Fermentative Conversion of Plant Biomass to Fuels and Commodity Products

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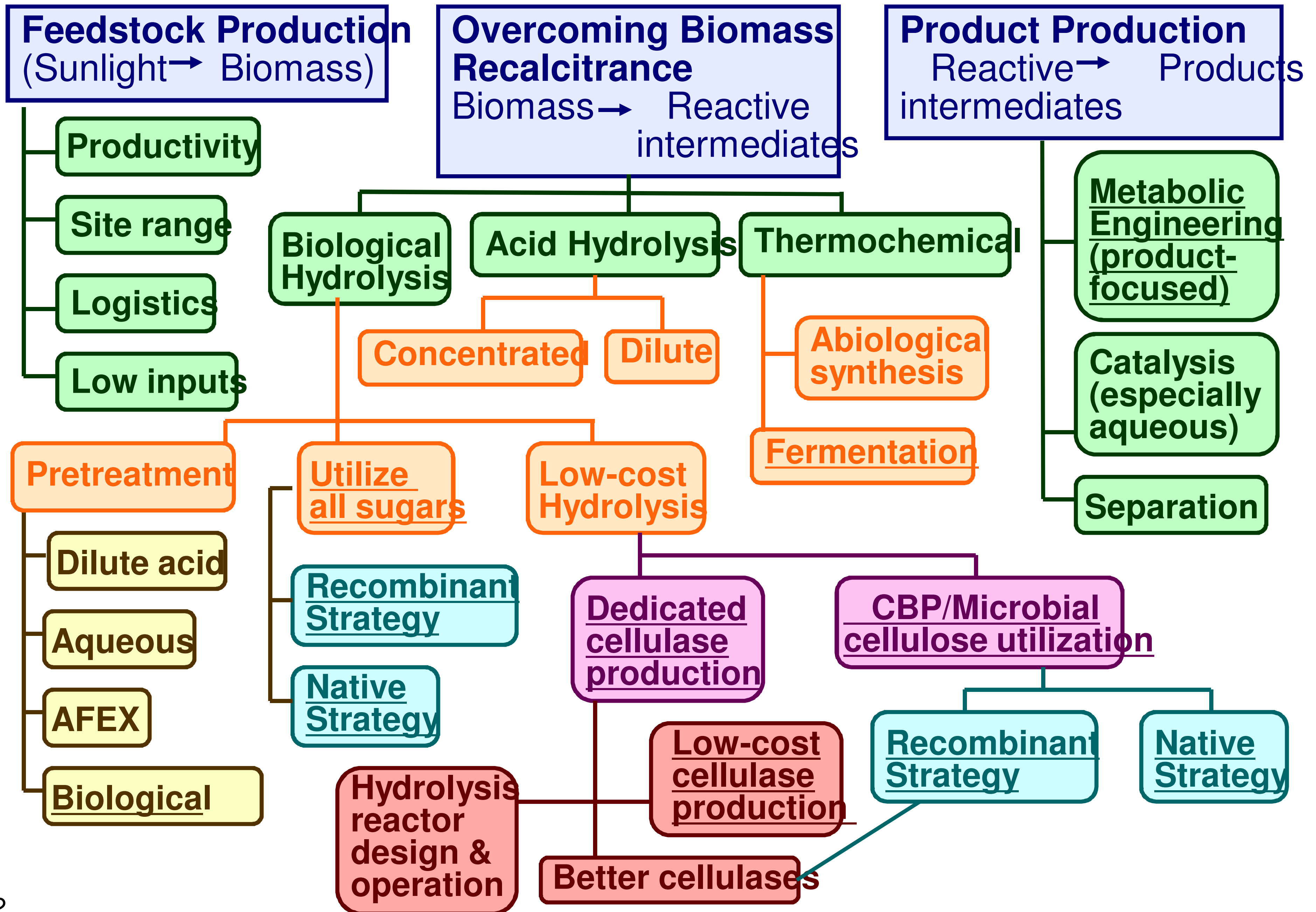
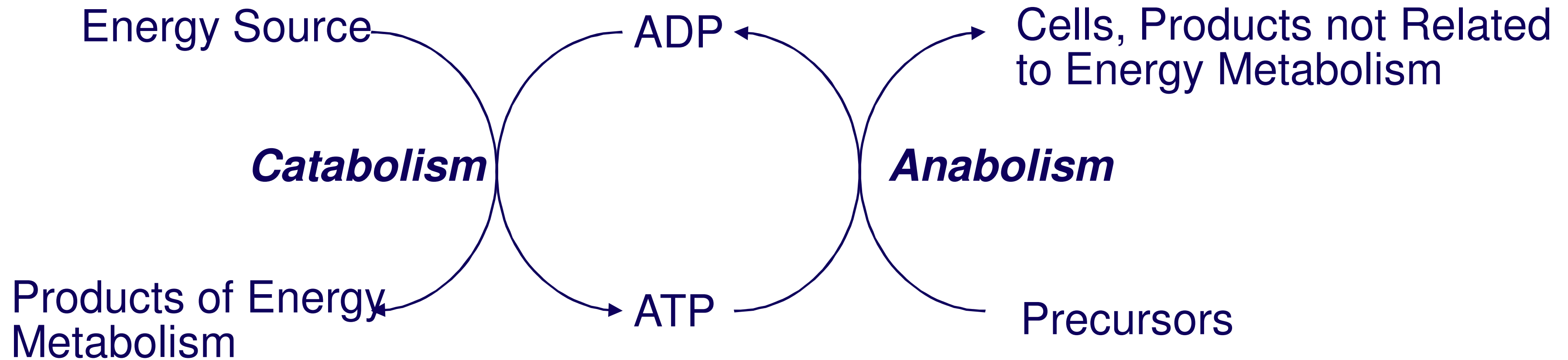
Overview of Fundamentals	**Lignocellulosic Processing**
Metabolic context	Process configurations
Tools	Fermentor design
Feasibility criteria	Organism design
Performance metrics	Economic drivers

Biofuels R&D Landscape (fermentations underlined)



Metabolic Context

Metabolism: The sum total of cellular processes resulting in cell maintenance & gro



Catabolic products

Often end-products of anaerobic, or effectively anaerobic, metabolism - potentially genetic manipulation

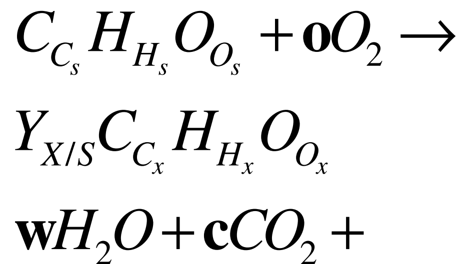
e.g. Alcohols (ethanol, butanol…), organic acids (lactic, acetic, succinic…), meth

Anabolic products

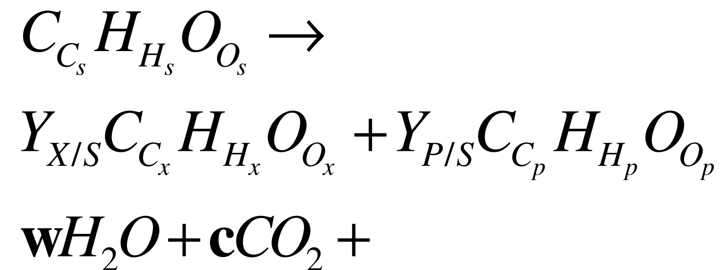
Amino acids, protein(s), therapeutics, probiotics, vitamins, antibiotics, secreted intermediates (e.g. shikimic acid), fuels (butanol from amino acid metabolism, Lia et. al.; isoprenoid derivatives, Keasling, Amyris)

Products of combined biological and abiological synthesis (many)

	Oxidative metabolism	***Non-oxidative metabolism***



$$C_{C_s}H_{H_s}O_{O_s} + \mathbf{o}O_2 \rightarrow Y_{X/S}C_{C_x}H_{H_x}O_{O_x} \; \mathbf{w}H_2O + \mathbf{c}CO_2 +$$



$$C_{C_s}H_{H_s}O_{O_s} \rightarrow Y_{X/S}C_{C_x}H_{H_x}O_{O_x} + Y_{P/S}C_{C_p}H_{H_p}O_{O_p} \; \mathbf{w}H_2O + \mathbf{c}CO_2 +$$

	Oxidative metabolism	Non-oxidative metabolism
ATP synthesis:	Respiration, 36 ATP/glucose	Substrate-level phosphorylation, 1 to 4 ATP/glucose
Cell yield, $Y_{X/S}$:	~ 0.5	~ 0.1
Product yield, $Y_{P/S}$:	~ 0	0.5 to 0.9 common
O_2 transport:	Major design, scale-up issue	Not needed, easily prevented
Fate of feedstock energy/reducing power:	Heat, cells	> 90% typically in organic produ
Heat production:	~0.5 x Feedstock heating value	~ 10% feedstock heating value

Oxidative and non-oxidative metabolism can be combined, but not without decreasing product yield.

