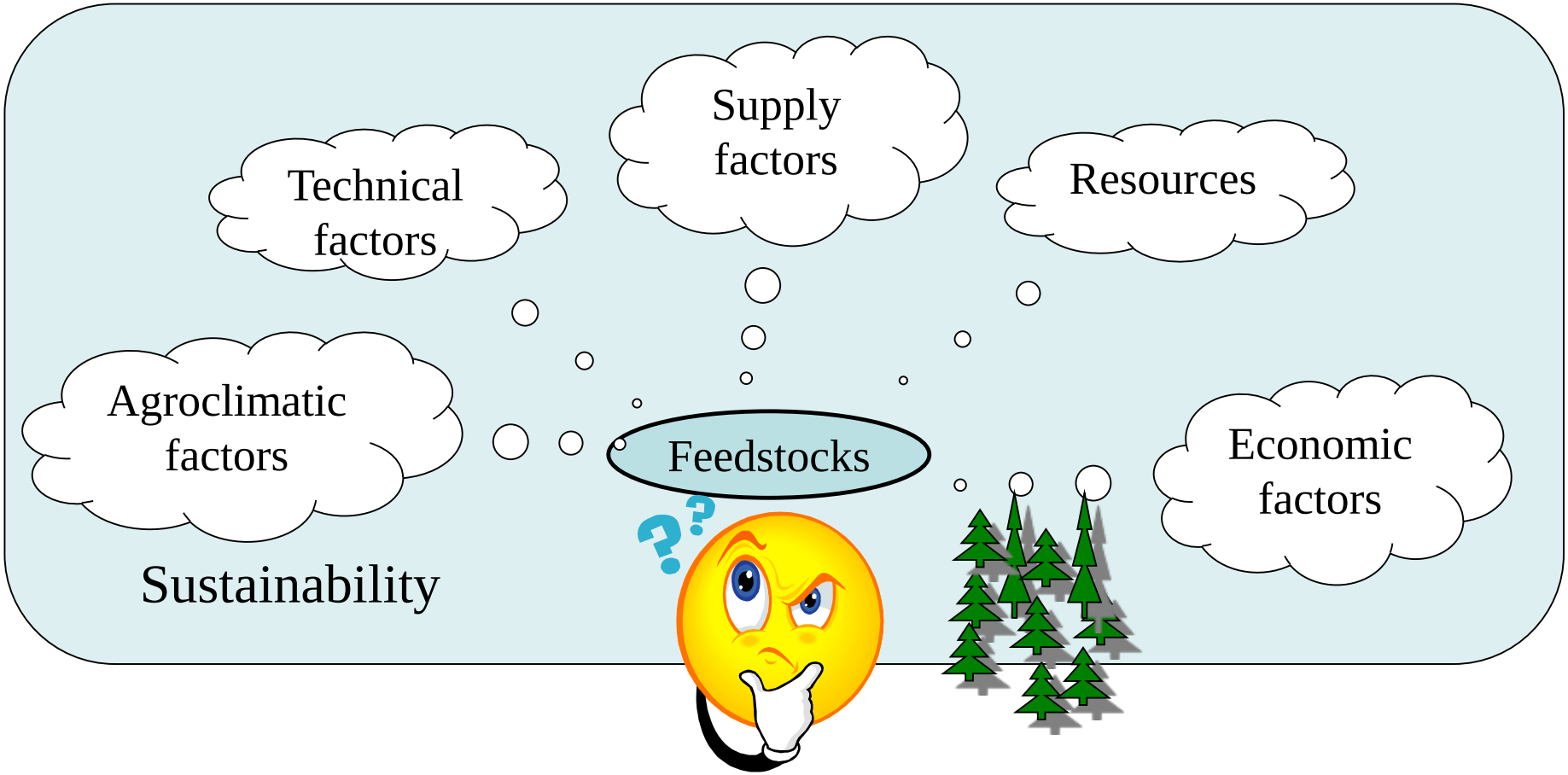
Topic Four

Lecture Six

Fermentation Technology: Yeast

Summary of Lecture Five

What are the critical factors that must be considered in evaluating/comparing feedstocks?



Ethanologenic Organisms

Organism	Natural sugar utilization pathways					Major products	
	Glu	Man	Gal	Xyl	Ara	EtOH	Others
Anaerobic bacteria	+	+	+	+	+	+	+
E. coli	+	+	+	+	+	−	+
Z. mobilis	+	−	−	−	−	+	−
S. cerevisiae	+	+	+	−	−	+	−
P. stipitis	+	+	+	+	+	+	−
Filamentous fungi	+	+	+	+	+	+	−

Organism	Tolerance			O_2 needed	pH range
	Alcohols	Acids	Hydrolysate		
Anaerobic bacteria	−	−	−	−	Neutral
E. coli	−	−	−	−	Neutral
Z. mobilis	+	−	−	−	Neutral
S. cerevisiae	++	++	++	−	Acidic
P. stipitis	−	−	−	+	Acidic
Filamentous fungi	++	++	++	−	Acidic

Ref: Hagerdal, et al. (2007)

Yeast

• Yeast is a eukaryotic microorganism. *Saccharomyces cerevisae* is the most common yeast used in ethanol fermentations.

• Yeast can switch to complete anaerobic or aerobic respiration depending on the environmental conditions.

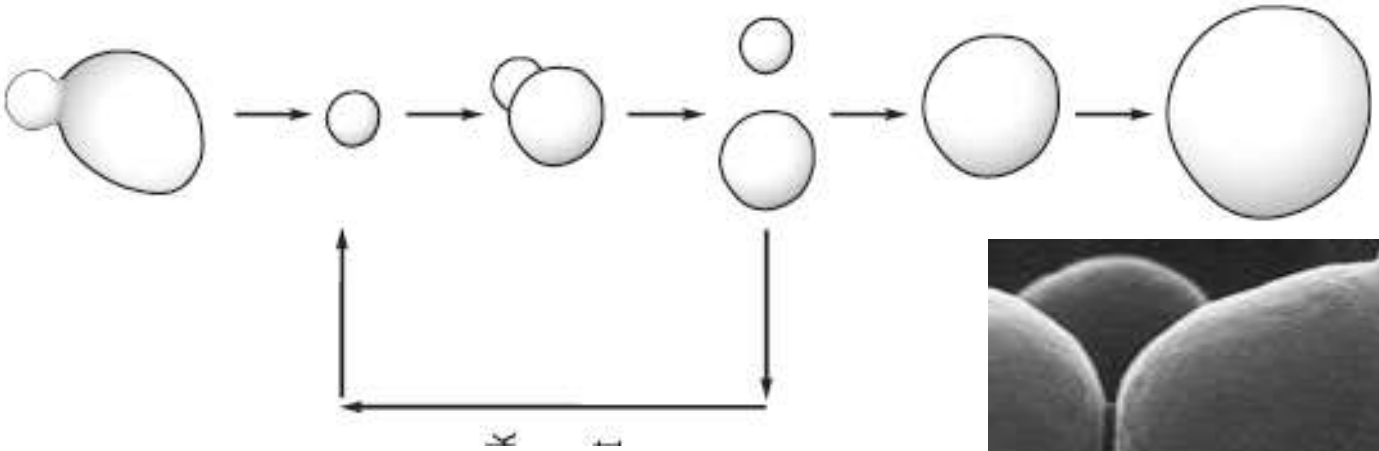
• Size of yeast cell varies between 5-10μm.

