Liming"**
  - **Leonard and Hajny, 1945**
- **Solid Extraction**
  - **Ion-Exchange**
  - **Adsorption resin**
  - **Activated charcoal**
- **Liquid Extraction**
- **Bio-remediation**
  - **Laccase for removal of phenolic acids**
  - **Fungal removal**
- **Other partial solutions: adapting/evolving cells, increasing size of inoculum, media, or diluting hydrolysate**

Bioabatement: inhibitors removed by *Coniochaeta ligniaria* C8

Grew in Mineral Medium On :

Furfural, HMF, *p*-hydroxybenzaldehyde, ferulic acid, acetic acid, *p*-hydroxybenzoate, catechol, gallic acid, syringaldehyde, coniferyl alcohol, vanillin, and vanillic acid.

Did not Grow on:

Levulinic acid

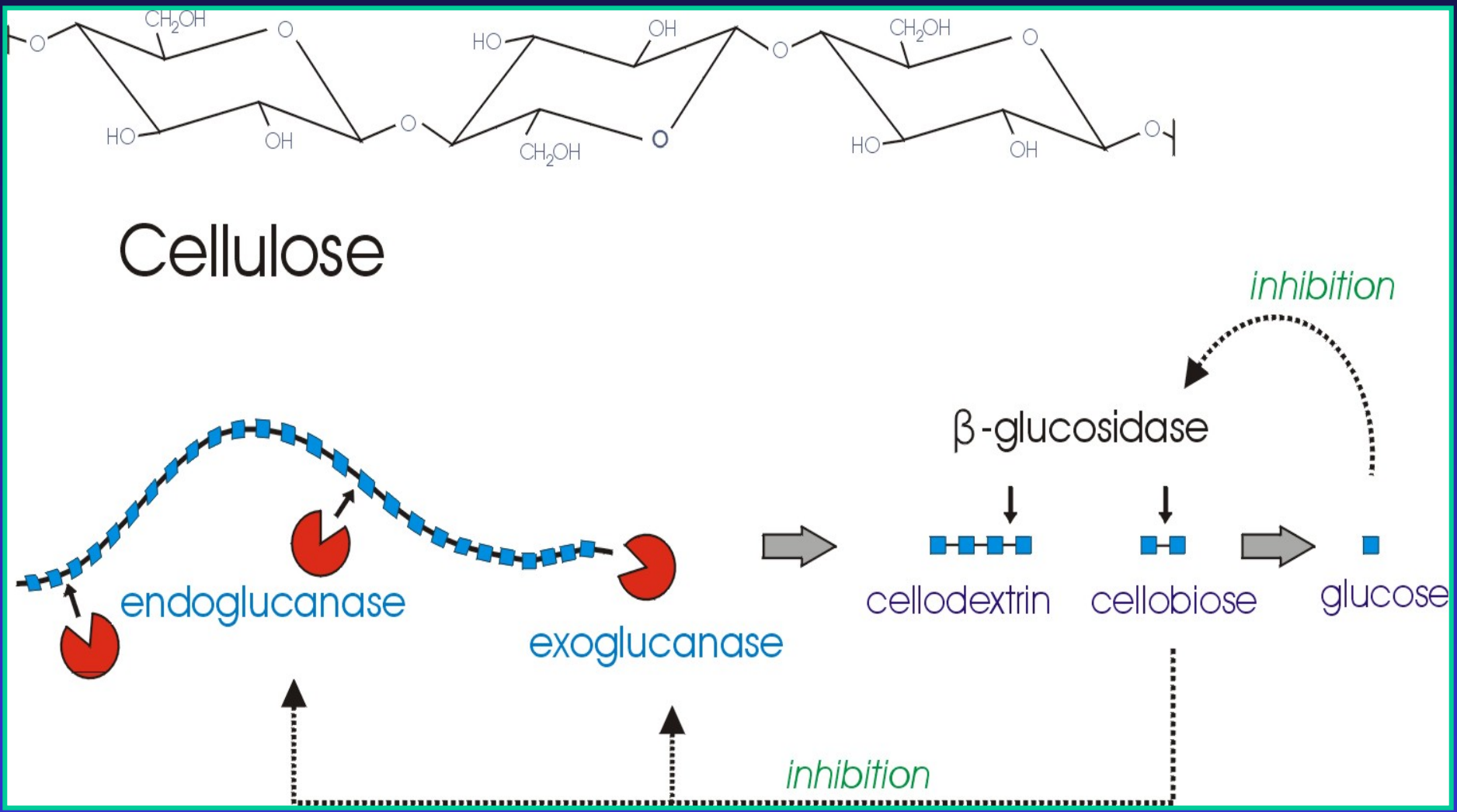
N.N. Nichols & M. Lopez, 2004

Enzymes

Enzymes important for processing biomass

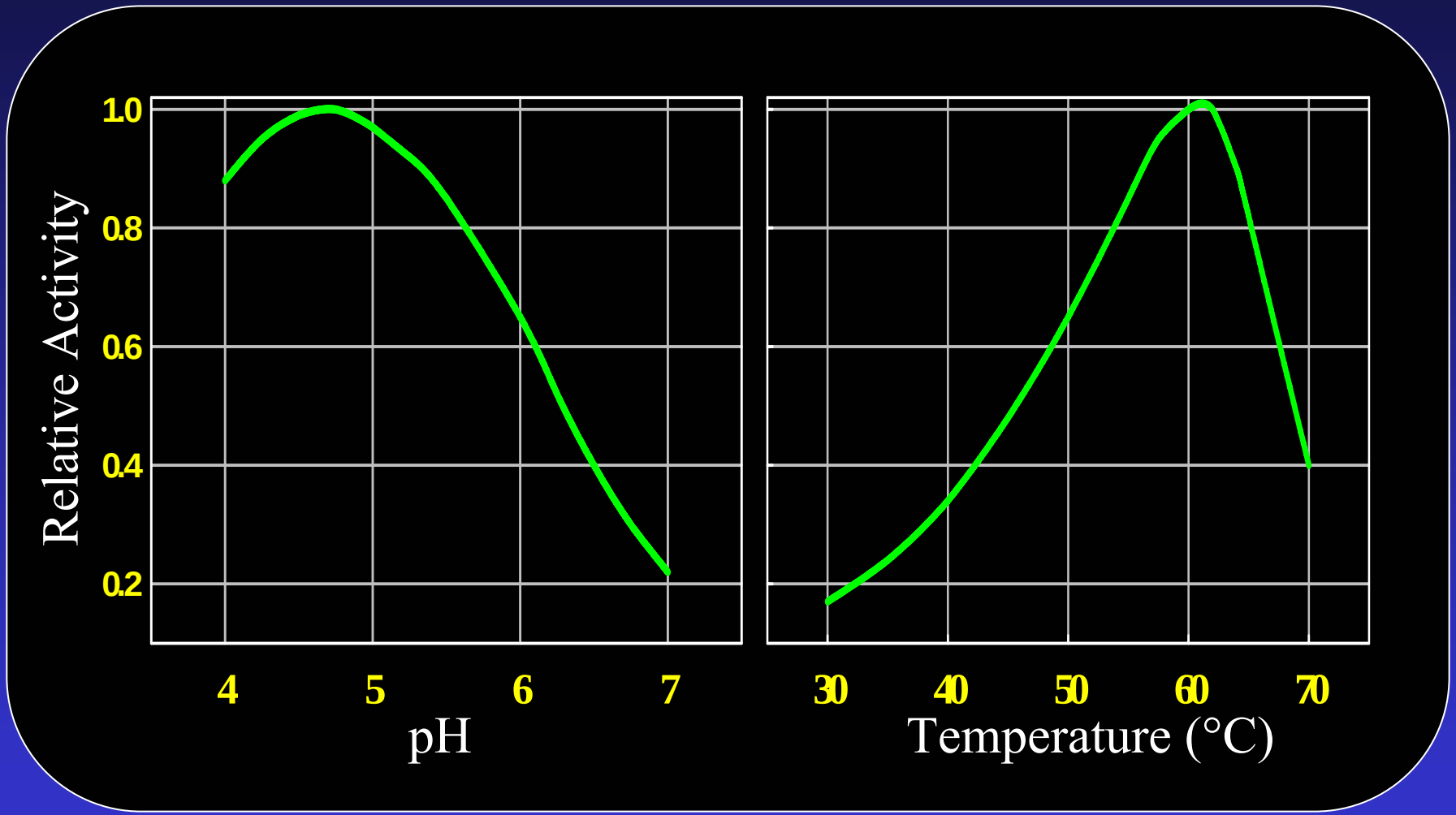
- Cellulases
  endo-1,4- β -D-glucanase , exo-1,4- β -glucanase (exocellobiohydrolase) and β -D-glucosidase (cellobiase).
- Hemicellulases
  endo-1,4-β -D-xylanase, EC-3.2.1.8), β -xylosidase (EC-3.2.1.37), other carbo-hydrolytic and esterases
- Ligninases
  Lignin peroxidase, manganese peroxidase, and laccase

How does cellulase work?



Genencor & Novozyme Price goal: 10-15¢/gal

What are the properties of *Trichoderma* cellulase?