Converting a large fraction of the feedstock mass & energy to organic products requires that most or all metabolism proceed non-oxidatively.

Key feature of non-oxidative metabolism: Conservation of reducing p

Formalized in terms of available electrons

Conceptual: Electrons that would be transferred to oxygen upon hypothetical oxid of an organic compound (or aggregation of compounds) to CO_2 and water.

Quantitative:

For formula $C_c H_h O_s$,

the number of available electrons per mole or fomula weight is given by:

$$\text{Av. e}^- \text{/mole (or FW)} = 4c + h - 2o$$

(derivation attached as an appendix)

Examples:

Glucose: $4*6 + 12 - 2*6 = 24$ av. e^- / mole

Xylose: $4*5 + 10 - 2*5 = 20$ av. e^- / mole

Ethanol: $4*2 + 6 - 2*1 = 12$ av. e^- / mole

Note that water and CO_2 both have zero available electrons

Calculations using available electrons

- Calculating theoretical product yields
- Checking data consistency based on conservation (e.g. % available electron recov
- Comparing oxidation states and determining stoichiometric coefficients of electron donors/acceptors

Illustrative example

Anaerobic catabolism of xylose to ethanol:

$$C_5H_{10}O_5 \rightarrow Y_{P/S} C_2H_6O + \mathbf{c} CO_2$$

Available electron balance:

$$20 \text{ av. e}^- \text{/mole xylose} = Y_{P/S} * 12 \text{ av. e}^- \text{/mole EtOH} \Rightarrow Y_{P/S} = 20/12 = 1.67$$

Carbon balance:

$$5 \rightarrow 1.67*2 + \mathbf{c} \Rightarrow \mathbf{c} = 5 - 3.33 = 1.67$$

So we have:

$$1 \text{ Xylose} \rightarrow 1.67\text{EtOH} + 1.67\text{CO}_2$$

$$\% \text{ substrate heating value in ethanol: } \eta = 100 \times \frac{1.67 * 295 \text{ kcal/mol EtOH}}{510 \text{ kcal/mol xylose}} = 96.6\%$$

Tools

Genetic Engineering (targeted cellular genome changes)

Most powerful for pathway creation - can in principle enable production of any stoichiometrically & bioenergetically feasible product at theoretical yields

Less powerful for intrinsic properties determined by multiple genes - e.g. product tolerance, growth at high temperatures, low pH.

Tools for genome manipulation are available for only a few hosts - a much bigger limitation for commodity products than biopharmaceuticals

Protein Engineering (targeted protein changes)

Dramatic examples of changing enzyme function

Fewer, less dramatic, examples of improving best-in-class performance

Changing a protein is the easy part, knowing what to change is the challenge

Interfaces between sequence, structure and function far from understood

Non-rational approaches (find what you want in a variable population)

Proteins - directed evolution enabled by high throughput screening

Cells - “evolutionary biotechnology” enabled by selection in the lab

Tools, continued…

Systems biology - measure/observe many things at once

Genes (genome)

Proteins (proteome)

Metabolites (metabalome)

Reactions (fluxome)

Bioinformatics - insights from sequence information

Quantitative Analysis

Design/prediction/optimization

Structure & test understanding

Applied to

Hydrolysis kinetics

Metabolic stoichiometry

Metabolic reaction rates (metabolic control theory, stuctured modelling)

Synthetic biology

Biocatalyst design (rather than modification) from first principles

Feasibility Criteria

To make a desired product

A pathway (set of enzymatically-mediated steps connecting feedstock & product)

Regenerated electron carriers

For cell growth

Net production of ATP

Availability of precursors for cell synthesis

Growth-compatable conditions (temperature, pH, tolerable inhibitors)…

Performance Metrics

Product titer, *P* (moles or mass per unit volume)

Determinant of separation costs, yield, and productivity

Often limited by the tolerance of the biocatalyst

Tolerance to added product may be > than the maximum concentration Produced at the start of process development. Available examples suggest that this discrepancy can usually be remedied with sufficient effort.

Performance Metrics, continued…

Product yield, $Y_{P/S}$ (dimensionless)

Commonly reported two ways

Product produced per substrate fermented

Product produced per substrate present initially (batch) or fed (continuous)

$$Y_{P/S} = \frac{P - P_o}{S_o - S}$$

P = product (moles, concentration, or mass, as appropriate)

S = substrate (moles, concentration, or mass, as appropriate)

Subscript o denotes initial (batch) or entering (continuous)

Critically important when the margin between product value & feedstock cost is small - commodity products in general, fuels in particular

Performance Metrics, continued…

Productivity, P (moles or mass product per unit volume per unit time)

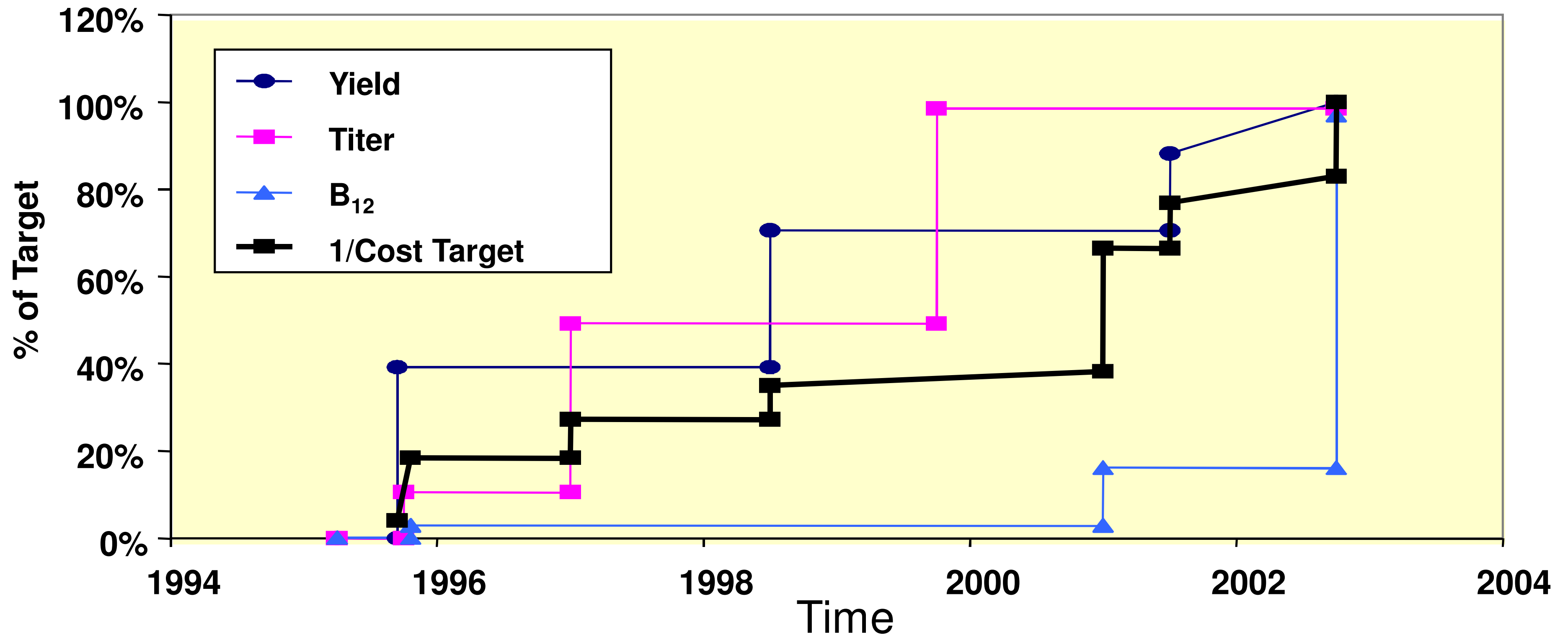
$$\text{P} = \frac{P - P_o}{\tau} = \text{Y}_{P/S}\,\overline{r_S}$$

= time (batch) or residence time (continuous) over which product is produced

$\overline{r_S}$ = time-average reaction rate

Determinant of reactor volume (V = R/ P, where R = rate of production)

Illustrative Product Development Trajectory: 1,3-Propane Dic



- Variable application of theoretical and empirical approaches
- Critical path determined by cost model
- Critical path dominated by Yield, Titer, B12 > Rate and CPI
- Multiple work streams were integrated into a converging strain lineage which ultimately provided a commercial strain
- Not all work streams made it into the commercial strain

Courtesy of John Pierce,DuPont

Fermentor Design: Performance Enhancement

	Soluble Substrates	Lignocellulose Particles
Productivity enhancement	↑ Cell concentration (cell recycle, immobilization)	↑ Hydrolysis rates, yields (↑ enzyme, [substrate], substrate reactivity)
	↓ Product inhibition (cascade reactors, product removal)	↓ Product inhibition (cascade reactors, product removal, keep [sugar] low)

↑ Product production (↑ S_o, $Y_{P/S}$)

Yield enhancement

↑ Hydrolysis yields

↓ co-product production
(metabolic engineering, strain choice)

↓ cell production
Limit nutrients, strain selection

Utilize all sugars present
Metabolic engineering, strain choice

Fermentor Design: Scale-up

Extensive literature on scale-up of aerobic processes, very little publically-available information - empirical or analytical - for anaerobic scale-up.