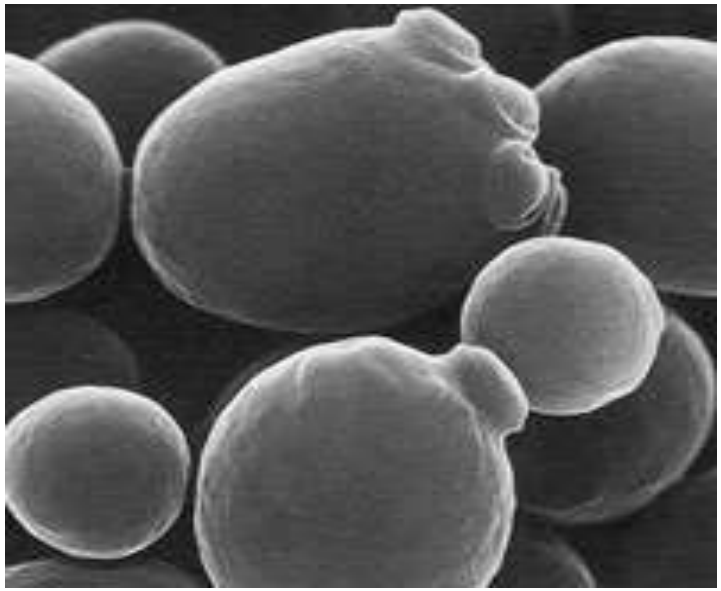
• Yeast reproduces asexually by budding although sexual reproduction also occurs in nature.

• Exhibits diauxic growth pattern.

Yeast

As yeast cells grow, the mother cell accumulates bud scars and increases in size. After a number of cell divisions (Hayflick limit; usually 10-33), the mother cell enters senescent phase and ultimately dies.

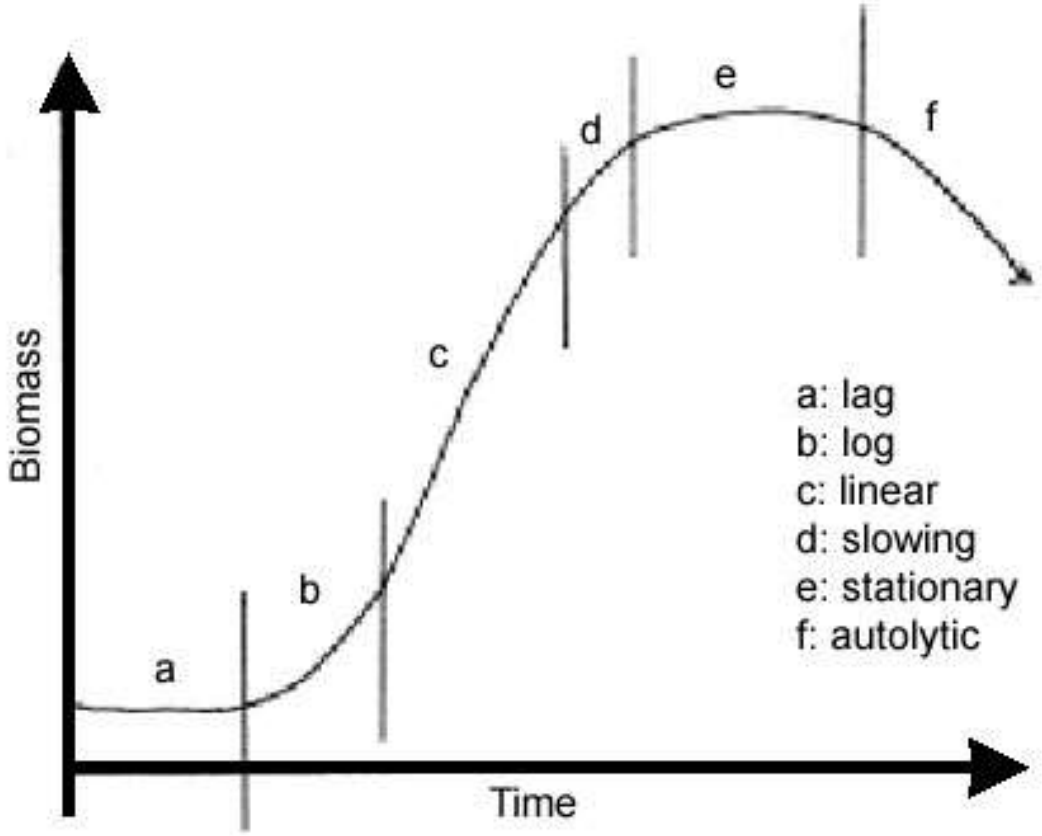




Ref: Russell (Yeast fundamentals : Alcohol Text book)

Yeast

Yeast growth phases: Yeast in maintained in log phase during ethanol fermentations as yeast can produce about ethanol 33 times faster in log phase than in stationary phase.



Ref:http://www.microbiologybytes.com/introduction/myc1.html

Yeast

Yeast nutrient requirements:

• Water: Yeast require at least 15% water.

• Carbon source: mono and disaccharides (sucrose, glucose, fructose, maltose)

• Nitrogen source: Inorganic nitrogen (as urea, ammonia), small chain peptides and amino acids (from protein degradation)

• Lipids/oxygen: Lipids or oxygen for lipid production. Lipids are integral components of cell membranes.