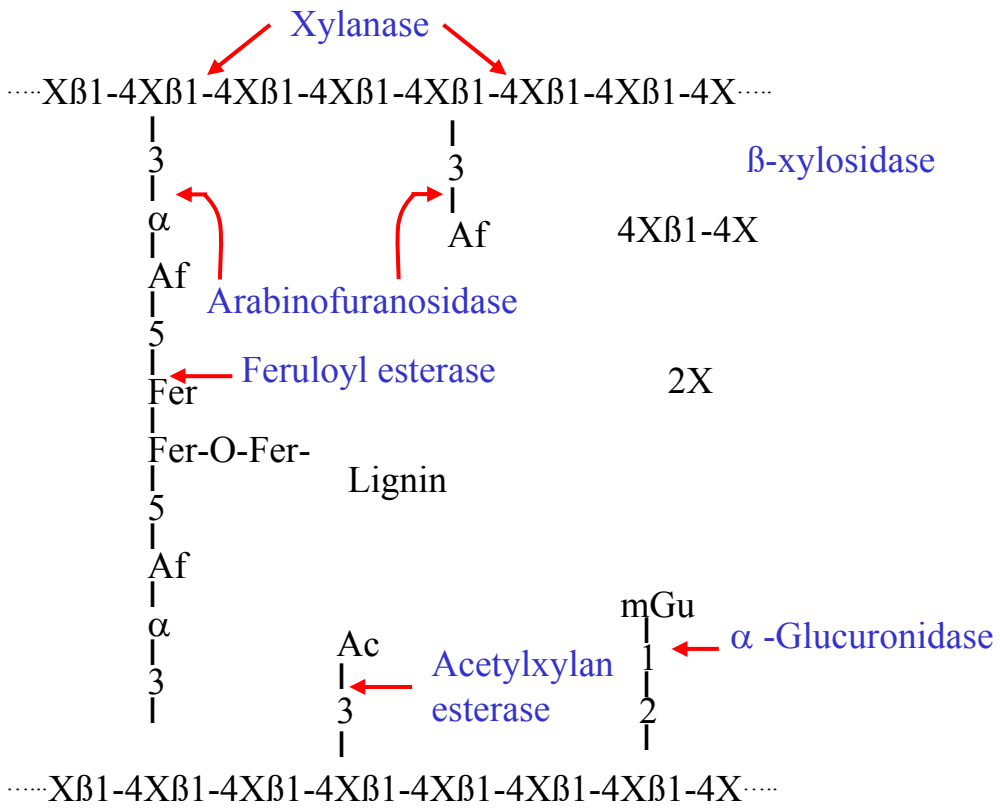


Tolan & Foody, 1999

Importance of hemicellulases for biomass conversion

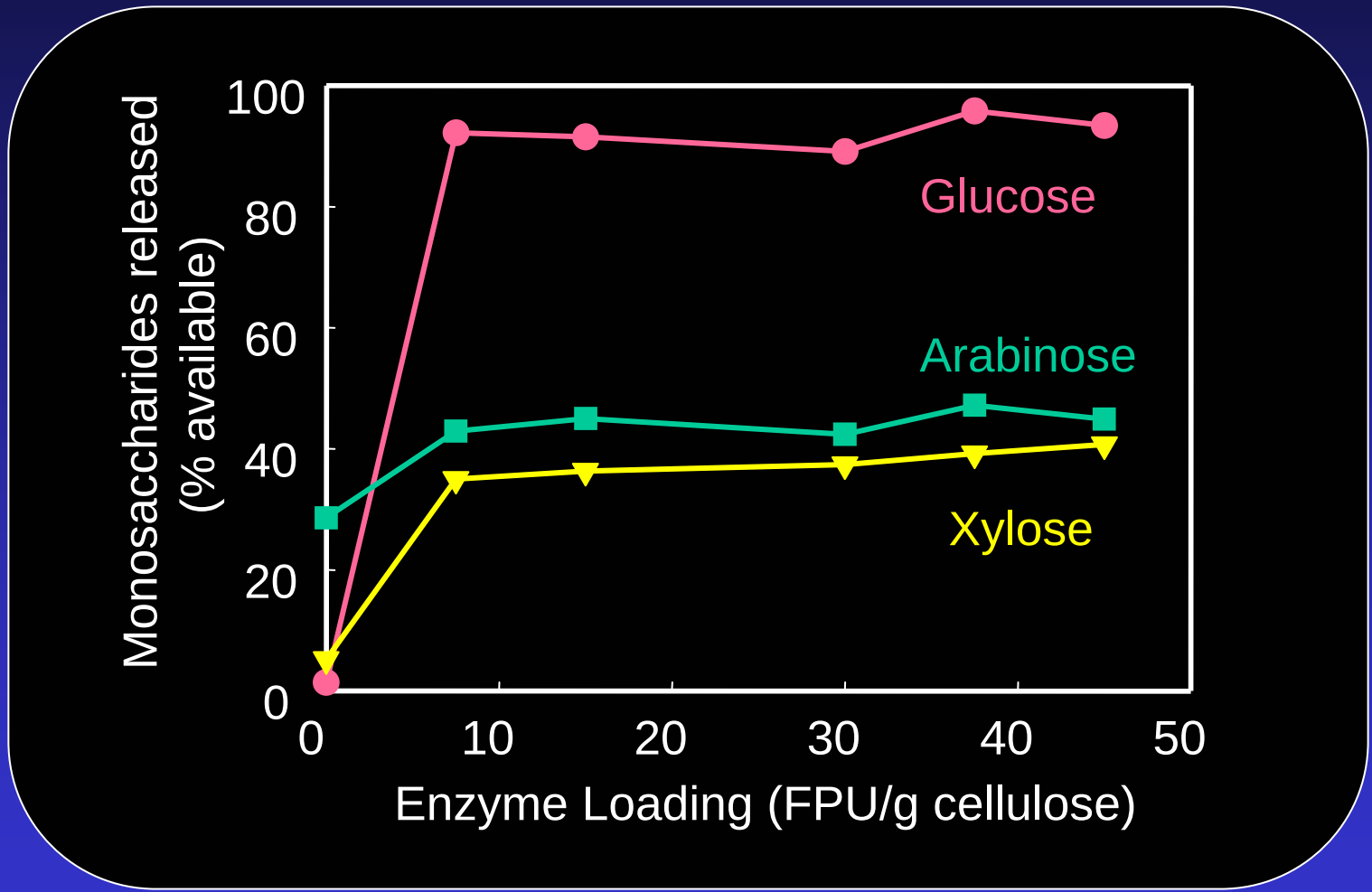
- An integral component of commercial cellulases – xylan removal a strong predicator of efficiency for glucose recovery.
- Most pretreatments do not convert hemicellulose to arabinose, xylose, etc. Hemicellulases are needed to complete saccharification.

Complex mixture of enzymes needed to degrade arabinoxylan



Selinger et al., 1996

Ineffectiveness of using a cellulase preparation on hot-water treated DDGS



Just adding GC220 Cellulase & Novo188 (40 U/g cellulose)

Activity comparisons for xylanases of various sources

Organism	Specific Activity * (μmol/min/mg)	Reference
Orpinomyces XynA	4,500	X. Li, 2005
Bacillus sp. strain T-6	288	Khasin et al., 1993
Clostridium sp. strain SAIV	36	Murty and Chandra, 1992
Fibrobacter succinogenes S85	34	Matte and Forsberg, 1992
Trichoderma longibrachiatum	130	Roger and Nakas, 1991
Aspergillus syndowii	204	Ghosh and Nanda, 1994
Aspergillus ficheri	588	Raj and Chandra, 1996

* All specific activities were compared on purified enzyme basis. The temperatures for assaying the enzymes were in the range of 40 to 50°C.

(X. Li, 2005)

Fermentation

Why Are Recombinant Microorganisms Needed for Ethanol Production?

Saccharomyces yeast do not ferment arabinose nor xylose.