Fundamentals indicate anaerobic scale-up is much more robust

$_{reactant}$, reactant residence time = $\dfrac{\text{Reactant Concentration (g/L)}}{\text{Reactant Reaction Rate (g/L*s)}}$

t_m, mixing time = time to achieve local concentrations within 5% of average

Aerobic

$_{O2}$, ~ 1 s in industrial bioreactors.

Spacial heterogeneity and locally oxygen-limited conditions arise because $_{O2} << t_m$.

Discrepancy between $_{O2}$ and t_m becomes greater at increasing scale.

Anaerobic

Sugars tend to be homogeneously distributed because $_{sugars} >> t_m$

Scale-up of anaerobic may be approached with little risk, large scale-increments.
Bioreactors for anaerobic processes are/will be much larger than for aerobic.
Caution: Experience indicates scale-up of processes with solids is challenging.

Organism Design

Considered here with respect to two goals

Utilization of non-glucose sugars

Consolidated bioprocessing

…and two organism development strategies

Native strategy - start with organisms that utilize desired substrates (non-glucose sugars, cellulose), modify to improve ethanol yield, titer

Recombinant strategy - start with organisms that produce ethanol at high yield and titer, modify so that desired substrates are utilized

Utilization of Non-Glucose Sugars

Primary targets

Xylose - main component of angiosperm hemicellulose

Arabinose - minor component of hemicellulose, major component of corn fiber

Native Strategy - start with bug that can use non-glucose sugars

Organism	Primary Modification	Lead Group
Enteric bacteria *(E. coli, K. oxytoca)*	Express ethanol-forming genes (Pet operon)	L. Ingram (U. Florida)
Xylose-utilizing yeasts (e.g. *Pichia stipitis*)	Disrupt respiratory function (*cyc*1, *sto*1)	T. Jeffries (FPL)
Xylose-utilizing thermophile (*T. saccharolyticum*)	Knockout genes associated with by-products (*ldh*, *ack/pta*)	Lynd (Dartmouth)

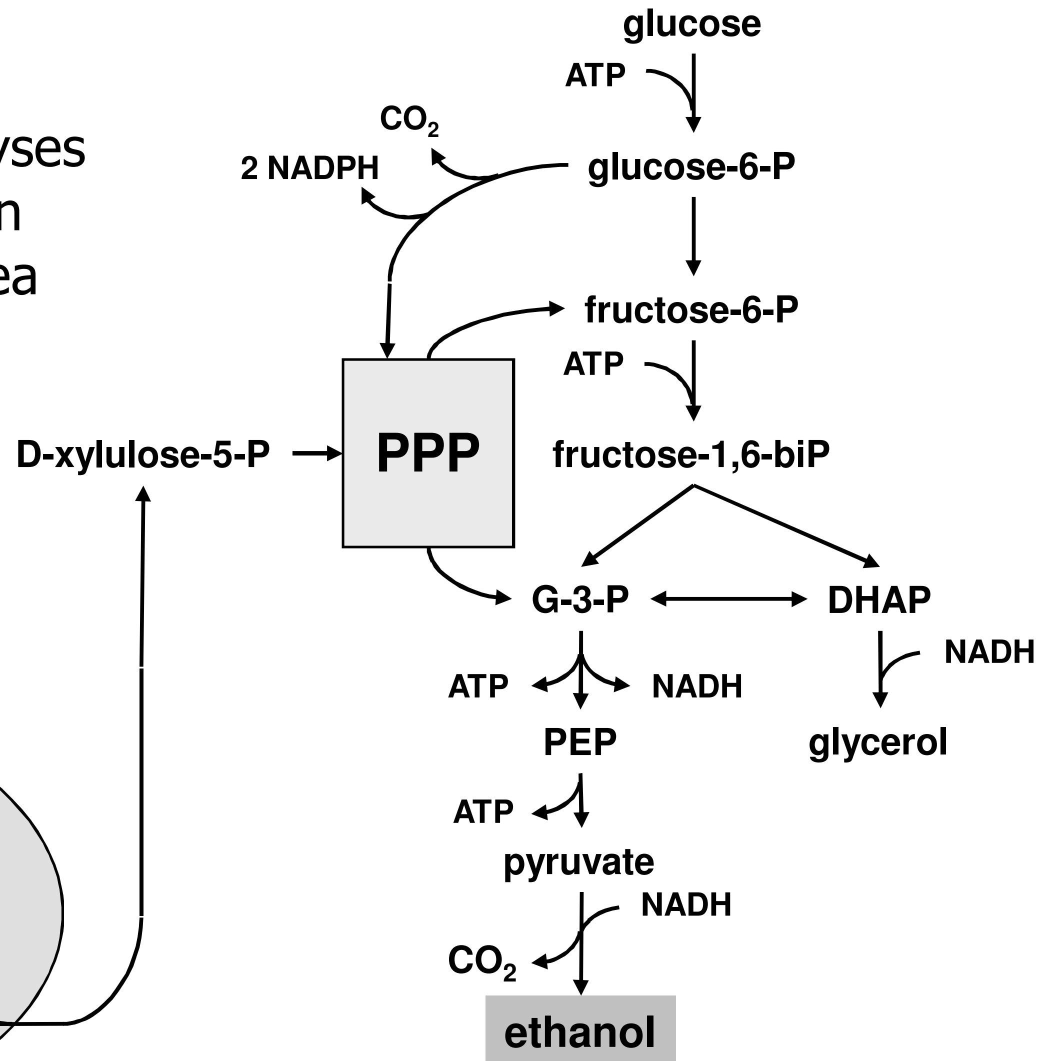
Utilization of Non-Glucose Sugars, continued…

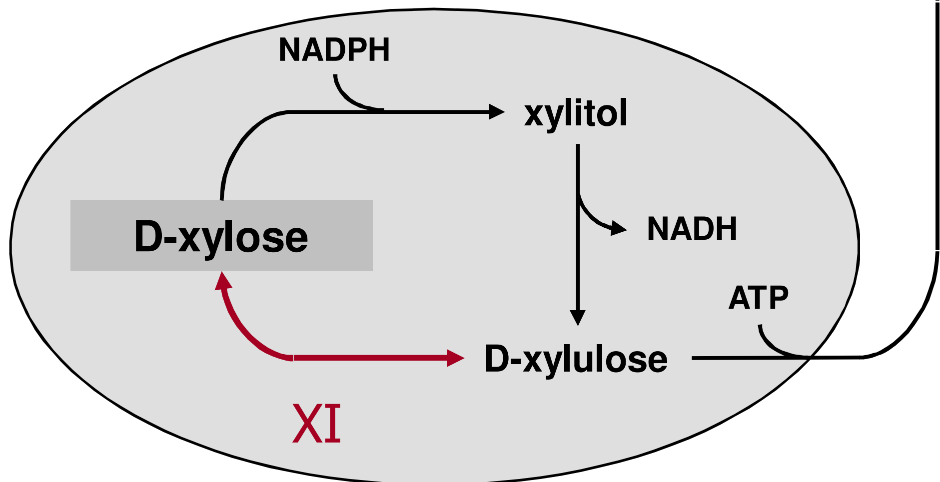
Recombinant Strategy - Start with bug that produces product well

Organism	Primary Modification	Lead Group
Zymomonas mobilis (mesophilic, anaerobe)	D-Xylose: + Xylose isomerase, xylulokinase, transaldolase, transketolase L-Arabinose: + arabinose isomerase, ribulokinase, ribulose-5-phosphate-4-epimerase	M. Zhang & NREL team,
Saccharomyces sp.	Xylose reductase, xylitol dehydrogenase	N. Ho (Purdue)
	Xylose isomerase expressed in *S. cerevisiae*	Pronk, van Dijken (Delft)
	Xylose reductase, xylitol dehydrogenase in *S. cerevisiae*	Hahn-Hagerdal (Lund)
Klyveromyces sp.	Xylose isomerase expressed	Cargill Dow/ Nature Works

Alternative Pathways for Xylose Fermentation

- xylose isomerase (XI) catalyses xylose/xylulose isomerisation
- common in Bacteria, Archaea