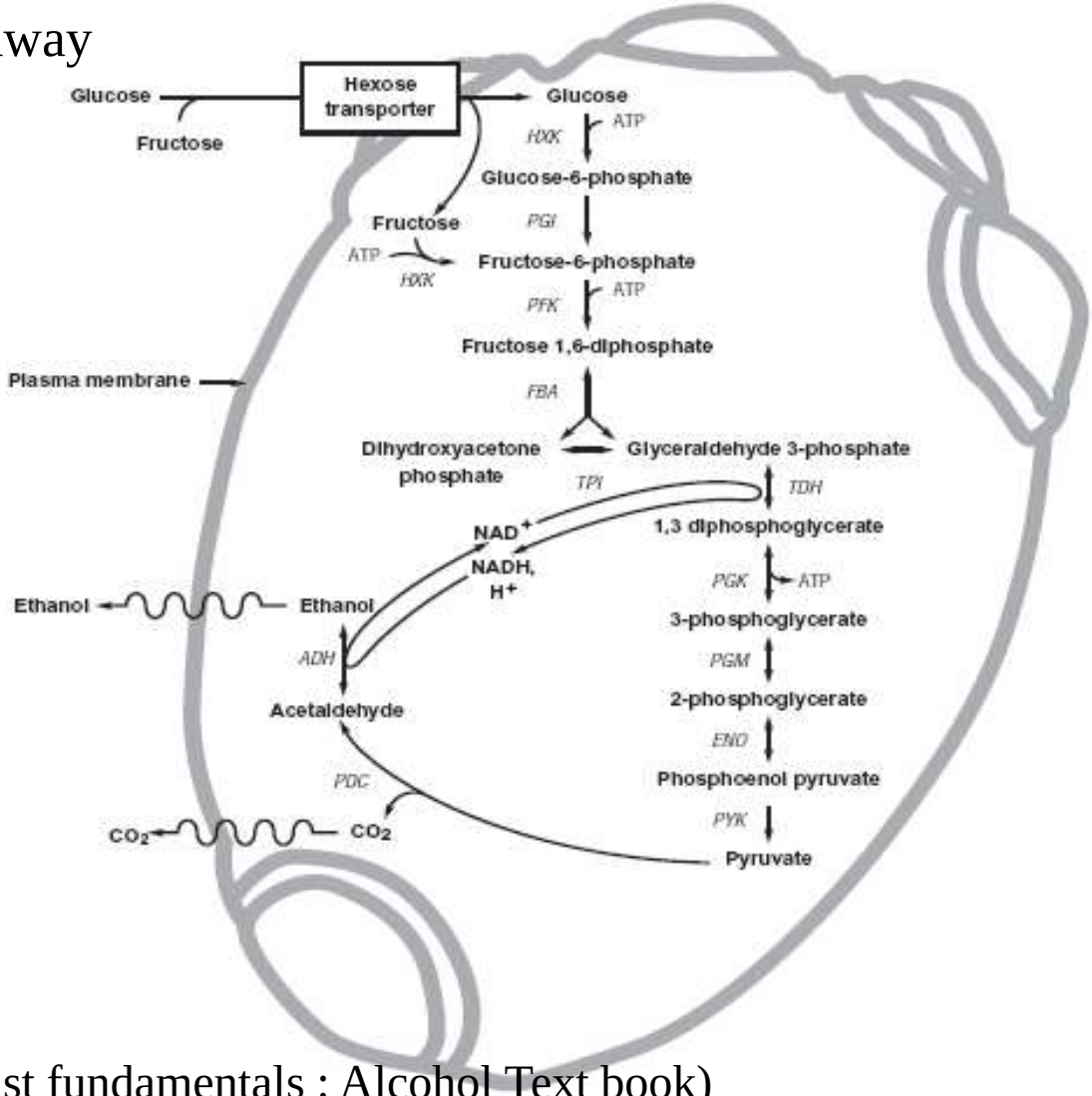
• Micronutrients: Vitamins, inorganic ions.

• pH: Yeast can tolerate a wide range of pH although 4.0-6.0 is optimum.

• Temperature: Optimum temperature 5- 35°C

Yeast

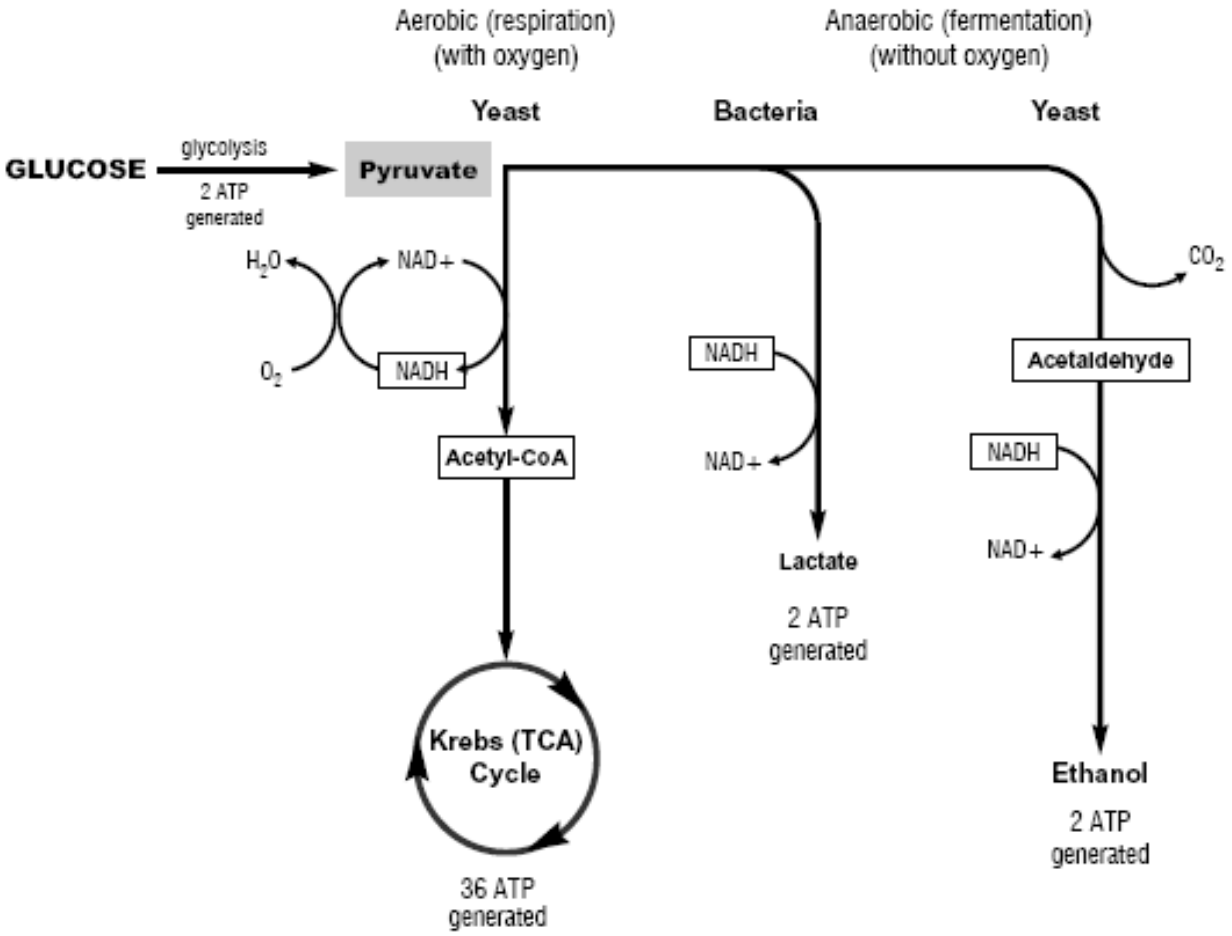
Glycolytic pathway



Ref: Russell (Yeast fundamentals : Alcohol Text book)