Strains available for fermentation of glucose and pentoses

I. rec *Escherichia coli*

II. rec *Klebsiella oxytoca* (not discussed)

III. rec *Zymomonas mobilis*

IV. rec *Saccharomyces & other yeast*

V. rec Thermophiles (not discussed)

Two Major Strategies

- Efficient ethanol producer

  →Engineer to metabolize pentoses

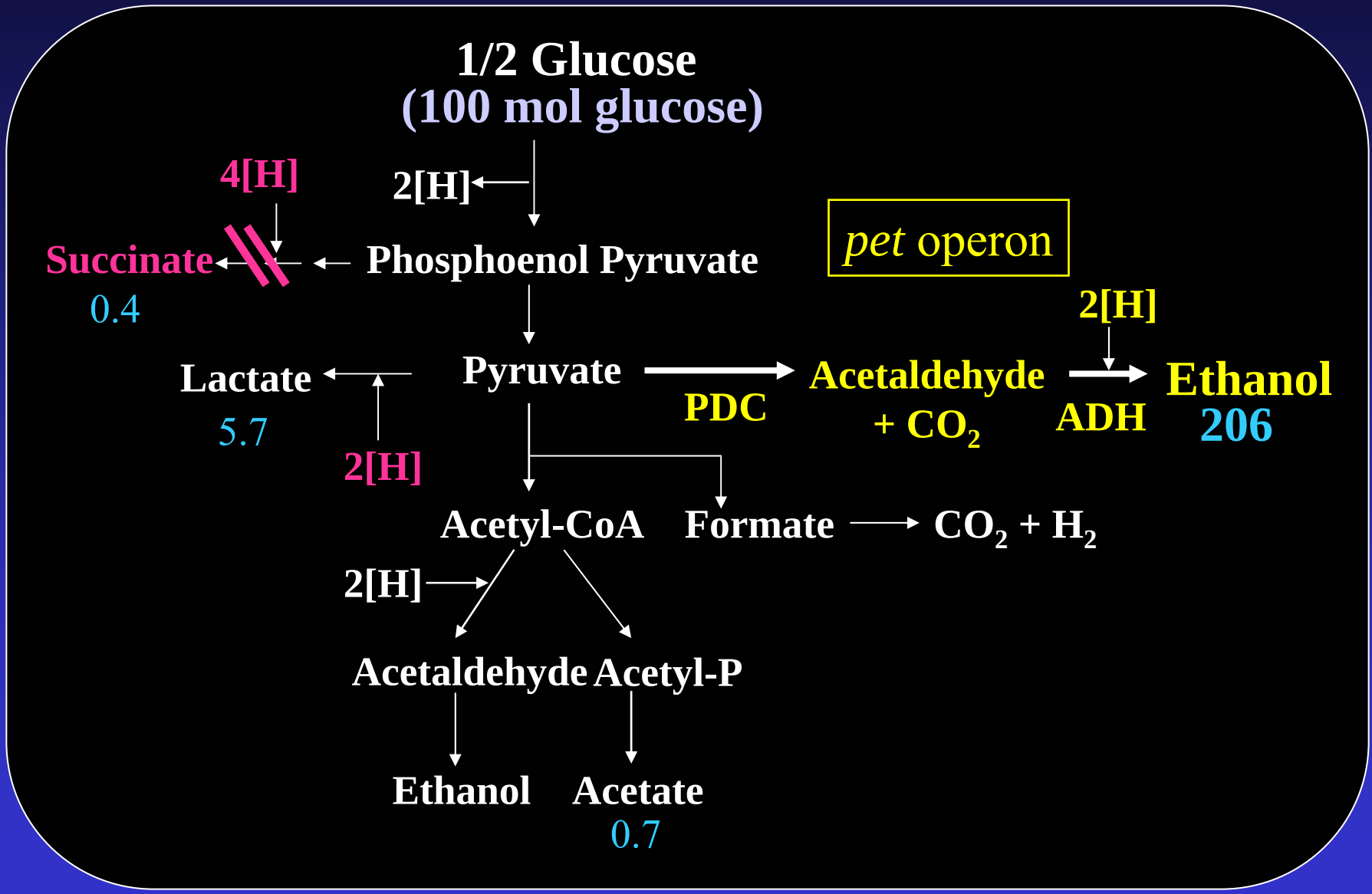
- Able to use wide-spectrum of sugars

  →Engineer to only produce ethanol

Metabolic engineering *E. coli* for efficient production of ethanol

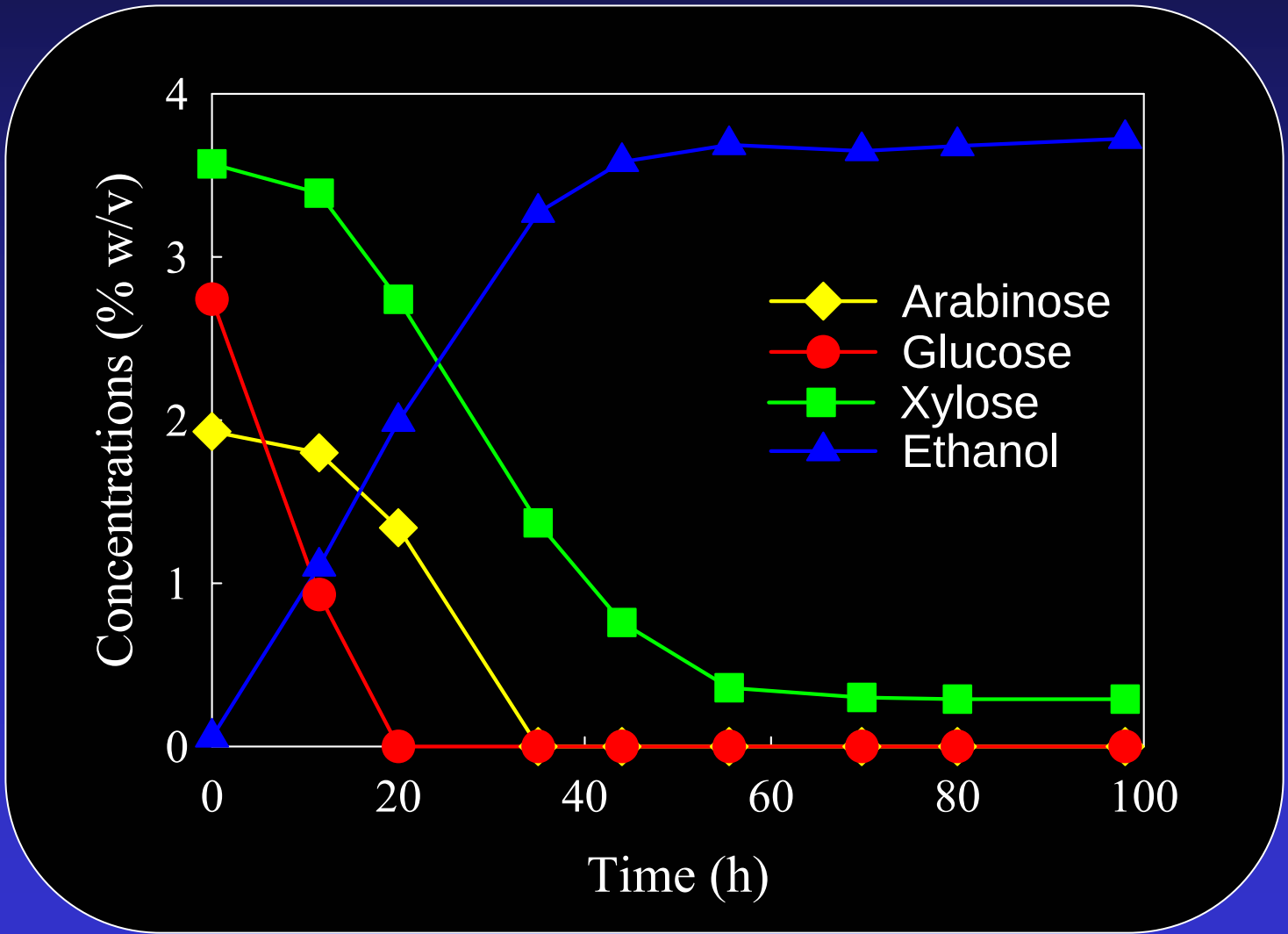
- First recombinant microorganism successfully developed for conversion of pentose sugars (arabinose and xylose) to ethanol (Ingram et al., AEM, 1987).
- *E. coli* ferments arabinose, glucose, mannose, and xylose.
- But, wild-type strains produce a mixture of fermentation products with little ethanol.