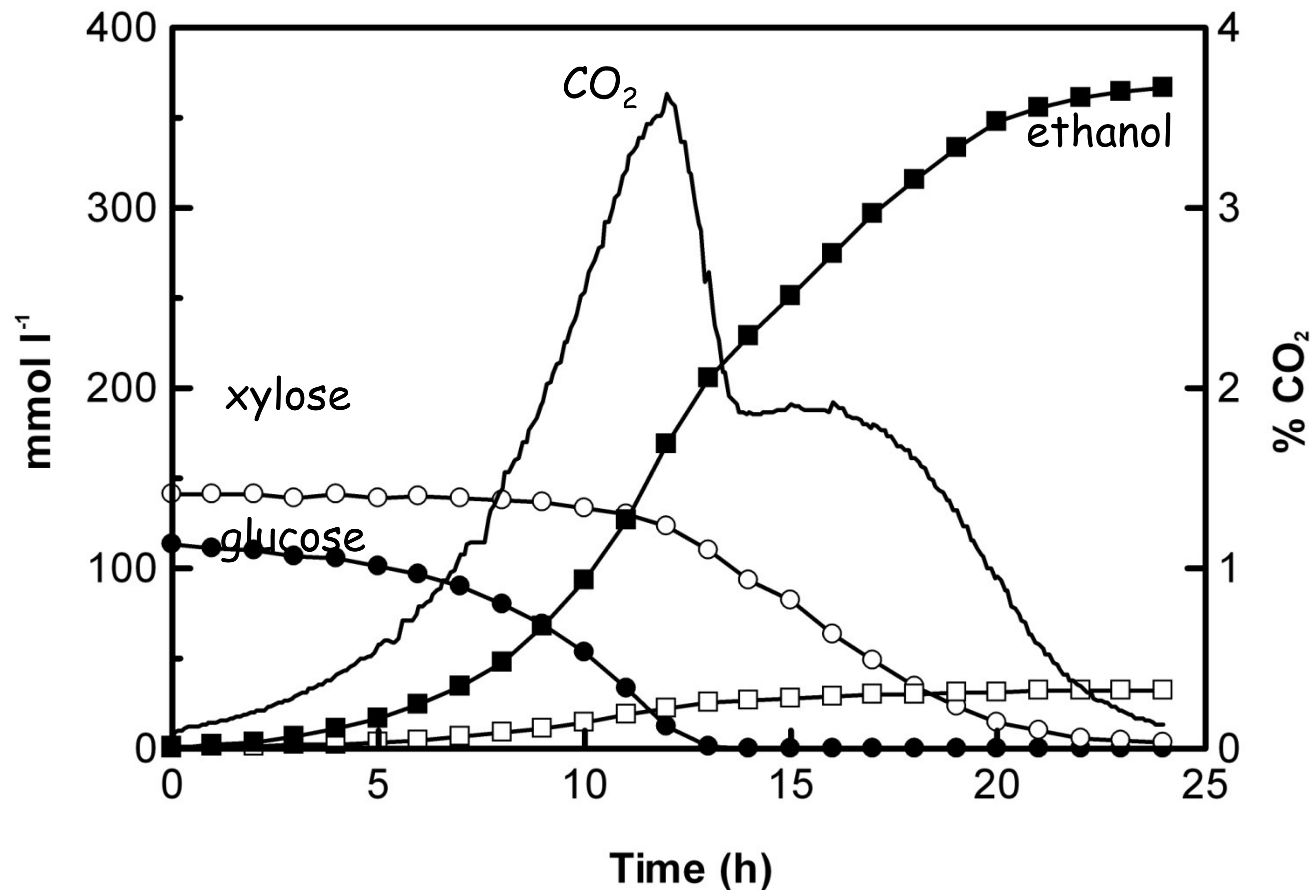




Courtesy of Jack Pronk, TU Delft

Anaerobic Fermentation of a Glucose-xylose Mixture by Strain RWB218

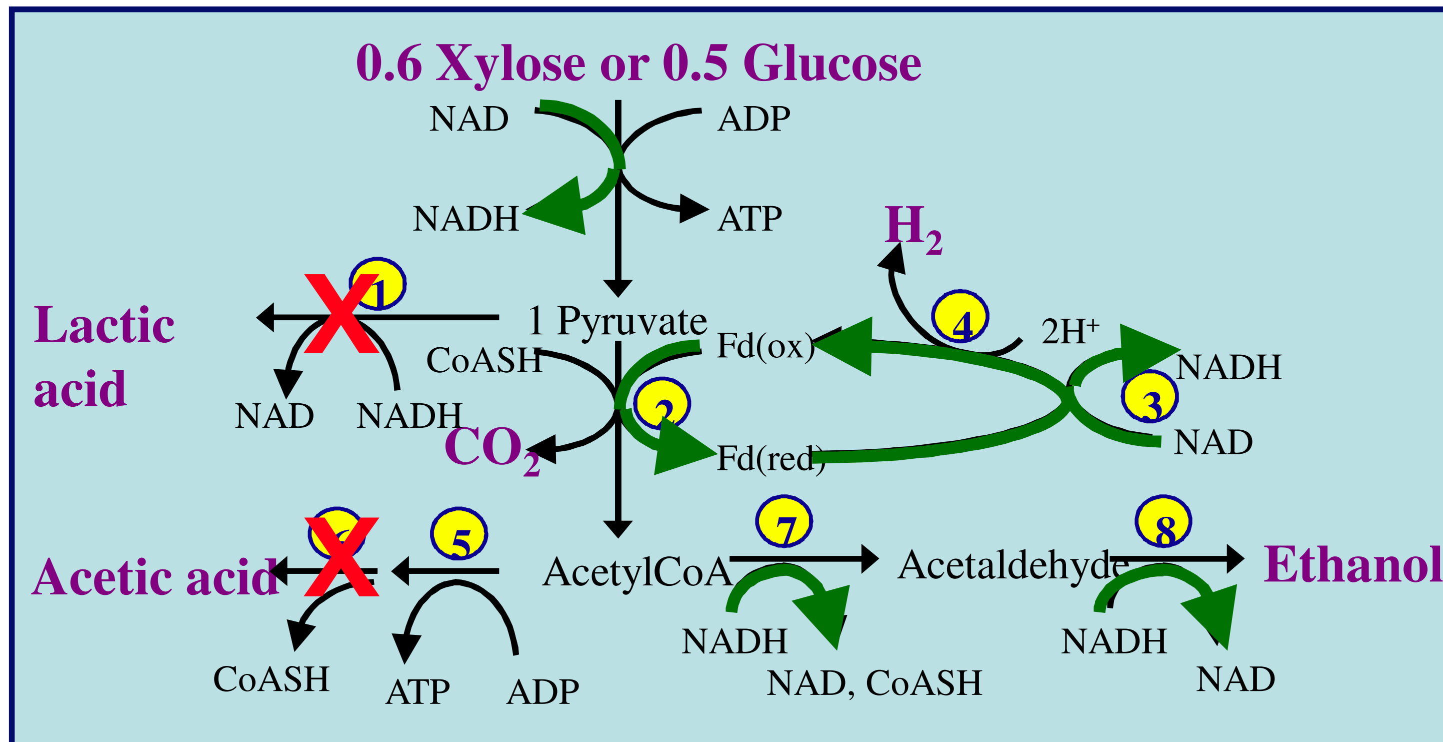
(evolved in chemostat and SBR cultures)



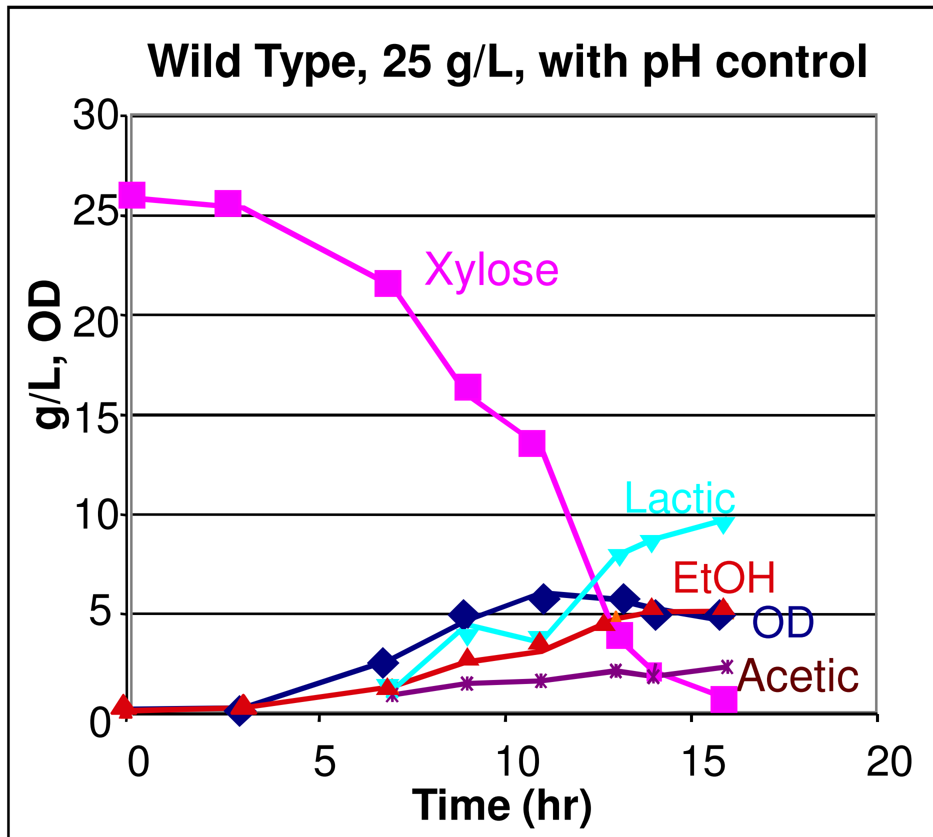
Kuyper *et al*. 2005
FEMS Yeast Research **5**: 925-934