Yeast

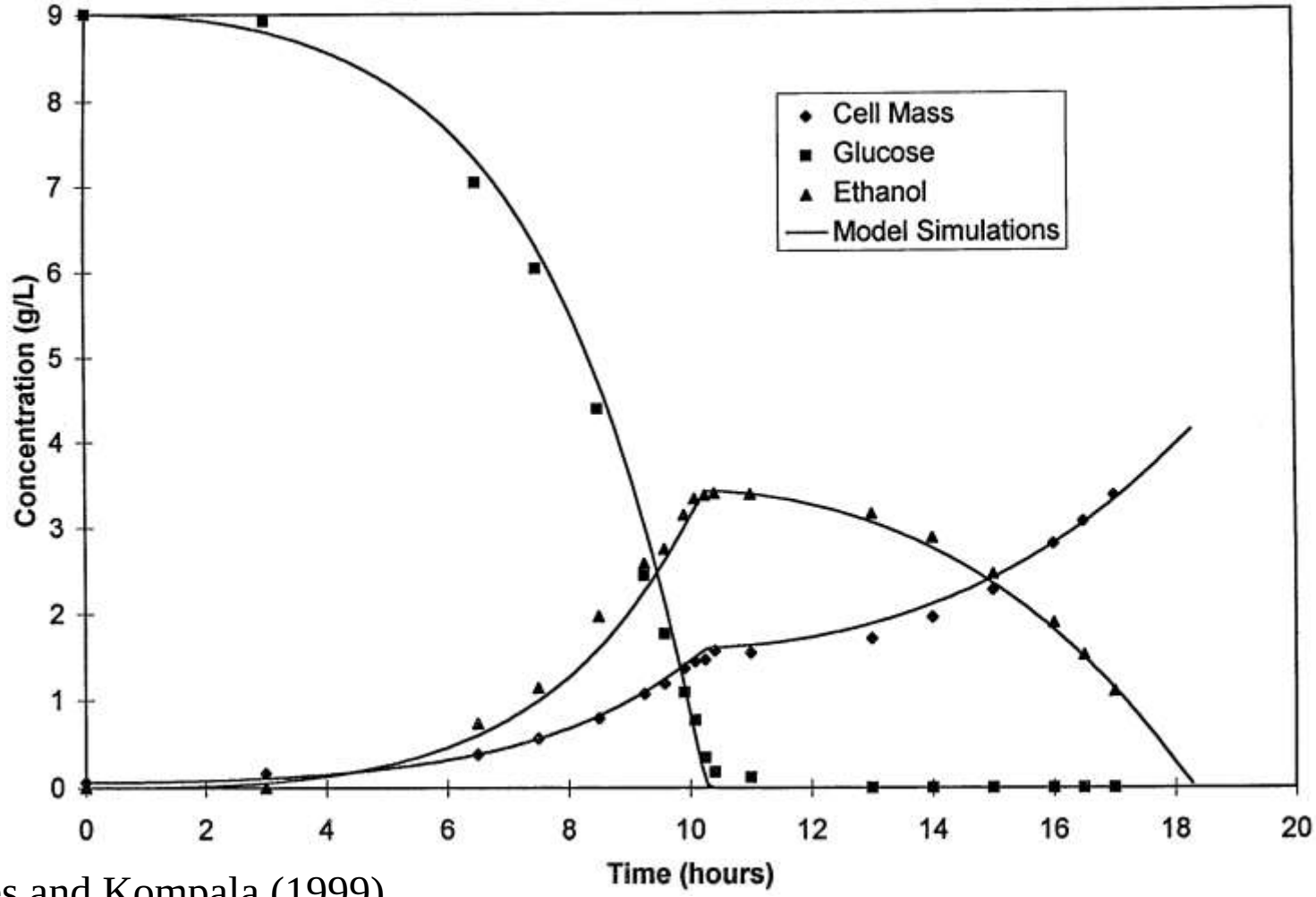
Anaerobic and aerobic metabolism



Ref: Russell (Yeast fundamentals : Alcohol Text book)

Yeast

Diauxic shift in yeast metabolism



Ref: Jones and Kompala (1999)

Yeast

Yeast cell viability and vitality are two important indicators of overall yeast health.

• Viability refers to the capacity of the yeast cells to form daughter cells. It is indicative of the . Viability is measured using methylene blue test, budding index and capacitance measurement.

• Vitality is a measure of how fast the cell is growing. It is an indicator of overall health of the yeast cell. It does not indicate the budding capacity of a yeast cell.

Yeast

Yeast cell response to external fluctuations.

• Heat and cold shock: Yeast synthesize stress response proteins and a disaccharide Trehalose in response to rapid temperature changes.

• Glycerol is also produced by yeast for osmoregulation.

• Cell membrane of yeast undergoes changes in response to increasing extracellular ethanol concentration.