Construction of ethanologenic *E. coli* K011



Ohta et al., 1991

Ethanol fermentation of corn fiber hydrolysate by *E. coli* FBR5



Zymomonas mobilis

Anaerobic gram-negative bacteria that ferments glucose and fructose to ethanol with high yields and to high concentrations.

Traditionally used to produce Pulque and Palm Wine

High ethanol tolerance > 15% v/v & Glucose > 25% w/v

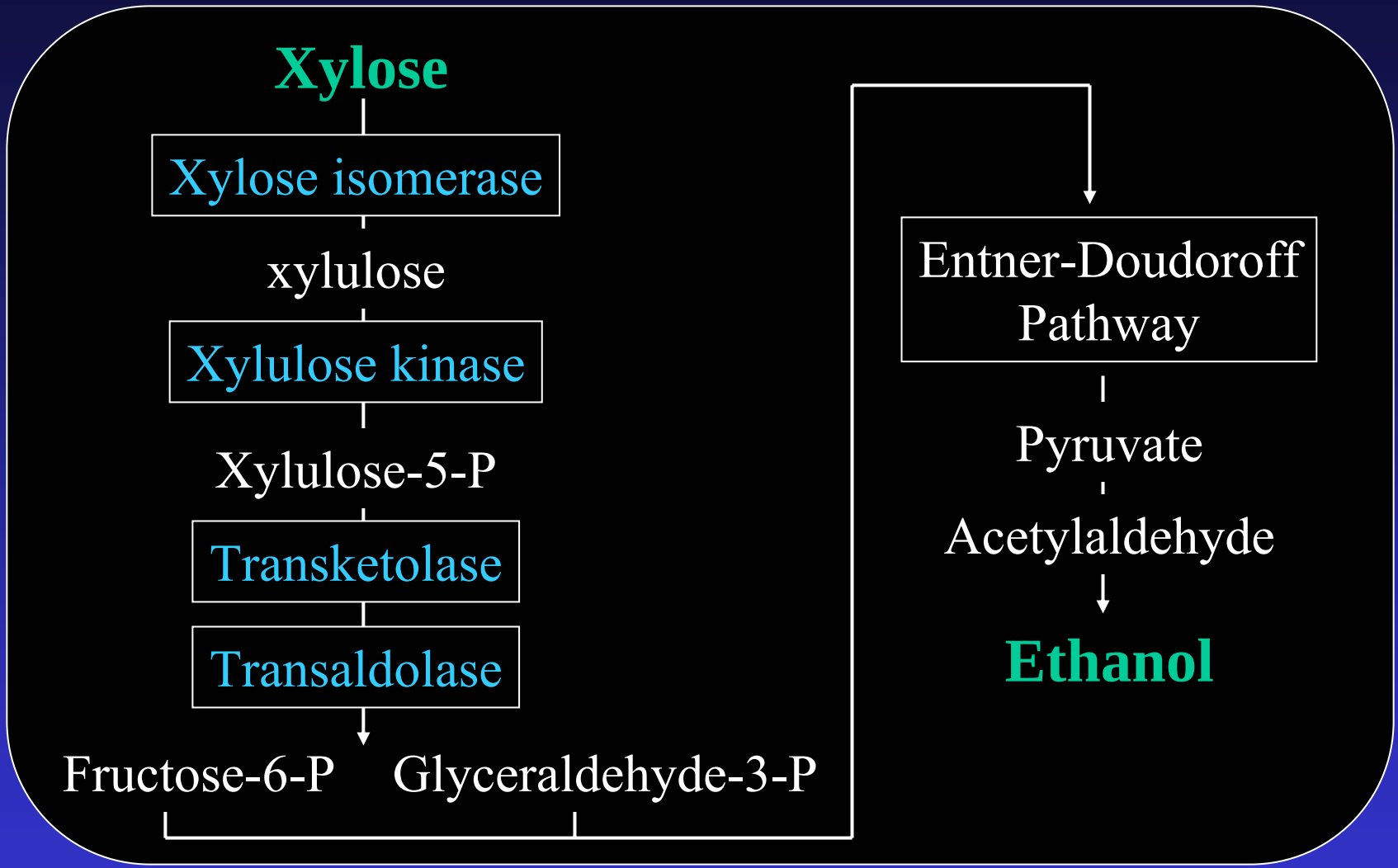
Higher specific glucose uptake rate and ethanol productivity (5x) than *Saccharomyces*

Grows at pH 3.8 – 7.5 & 25-35°C

Only microbe that used Entner-Doudoroff for anaerobic growth, which allows for higher ethanol yields than *Saccharomyces* (+5%)

Related to *Gluconobacter* and *Acetobacter*

Zymomonas Xylose Engineered Pathway



$$3\text{Xylose} + 3\text{ADP} + 3\text{Pi} \rightarrow 5\text{EtOH} + 5\text{CO}_2 + 3\text{ATP} + 3\text{H}_2\text{O}$$