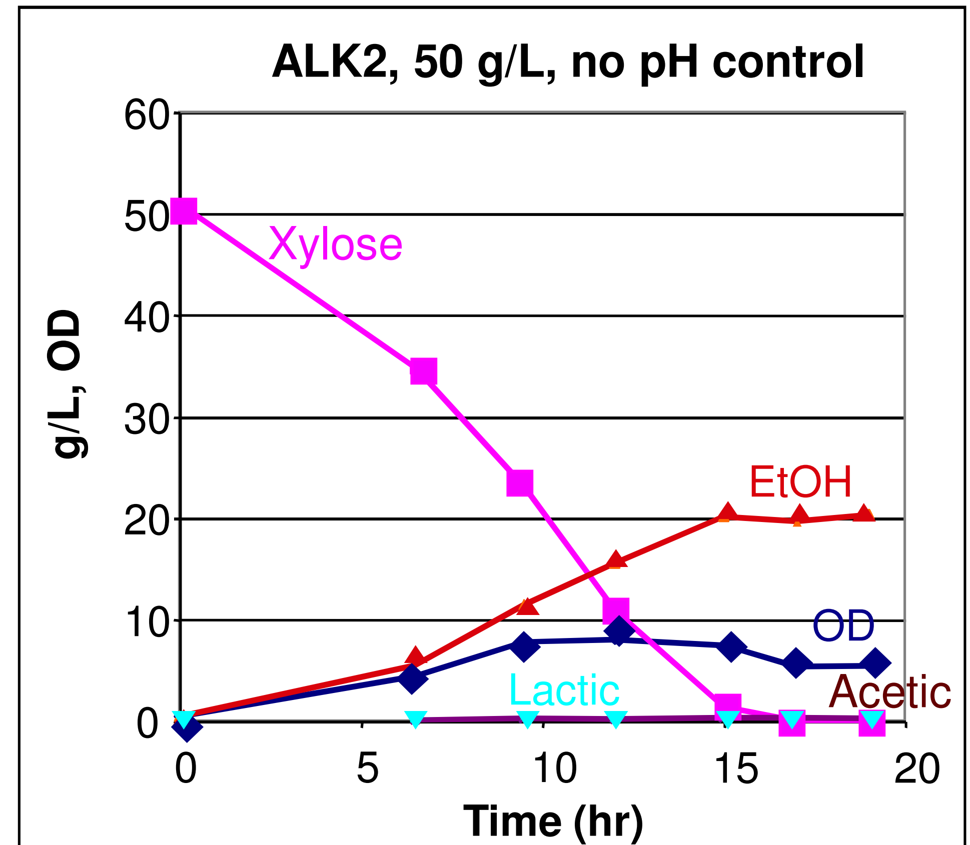
TU Delft

End-Product Metabolism of Thermophilic, Ethanol-Producing Bac



Batch Fermentation Results: Wild-Type and ALK2





Experiments carried out in 1L fermentors in MTC media + 10 g/L YE, 5 g/L tryptone

Utilization of Non-Glucose Sugars: Assessment

A tractable problem with multiple solutions.

Both native & recombinant strategies seem likely to work.

Although some further improvements can & will be realized, much of the potential economic benefit has been realized compared to a process utilizing only glucose.

While non-glucose sugar utilization has been a central focus for > 15 years, the magnitude of resources expended has been quite small (e.g. as compared to biopharm). Much higher rates of progress are possible with larger resources & today's tools.

Consolidated Bioprocessing

Primary targets

Cellulose - main carbohydrate in most cellulosic feedstocks

Xylan - main component of angiosperm hemicellulose, may or may not remain after pretreatment

Organism	Primary Modification	Lead Group
Native Strategy		
C. thermocellum	Characterize cellulosome, cellulose-utilization Develop & apply genetic tools	Lynd (Dartmouth)
Recombinant Strategy		
E. coli	Express endoglucanase, -glucosidase from *Erwinia*	Ingram (U. Fl.)
	Growth on amorphous cellulose	
S. cerevisiae	Saccharolytic enzymes expressed	Many
	Growth on amorphous cellulose enabled by heterologous cellulase expression (…)	van Zyl (U. Stellenbosch) & Lynd