Yeast

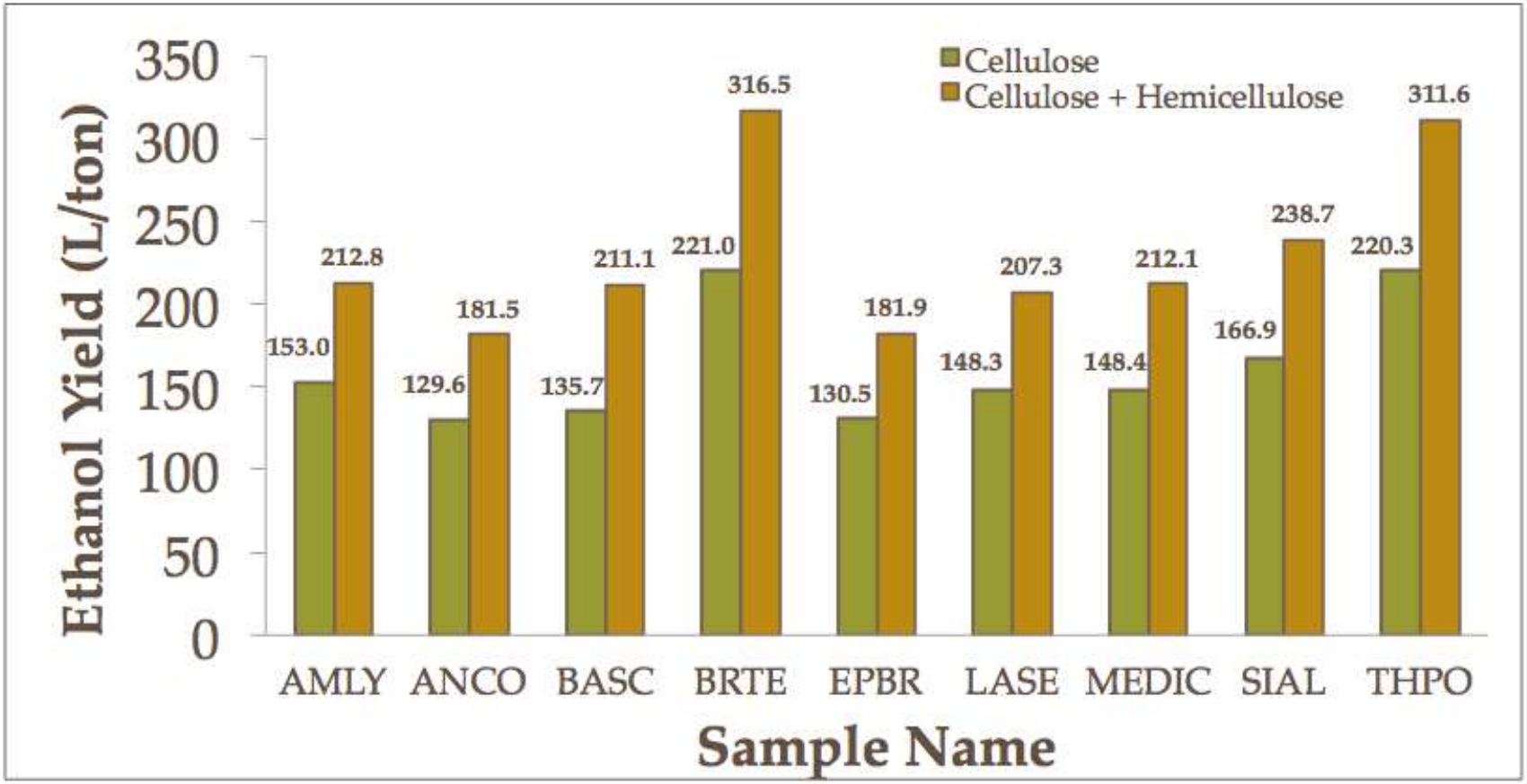
Some issues in yeast fermentations.

• Killer yeast: Wild strains of yeast that produce toxic compounds. The strains of yeast that are affected by these toxins are sensitive yeasts.

• Lactic and acetic acid bacteria: They inhibit yeast growth by producing weak organic acids (lactic and acetic acid)

• Stuck fermentations: Under nutrient limited conditions such as nitrogen deficiency, fermentations can become ‘stuck’. In stuck fermentations, yeast does not grow even in presence of glucose.

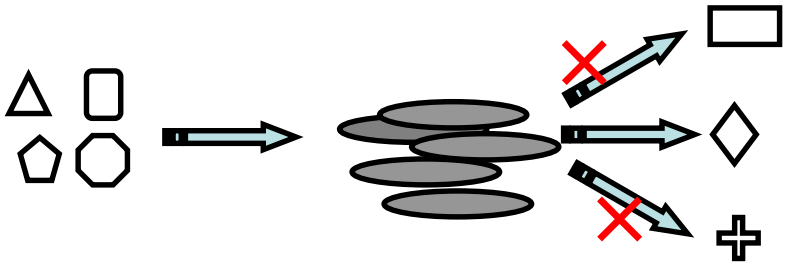
Importance of Pentoses



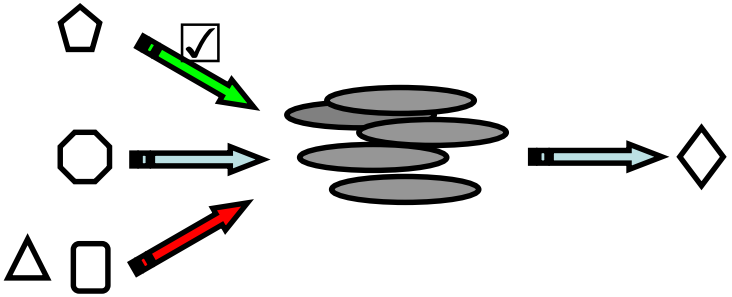
40% Yield Increase Using Both Five and Six Carbon Sugars – 22% price drop

Three Major Strategies

Efficient utilization of diverse sugars → Genetically engineer to produce only ethanol