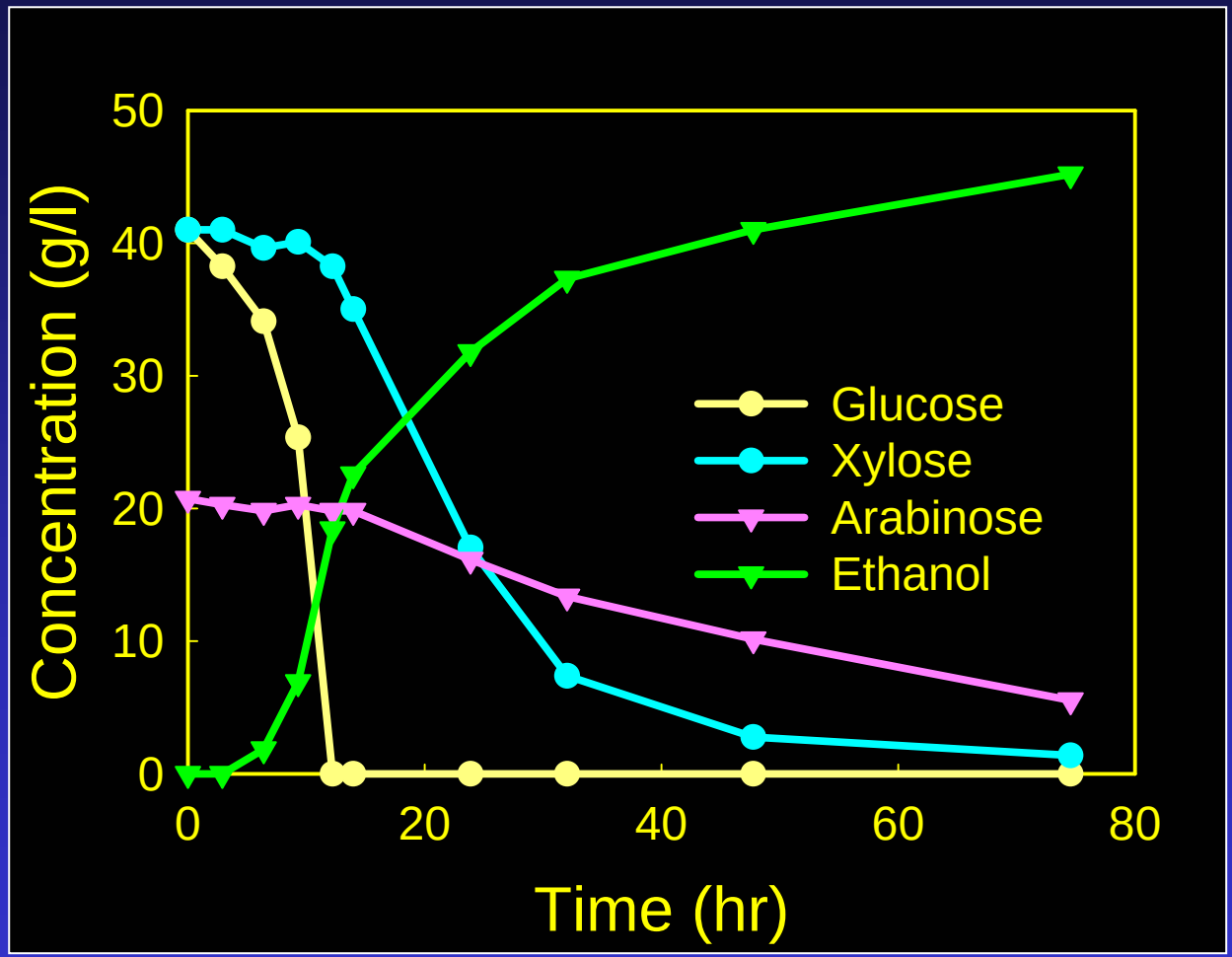
Zhang et al., 1995

Recent progress on *Z. mobilis*

- Strain AX101 (derived from ATCC 39676)
  - *Genes for Ara & Xyl utilization integrated
  - *Phenotypically/Genetically stable after 160 generations growing on glucose
  - *Produces less side-products
- Strain 8b (derived from ZM4/Ac^R; ATCC 31821)
  - *Genes for Xyl utilization integrated
  - * tolerates acetic acid > 16 g/l at pH 6
  - *85% EtOH yield on over-limed corn stover hydrolysate
- Genome sequence for ethanologenic bacterium *Zymomonas mobilis* ZM4 completed (Seo, J.-S et al. 2005)

(Zhang, Mohagheghi, Lawford and co-authors)

Fermentation of sugar mixture by AX101



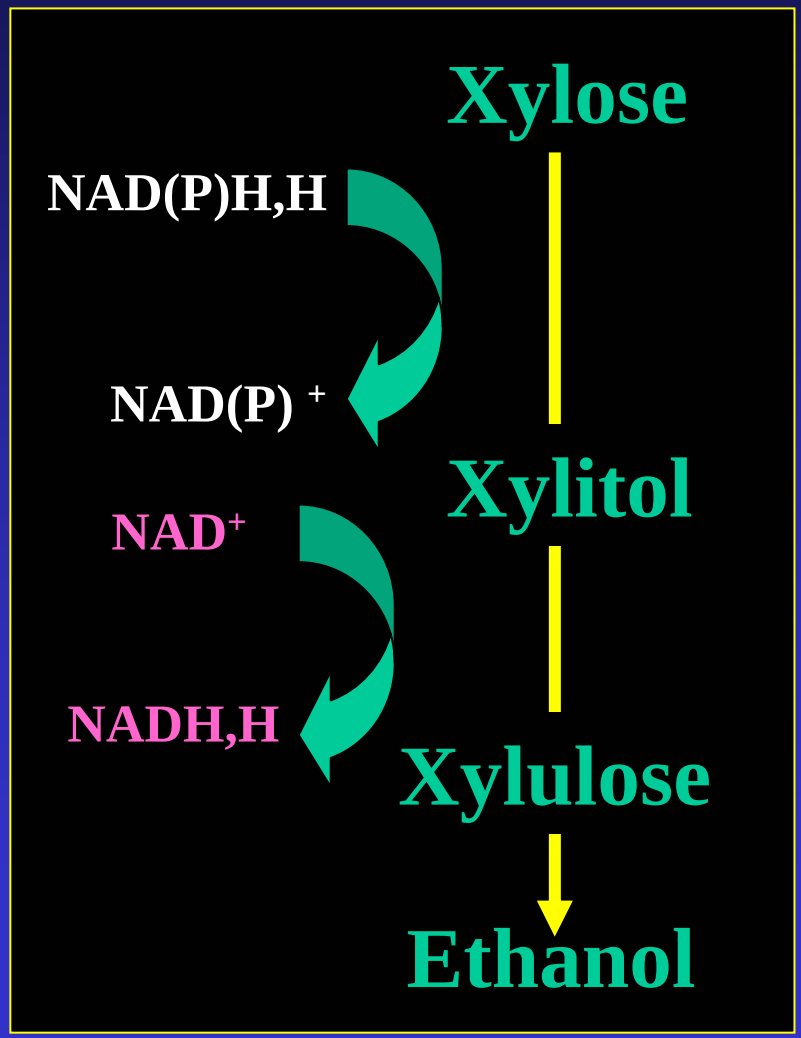
Data for pH 5.0	
μ_{max}(1/h)	0.34
q_p (g/g/h)	0.32
Q_p (g/L/h)	0.59
Yield (%)	82.6
Ac Tol (g/l)	2.5

(Mohagheghi ABB,2002 & Lawford ABB, 2002)