Evolution of Biomass Processing Featuring Enzymatic

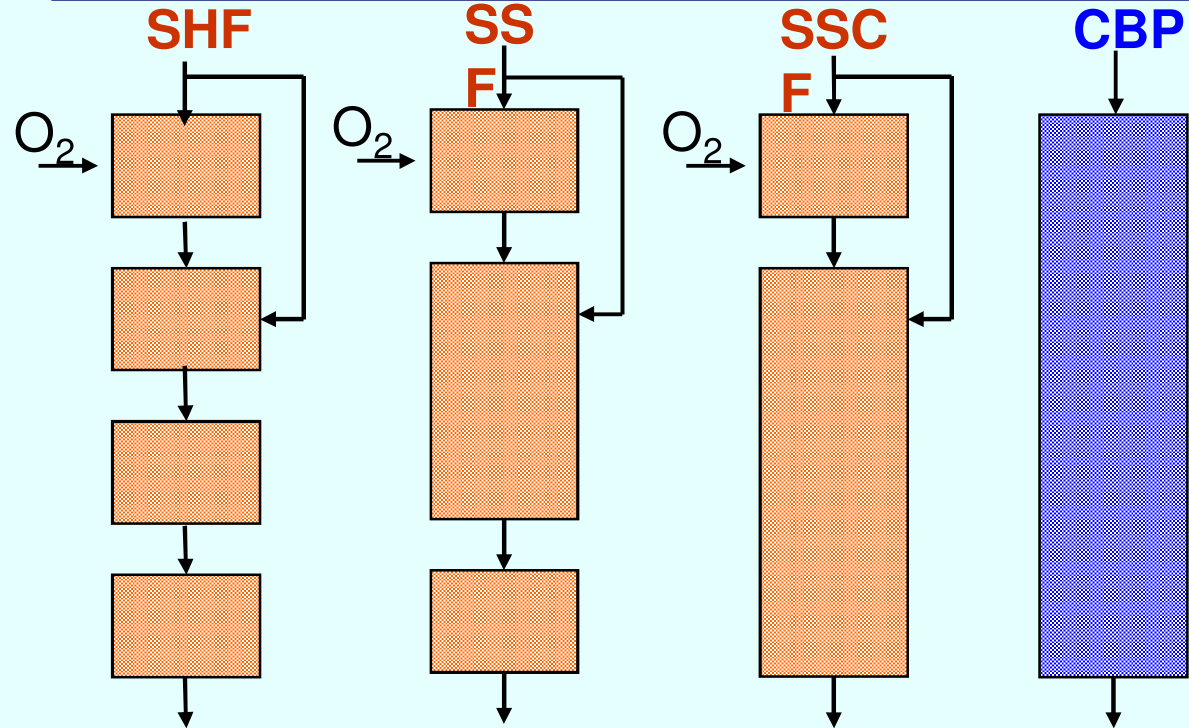
Biologically-Mediated Event

Cellulase production

Cellulose hydrolysis

Hexose fermentation

Pentose fermentation



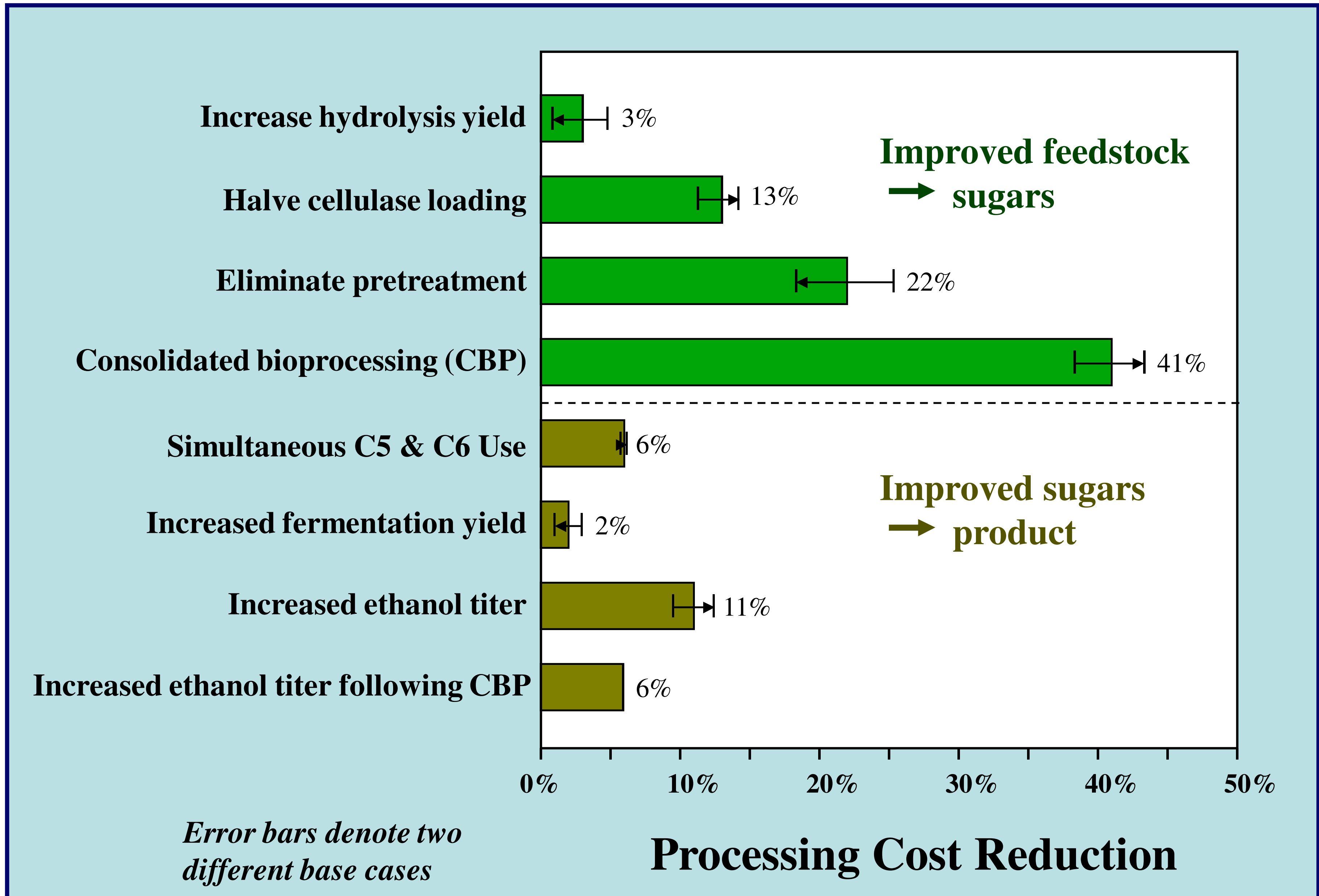
SHF: Separate hydrolysis & fermentation

SSF: Simultaneous saccharification & fermentation

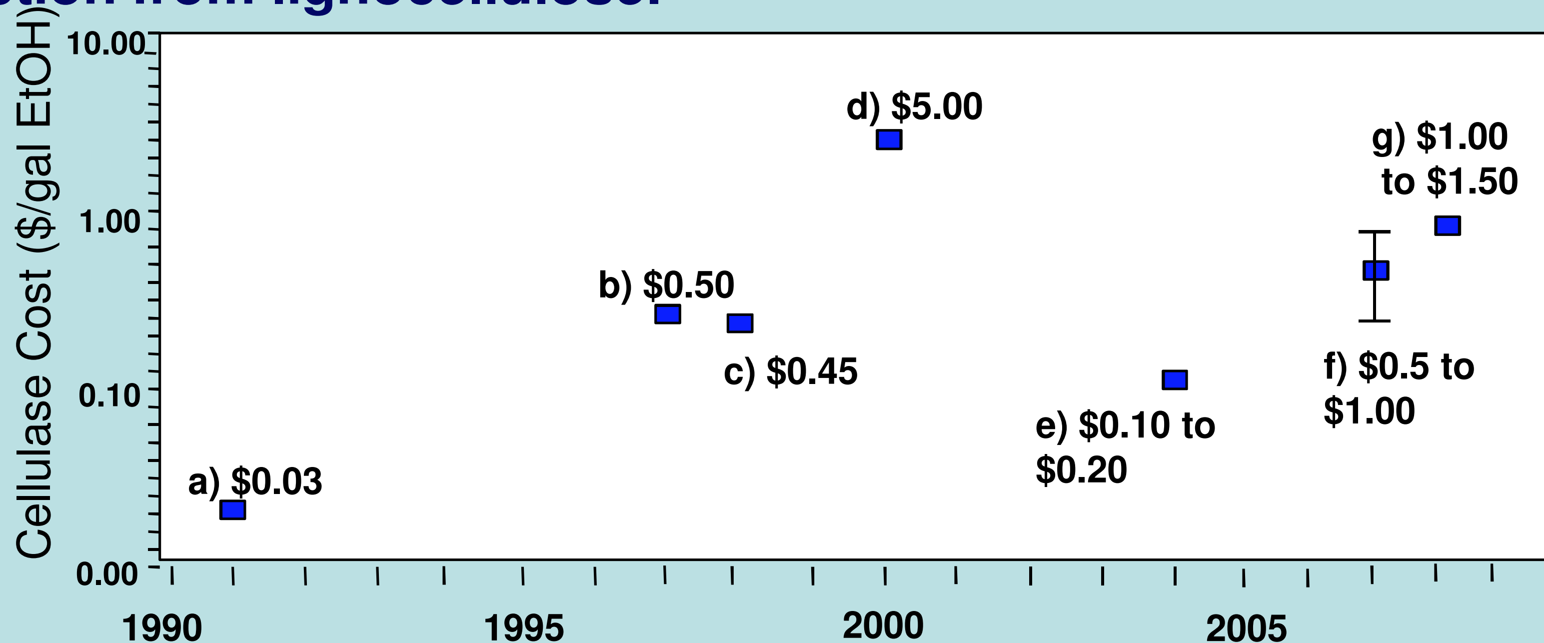
SSCF: Simultaneous saccharification & co-fermentation

CBP: Consolidated bioprocessing

Economic Impact of Various R&D-Driven Improvements



Estimated cost of dedicated fungal cellulase production for ethanol production from lignocellulose.



a) Hinman et al. 1991. Appl. Biotechnol. Bioeng. 34/35:639-657

b) Hettenhaus & Glassner, 1997(http://www.ceassist.com/assessment.htm)

c) NREL, 1998. Bioethanol from the corn industry. DOE/GO-1009-577

d) Schell, 2004. ASM National Meeting; McMillan, 2004. DOE/NASULGS Biomass & Solar Energy Workshops

e) Genencor & Novozyme, 2004. Press releases (e.g. http://www.genencor.com/wt/groc/pr 1098313606

f) Petiot, Novozymes, Platts Cellulosic Ethanol & Second Generation Biofuels, 2007.

g) Cormac Sheridan, Nature Biotech, 2008

At 10 to 15 IU cellulase/g cellulose (~0.25 lb cellulase/gallon ethanol), ~3 $/lb protein (cost of amylase, an established industrial enzyme), the cost of cellulase is much too high at $0.75/gallon ethanol.

Consolidated Bioprocessing: Fundamentals

Process economic studies provided original motivation

Fewer process steps

Large potential cost savings from eliminating dedicated cellulase production

But is success realistic to expect, particularly in light of high ATP demand for cellulase synthesis & low ATP supply from anaerobic fermentation?

Studies with the naturally-occurring cellulolytic bacterium *C. thermocellum*:

- Cellulose-specific bioenergetic benefits (sources of ATP) identified
- Because of these benefits, there is MORE ATP available during growth on cellulose than on soluble substrates - even after allowing for cellulase synthesis

How does the effectiveness of cellulose hydrolysis compare for CBP relative to a process featuring cellulase acting independently of microbes

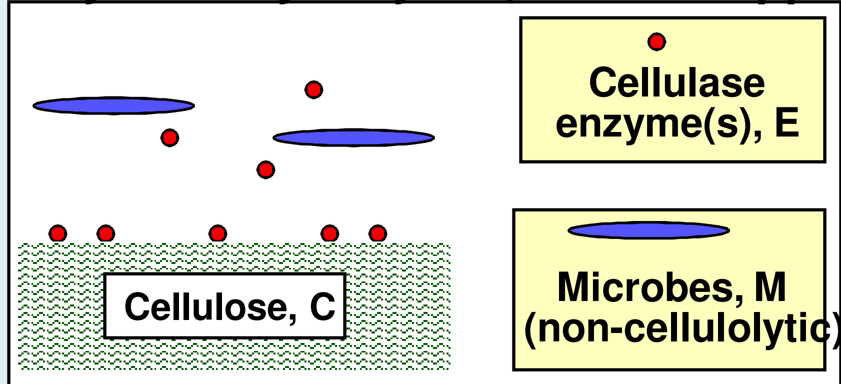
Laboratory substrates, low concentration: 3 to 5x more higher for CBP conditions - "*enzyme-microbe synergy*"

As more is learned about microbial cellulose utilization, biological considerations provide added CBP impetus beyond process economics