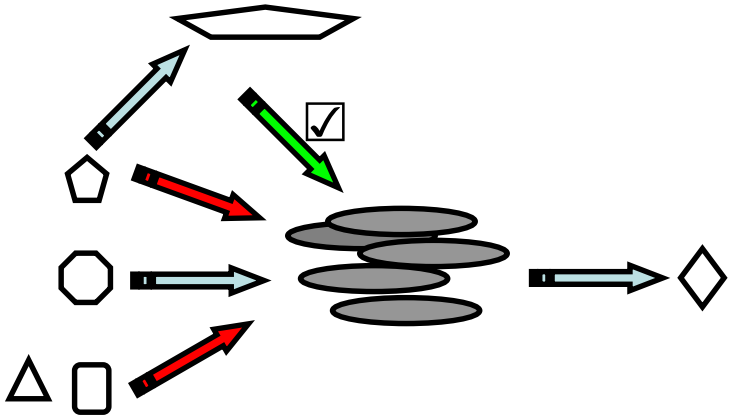


Efficient ethanol producer → Genetically engineer to metabolize pentoses



Three Major Strategies

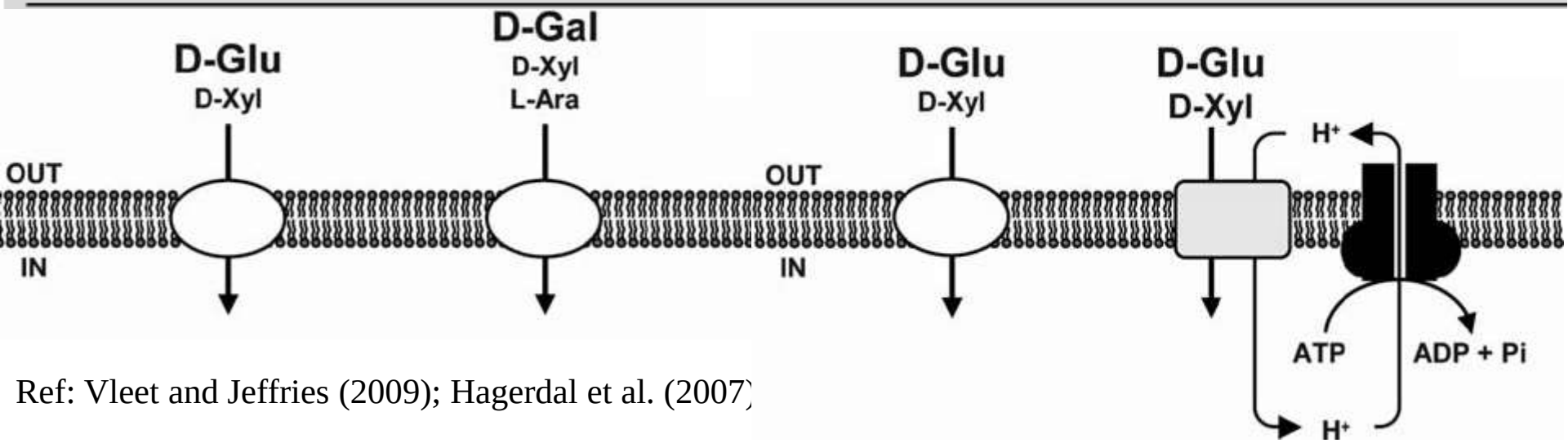
Convert the sugars into a metabolizable form → Xylulose production by xylose isomerization.