Approaches for developing xylose fermenting yeasts

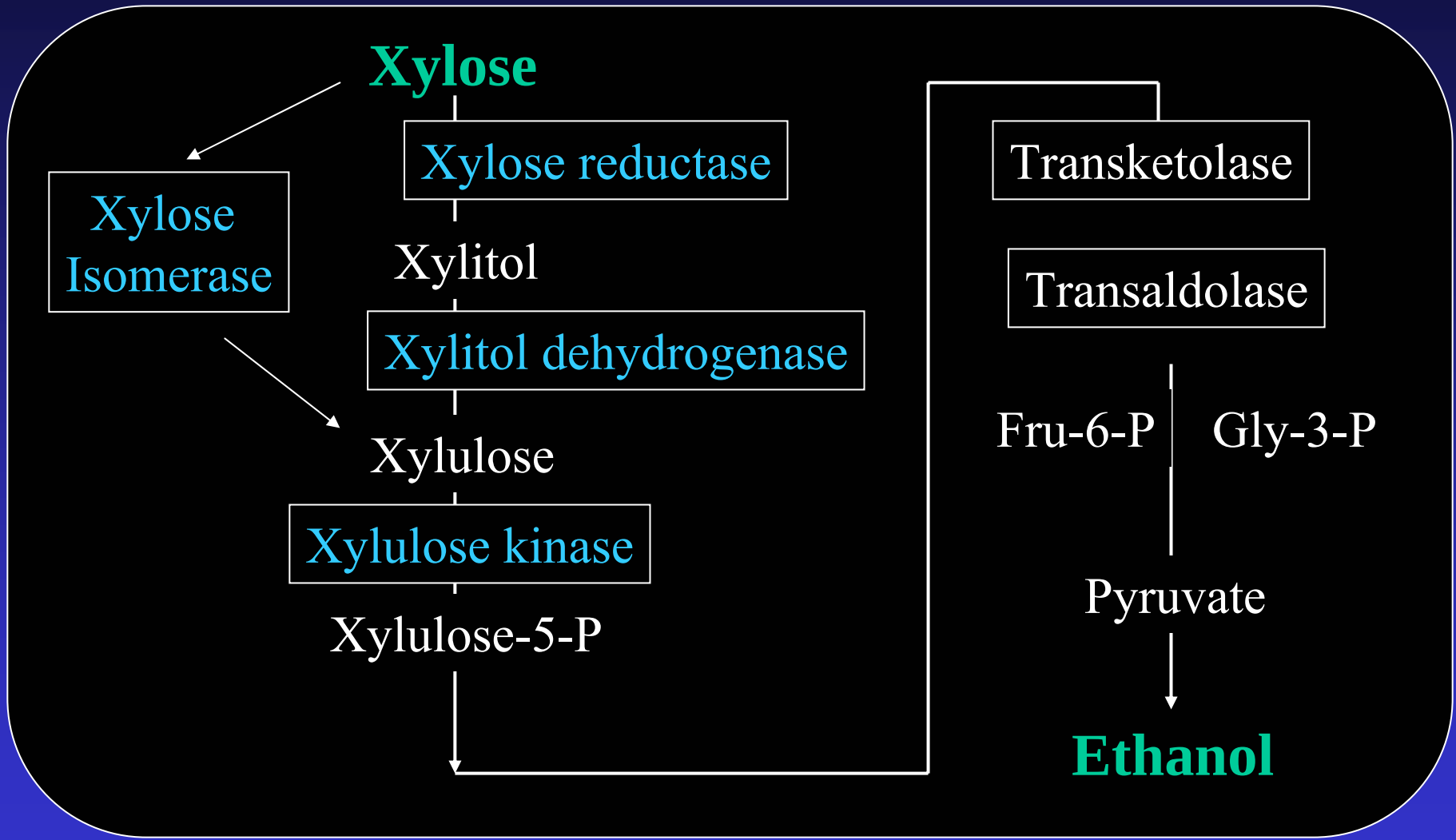
- Express xylose metabolic genes from natural xylose fermenting yeast strain *Pichia stipitis* in *Saccharomyces* (N. Ho, B. Hahn-Hägerdal, & L. Olsson)
- Express xylose isomerase gene from anaerobic fungus *Piromyces* sp. E2 in *Saccharomyces* (J.T. Pronk)
- Directly evolving *Saccharomyces* that ferment xylose via native pathway (van Zyl et al. 1989, Batt et al. 1986 and studied by Microbiogen & G. Sherlock)
- Engineering better native xylose fermenting *P. stipitis* (T. Jeffries)

Native xylose fermenting yeasts



- *Pachysolen tannophilus, Candida shehatae, and Pichia stipitis*
- Co-factor imbalance = xylitol (not *P. stipitis*)
- Min. O_2 for optimal ethanol
- Cytochrome C mutant increased ethanol yield by 21% (Shi et. al., Yeast, 1999)