Kinetic feasibility of CBP

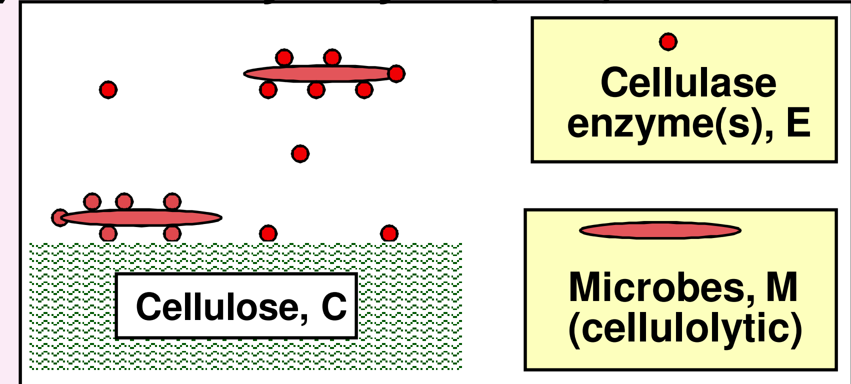
How do rates of cellulose hydrolysis compare for enzymatic & microbial conv

Enzymatic hydrolysis (classical approach)

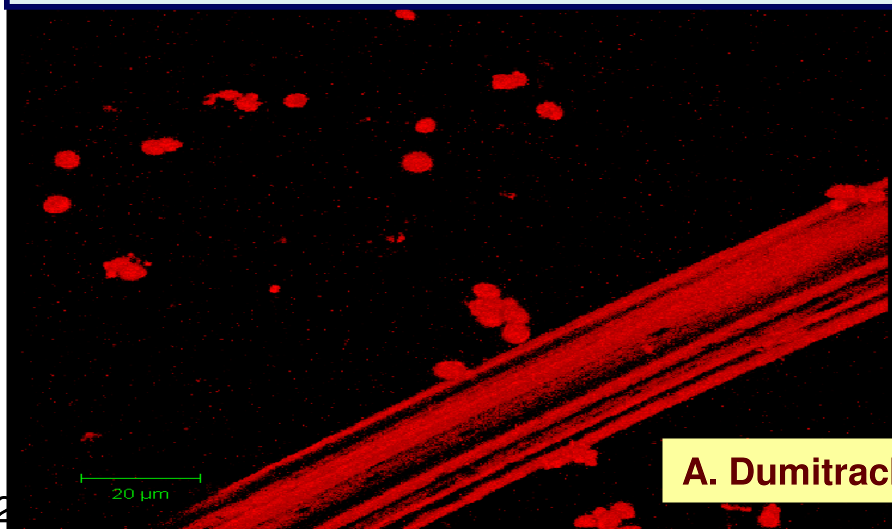


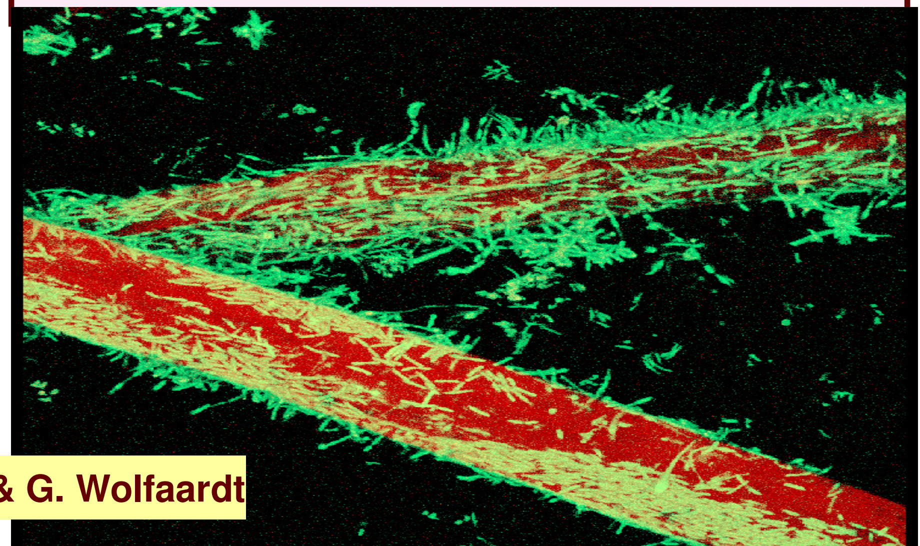
- Hydrolysis mediated by CE complexes
- Enzymes (several) both bound & free
- Cells may or may not be present

Microbial hydrolysis (CBP)



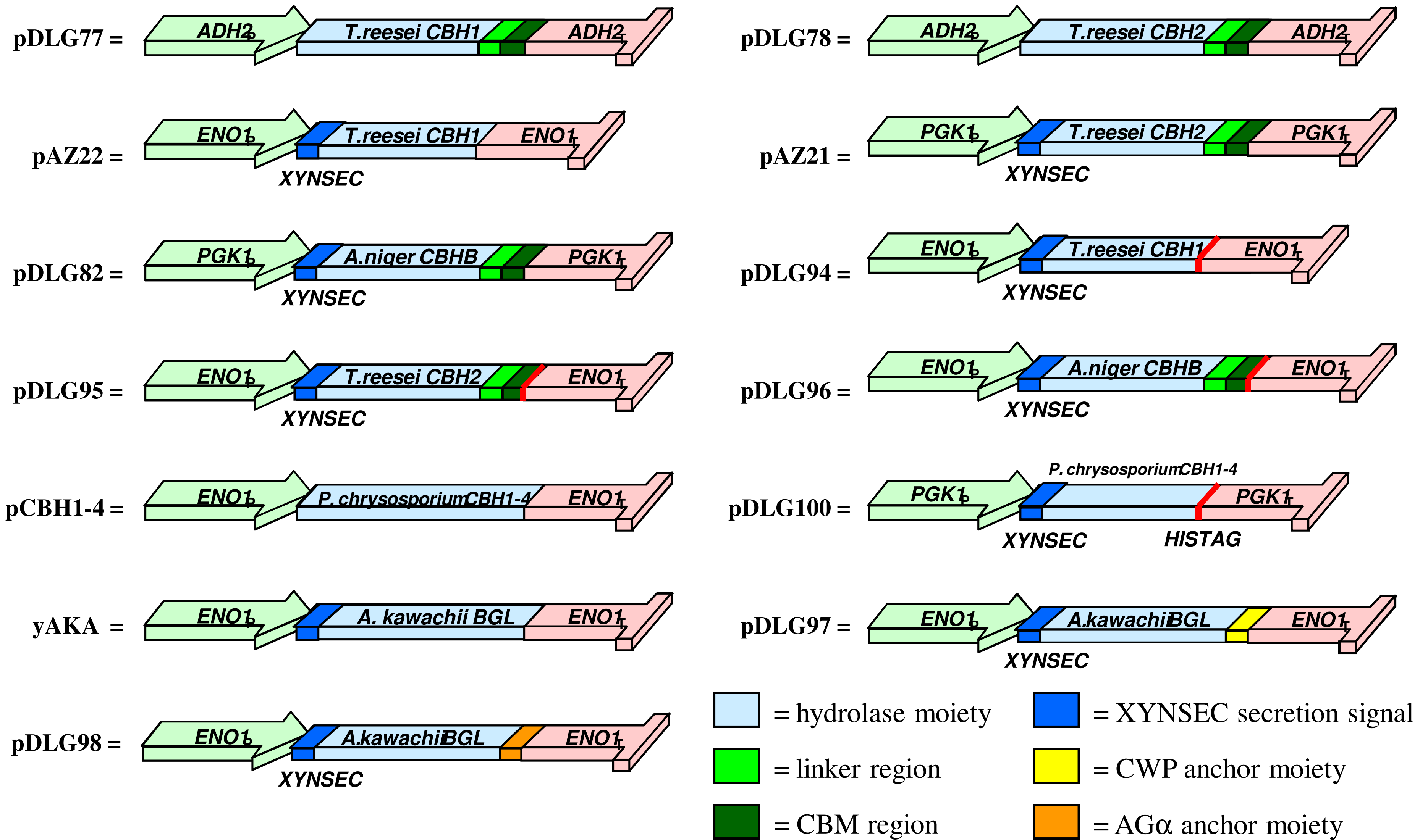
- Hydrolysis mediated mainly by CEM complexe
- Enzymes both bound & free
- Cells both bound & free





A. Dumitrache & G. Wolfaardt

Cellobiohydrolase constructs for expression in *S. cerevisiae*



Courtesy of Emile van Zyl, University of Stellenbosch

Energy Carrier	Price: Common Units	Price: $/GJ
Fossil		
Petroleum	$50/bbl	8.7
Natural gas	$10/kscf	11.0
Coal	$55/ton	2.5
w/ carbon capture @ $150/ton C		6.5
Electricity	$0.045/kWh	11.3
Biomass		
Soy oil	$0.50/lb	30.0
Corn kernels	$5/bu	14.4
Cellulosic crops[a]	$60/ton	4.0
Cellulosic residues		Some < 0

[a] e.g. switchgrass, short rotation poplar

Modified from Lynd et al., Nature Biotech., 2008

At $4/GJ, cellulosic biomass purchase price competitive with oil at $23/bbl.

Cellulosic biomass: The cheapest GJ in a carbon-constrained world.

The cost of processing, not feedstock, has prevented industry emergence.