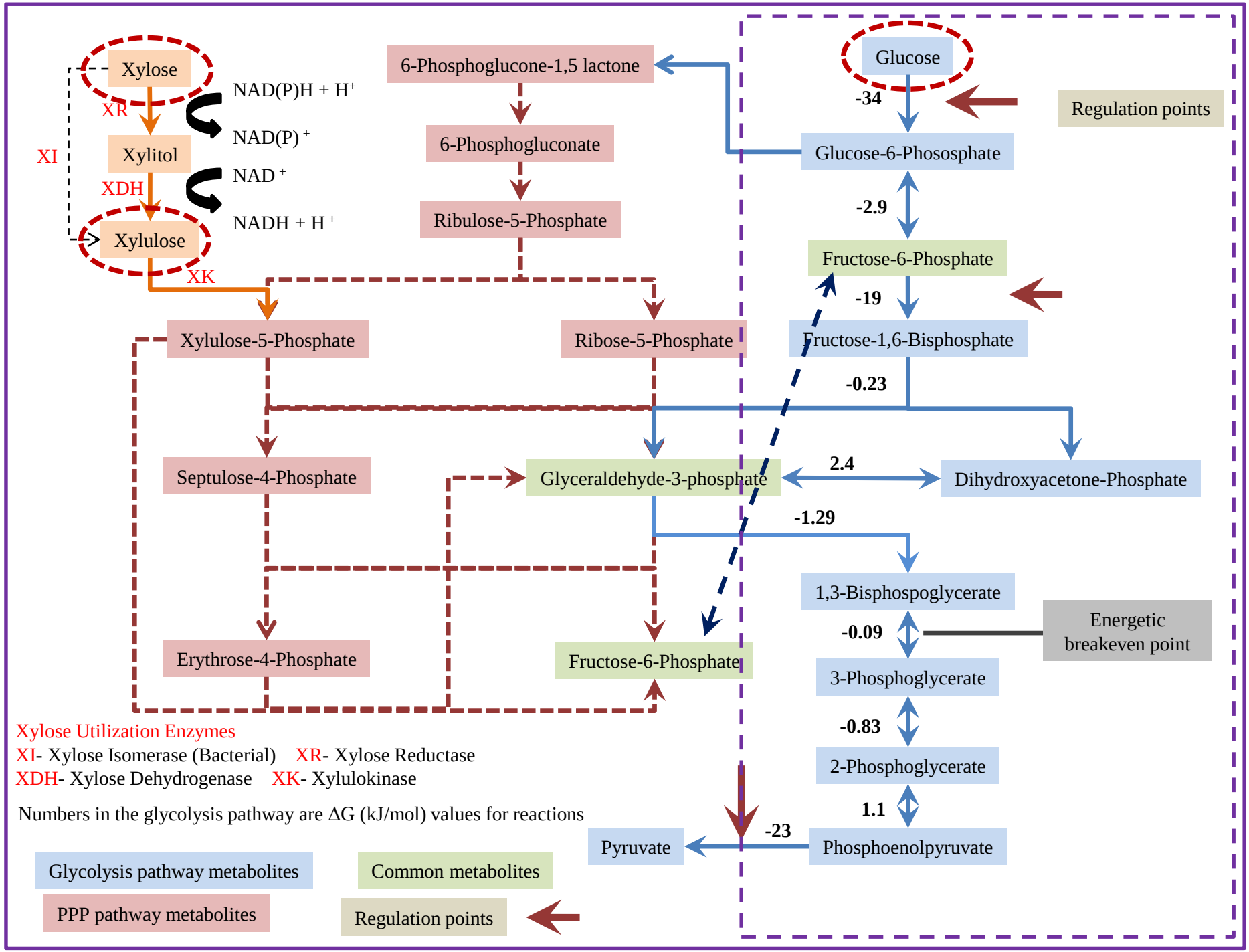
Sugar Assimilation Pathways

Kinetic parameters of yeast glucose/xylose transporters

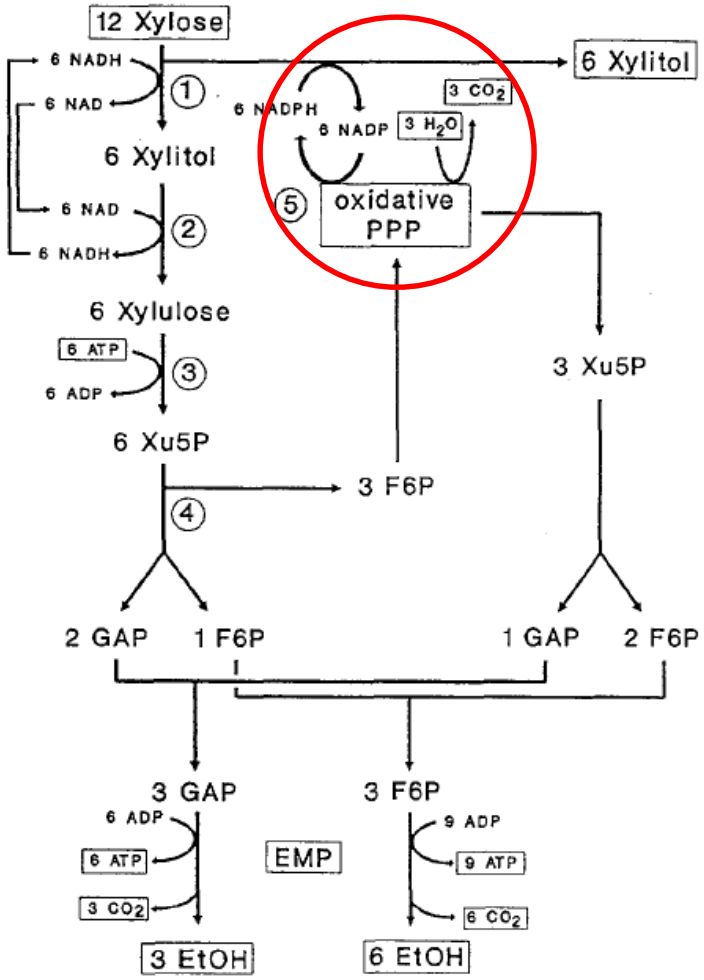
Transporters	K_m (mM)		V_{max} (nmol min^{-1} mg dw^{-1})	
	Glucose	Xylose	Glucose	Xylose
ScHxt1	107 ± 49^a	880 ± 8^b	50.9 ± 3.7^a	750 ± 94^b
ScHxt2	2.9 ± 0.3^a	260 ± 130^b	15.6 ± 0.9^a	340 ± 10^b
ScHxt4	6.2 ± 0.5^a	170 ± 120^b	12.0 ± 0.9^a	190 ± 23^b
ScHxt7	1.3 ± 0.3^a	130 ± 9^b	11.7 ± 0.3^a	110 ± 7^b
CiGxf1	2.0 ± 0.6	48.7 ± 6.5		
CiGxs1	0.012 ± 0.004	0.4 ± 0.1		



Ref: Vleet and Jeffries (2009); Hagerdal et al. (2007)


Xylose
XR
NAD(P)H + H+
NAD(P) +
Xylitol
XI
XDH
NAD +
NADH + H +
Xylulose
XK
6-Phosphoglucone-1,5 lactone
6-Phosphogluconate
Ribulose-5-Phosphate
Xylulose-5-Phosphate
Ribose-5-Phosphate
Septulose-4-Phosphate
Erythrose-4-Phosphate
Fructose-6-Phosphate
Glucose
-34
Regulation points
Glucose-6-Phososphate
-2.9
Fructose-6-Phosphate
-19
Fructose-1,6-Bisphosphate
-0.23
Glyceraldehyde-3-phosphate
2.4
Dihydroxyacetone-Phosphate
-1.29
1,3-Bisphospoglycerate
-0.09
Energetic breakeven point
3-Phosphoglycerate
-0.83
2-Phosphoglycerate
1.1
-23
Pyruvate
Phosphoenolpyruvate
Xylose Utilization Enzymes
XI- Xylose Isomerase (Bacterial) XR- Xylose Reductase
XDH- Xylose Dehydrogenase XK- Xylulokinase
Numbers in the glycolysis pathway are ΔG (kJ/mol) values for reactions
Glycolysis pathway metabolites
Common metabolites
PPP pathway metabolites
Regulation points

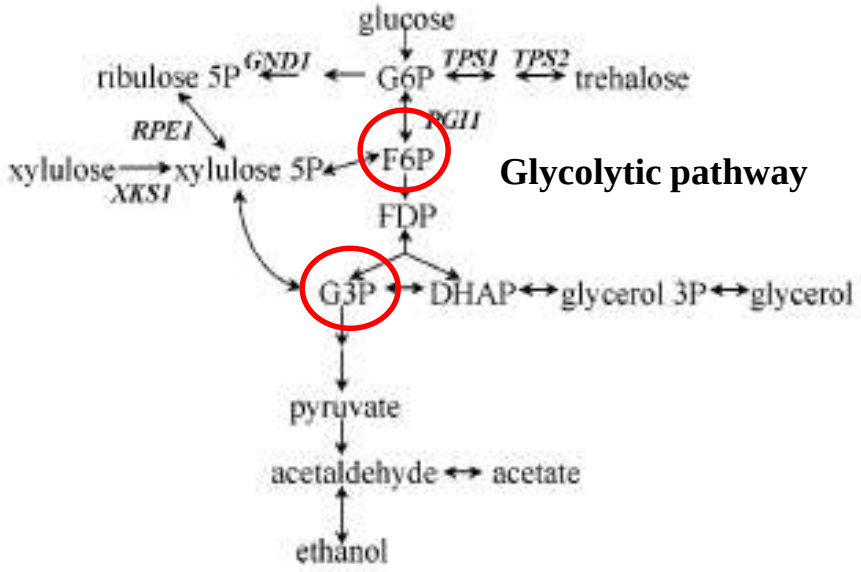
Challenges in Xylose Utilization Scheme in Yeast



Ref: Kotter and Ciricacy (1993)