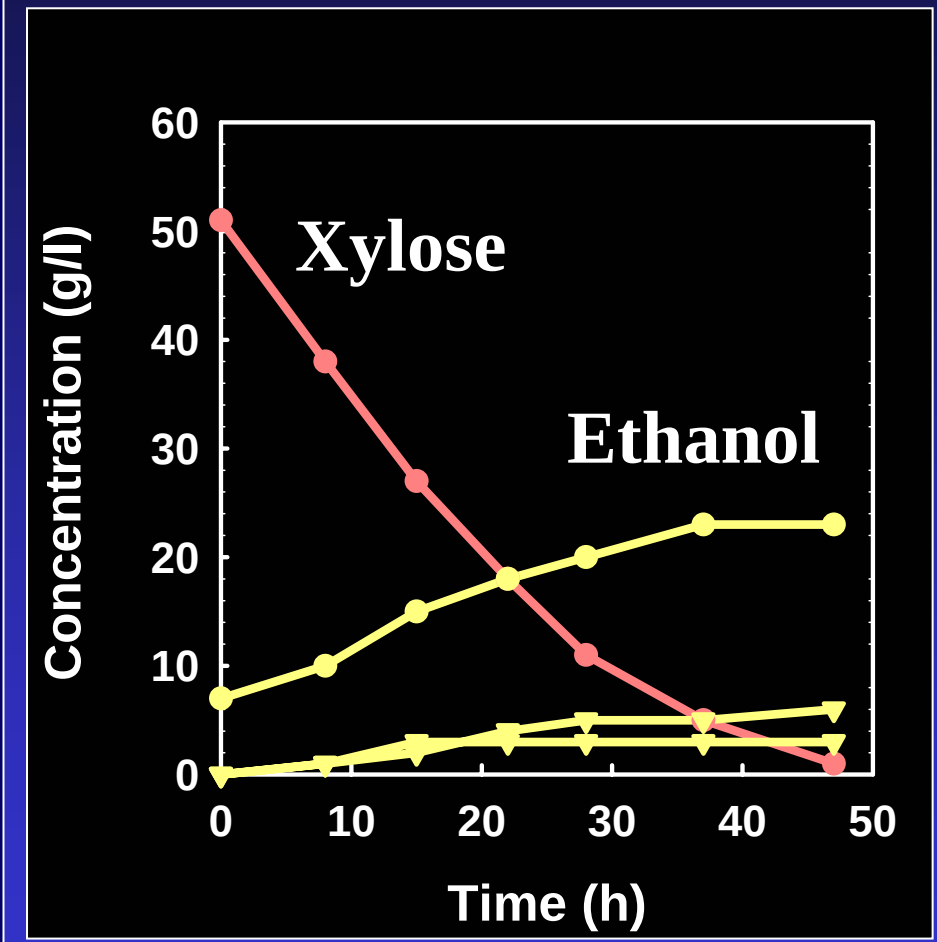
Saccharomyces Xylose engineered pathway



XI: *Piromyces sp. E2* / XR & XH: *P. stipitis*

Saccharomyces 1400 engineered for fermenting Xylose

- Yeast strain: thermotolerant *Saccharomyces* sp. strain 1400 (fusion of *S. diastaticus* & *S. uvarum*).
- Plasmid: XR and XDH from *P. stipitis* & XK from *S. cerevisiae (pLNH32).*
- Integration: multiple copies of each gene. Stable for 50+ generations on non-selective medium (LNH-ST).
- Licensed by Iogen. Strains selected for inhibitor tolerance.



(Ho et al., AEM, 1998)

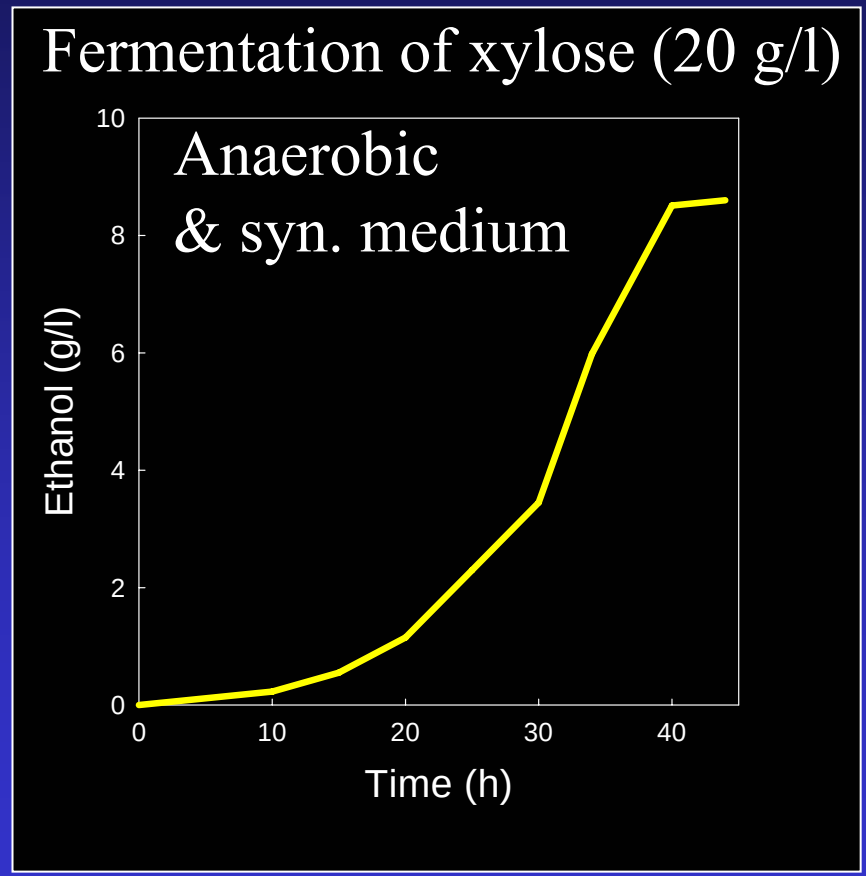
Saccharomyces cerevisiae engineered with *Piromyces* sp. E2 xylose isomerase

❖*S. cerevisiae* RWB217:
XI & XK expressed on plasmid
Over-expression of all 4 redox neutral PPP enzymes
Aldose reductase (GRE3) disrupted (↓xylitol)

Results: EtOH Yld = 0.43g/g, xylitol = 0.4 mM; Tgen = 7.7 h

❖RWB218: selected in chemostat for improved xylose transport

Results: EtOH Yld = 0.41g/g; xylitol = 0.2 mM, Tgen = 5.8 h (ferments G/X mixtures 2x faster)



(Kuyper et al., FEMS Yeast Res, 2005)

Comparison of traits

Host	Ara	Gal	Glu	Man	Xyl	Temp	pH
E. coli	+	+	+	+	+	35ºC	6.5
K. oxyotca	+	+	+	+	+	30 ºC	5.5
Z. mobilis	+	-	+	-	+	30 ºC	5.5
Saccharo-myces	-	+	+	+	+	30 ºC	4.5
P. stipitis	-	+	+	+	+	30 ºC	4.5

Comparison of performances

Host	Max. EtOH (g/l)	EtOH Yield (%)	EtOH Prod. (g/l/h)
E. coli	50-64	86-100	0.70-1.0
K. oxytoca	47	84-95	0.40-1.0
Z. mobilis	130 (68)	83-98	0.6-1.1
Saccharomyces	>150 (70)	64-88	0.5-0.6
P. stipitis	47	66-75	0.30

Where do we go from here?

- Feedstock: Breeding/engineering cultivars for higher ethanol yield as a quality trait
- Pretreatment: Role of cell wall structure & lignin for cellulose digestibility
- Enzymes: Designer *T. reesei* w/over-expressed native and xeno-enzymes (balance & synergy); Enzyme blends for high-temp/high-solids saccharification
- Biocatalysts: Improved *Saccharomyces* strains (redox balance, xylose transport); Improved inhibitor tolerance; Thermophilic homo-ethanol bacteria; Consolidated Processes
- Commercialization? Abengoa, Aventine, BCI, Dupont, Iogen etc.