Recalictrance of Cellulosic Biomass

Improved fuels, fuel production

• Ultimate advances in fuel production from soluble sugars will not enable a cellulosic biofuels industry

• New biofuels do not address factors limiting emergence of cellulosic biofuels, will add value once the industry is established, impact sector biofuels distinctively suited to.

Overcoming the recalcitrance of cellulosic biomass

Cellulosic biomass

Sugars

Fuels

• Most costly

• Greatest potential for R&D-driven cost reduction

• Advances necessary & sufficient to create cellulosic biofuels industry, generically enabling

APPENDIX: AVAILABLE ELECTRONS AND RELATED CONCEPTS

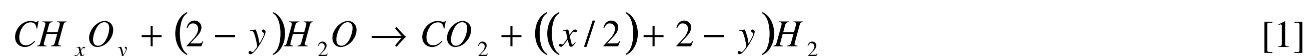
Utility. The concept of available electrons, used to calculate quantities such as degree of reductance, is useful in analysis of metabolism primarily for two reasons:

A metric of the electron-richness, or oxidation state, of metabolites. We might for example want to determine whether a given metabolite A is more or less reduced than a second metabolite B. This would in turn tell us whether we need an electron donor or an acceptor to convert A to B. The number of available electrons can also tell us how many electrons can be donated to ETP for a given molecule Š indeed, this second application is closely related to the definition of available electrons as we shall see.

A conserved quantity that can decrease the number of balance equations and is much more practical to experimentally verify as compared to alternative methods. Since elements are neither created nor destroyed, we can write Ņelemental balancesÓ for biological processes that provide insights into matters such as metabolic stoichiometry, theoretical yields, and data adequacy testing. As we shall see, available electrons are also a conserved quantity, and one can hence write an Ņavailable electron balanceÓ analogous to a carbon or nitrogen balance. Whereas an available electron balance is readily verified experimentally, it is almost impossible to experimentally verify elemental balances for H and O because of the difficulty of determining small amounts of produced or consumed water in an environment featuring 55M water. Secondarily, the available electron balance replaces *both* the H and O balances, reducing the number of equations that have to be solved (although this is seldom a practical constraint).

A related concept. In the wastewater treatment field, it is common to define the chemical oxygen demand (COD) . The COD is provides a composite measure of the concentration of an undefined mixture of organic compounds in terms of the amound of oxygen that would be consumed if that compound were completely oxidized to CO_2. Since wastewater typically contains a very large range of organic compounds, and since the ability of these compounds to consume oxygen is a key determinant of environmental impact in receiving waters, one can see why the concept of COD is useful.

To understand COD , and available electrons on a more quantitative basis, consider a hydrogen (electron) donating half reaction for oxidation of one or more organic compounds:



$$CH_xO_y + (2-y)H_2O \rightarrow CO_2 + ((x/2)+2-y)H_2 \quad [1]$$

Biologically-relevant hydrogen (electron) accepting reactions include:

$$2H_2 + O_2 \rightarrow 2H_2O \quad [2]$$

$$4H_2 + SO_4^{--} + 2H^+ \rightarrow H_2S + 4H_2O \quad [3]$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad [4]$$

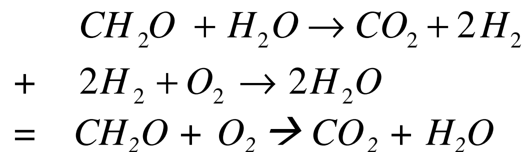
The COD corresponds to the mass of oxygen per volume necessary to oxidize organize organic matter, which corresponds to combining [1] and [2] to eliminate H_2.

Thus for example, for a waste water containing 2 mM/L lactic acid ($H_3CCHOHCOOH$, MW 46 g/mole), we have formula $C_3H_6O_3$, corresponding to a carbon-normalized formula of CH_2O/mole C (x = 2, O = 1). A hypothetical decomposition to H_2 and CO_2 based on equation [1] (also on a carbon-normalized basis) would have the following stoichiometry:

$$CH_2O + H_2O \rightarrow CO_2 + 2H_2 \quad [1e]$$

It may easily be verified that this equation is balanced with respect to C, H, and O.

If we add equation [2] to equation [1e], we obtain



$$CH_2O + H_2O \rightarrow CO_2 + 2H_2$$
$$+ \quad 2H_2 + O_2 \rightarrow 2H_2O$$
$$= \quad CH_2O + O_2 \rightarrow CO_2 + H_2O$$

So we see that or each mole of lactic acid carbon, we consume one mole of O_2 in a hypothetical complete oxidation with oxygen as the electron acceptor. Thus, for lactic acid at 2mM/L, the COD is:

(2mM lactic acid/L)*(3 mm C/mm lactic acid)*(1mm O_2/mm C) = 3 mmO_2/L

Conceptual definition of available electrons. As an alternative to COD, we could just as well define the hydrogen (or electron) donating potential, as the moles of hydrogen (or moles of electrons) in the course of organic matter oxidation that could be donated to an electron acceptor. While this is less directly relevant to receiving waters than COD, it is more directly relevant to metabolism.

These observations motivate the following definition of available electrons:

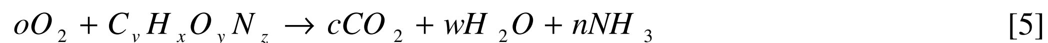
> Available electrons are defined as electrons that would be transferred to oxygen in the hypothetical oxidation of a compound to a specified reference state. The most common reference state involves oxidation to CO_2, H_20 and NH_3 (other reference states are possible, additional elements could be included if needed).

A conserved quantity. Available electrons are neither created nor destroyed in biologically mediated reactions, as is the case with mass and atoms. To see this, we note that electrons in all but the outermost orbitals are normally inaccessible to chemical reactions, and are thus conserved. Since the total number of electrons are conserved, and electrons in other than outermost orbitals are conserved, then available electrons (electrons in outermost orbitals that can participate in chemical reactions and covalent bonds) are also conserved

Calculating available electrons. Equation [2] can be rewritten as

$$4H^{+} + 4e^{-} + O_2 \rightarrow 2H_2O \qquad [2b]$$

Consider oxidation of an N-containing organic compound:



$$oO_2 + C_vH_xO_yN_z \rightarrow cCO_2 + wH_2O + nNH_3 \qquad [5]$$

We recognize that the available electrons per mole are equal to 4 times the parameter o.

Elemental balances for equation [5] yield:

C: v = c

N: n = z

H: x = 2w + 3n

O: 2o + y = 2c + w

These can be combined to give:

$$o = \frac{2v + \frac{x - 3n}{2} - y}{2} \qquad [6]$$

So available electrons per mole (= 4o) can be found from:

$$4o = 4v + x - 3n - 2y \qquad [7]$$

We see that, in general, atoms in an organic molecule contribute available electrons as follows:

C: +4
O: -2
H: +1
N: -3

Note: These assignments would change if the basis were changed (e.g. N ends up as NO_3 instead of NH_3).

EXAMPLE.

Repeated experiments involving an anaerobic fermentation indicate that 10 moles of methanol are converted to 3 moles of butyric acid (Lynd, L., R. Kerby, and J.G. Zeikus. 1983. Metabolism of H_2/CO_2, methanol, and glucose by *Butyribacterium methylotrophicum* J. Bacteriol 153:1415-1423.)

Are these results consistent with:

An available electron balance?

A carbon balane?

If you were to measure the concentration of an additional compound, what would you measure?

Hypothesize an overall stoichiometry for this reaction.

SOLUTION.

Available electrons per mole of compounds involved:

Methanol (CH_4O): $4 + 4 - 2 = 6$ available electrons per mole

Butyric acid ($C_4H_8O_2$): $4*4 + 8 - 2*2 = 20$ available electrons per mole

Carbon balance (10 methanol → 3 butuyric acid): 10 → 12 *Not balanced*

Available electron balance: 10 MeOH*6 av. e^-/MeOH → 3 Butyrate*20 av e^-/butyrate

60 → 60 *Balanced*

To have a balanced stoichiometry, we need a reactant that has carbon but no available electrons. The only such compound is CO_2.

Thus, we can hypothesize (and experiment confirmed in this case) a stoichiometry of:

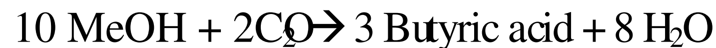
$$10\ MeOH + 2CO_2 \rightarrow 3\ \text{Butyric acid}$$

This satisfies carbon and electron balances. Since water has zero available electrons per mole, it may or not be involved. To find this, we can use elemental balances. It may be noted that elemental balances involving H and O are almost never verified experimentally because water production or consumption occurs in an aqueous environment with H_2O concentration of 55 molar.

H: 10*4 → 3*8 *Have 16 more H on reactant side than product side*

O: 10 +4 → 6 *Have 8 more O on reactant side than product side*

This can be reconciled by adding 8 waters to the product side:



$$10\ MeOH + 2CO_2 \rightarrow 3\ \text{Butyric acid} + 8\ H_2O$$