Challenges in Xylose Utilization Scheme in Yeast

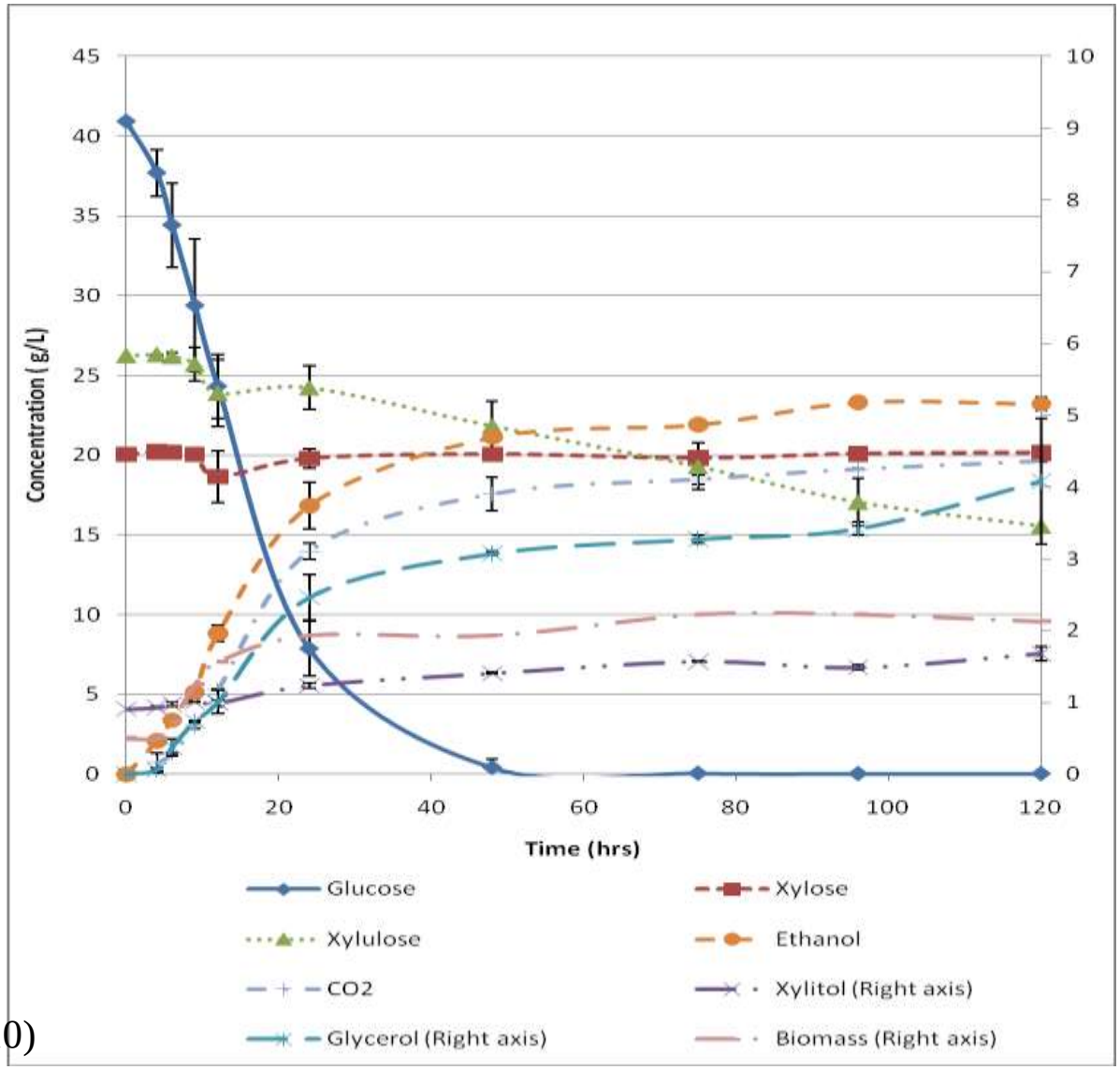
PPP



Glycolytic pathway

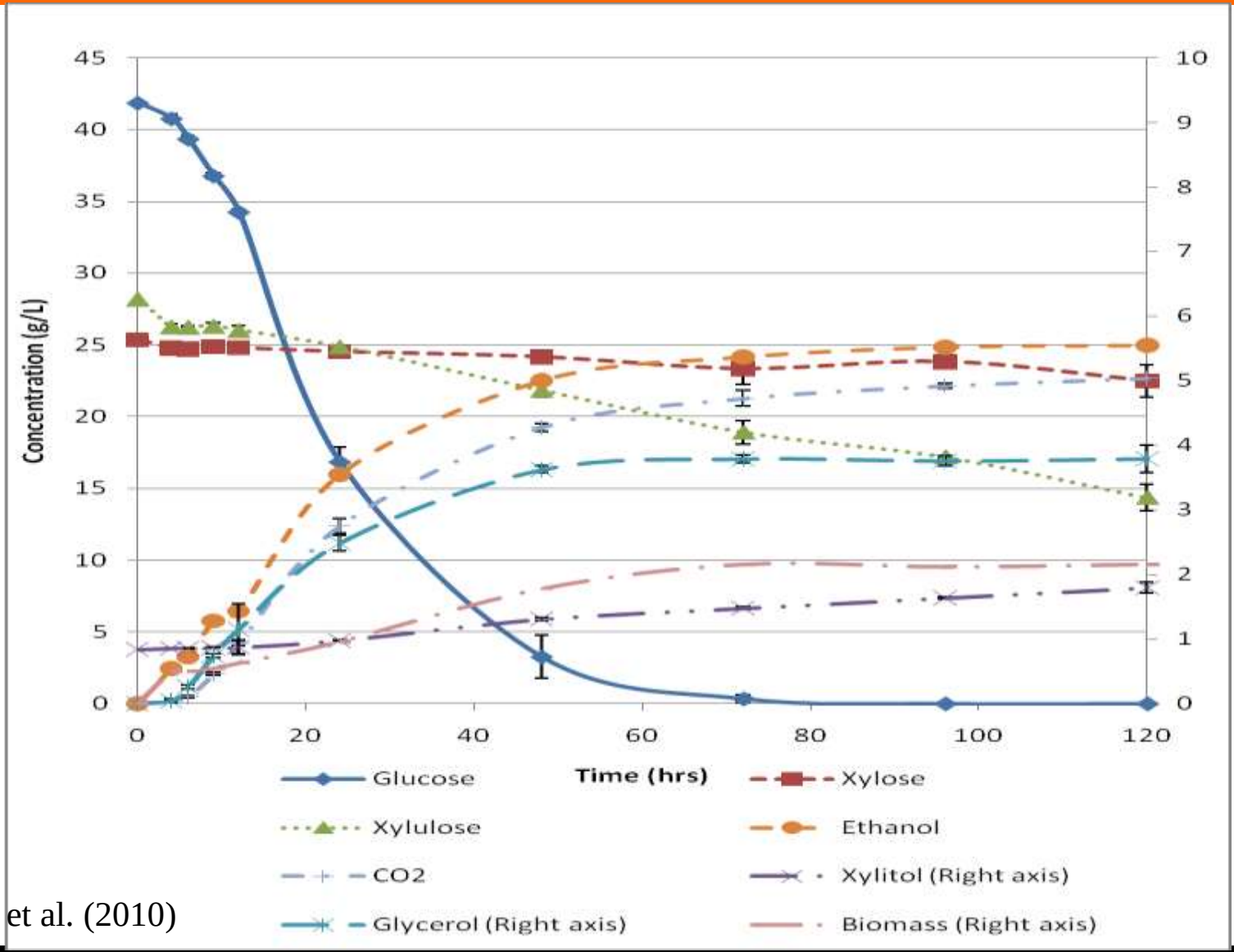
Ref: http: //www.rpi.edu/dept/chem-eng/Biotech-Environ/beer/biochem/biochem.htm; Eliasson et al. 2000

Xylose Fermentation in Yeast (*S. cerevisae*)



Ref: Avanasi et al. (2010)

Xylose Fermentation in Yeast (*Schizosaccharomyces pombe*)



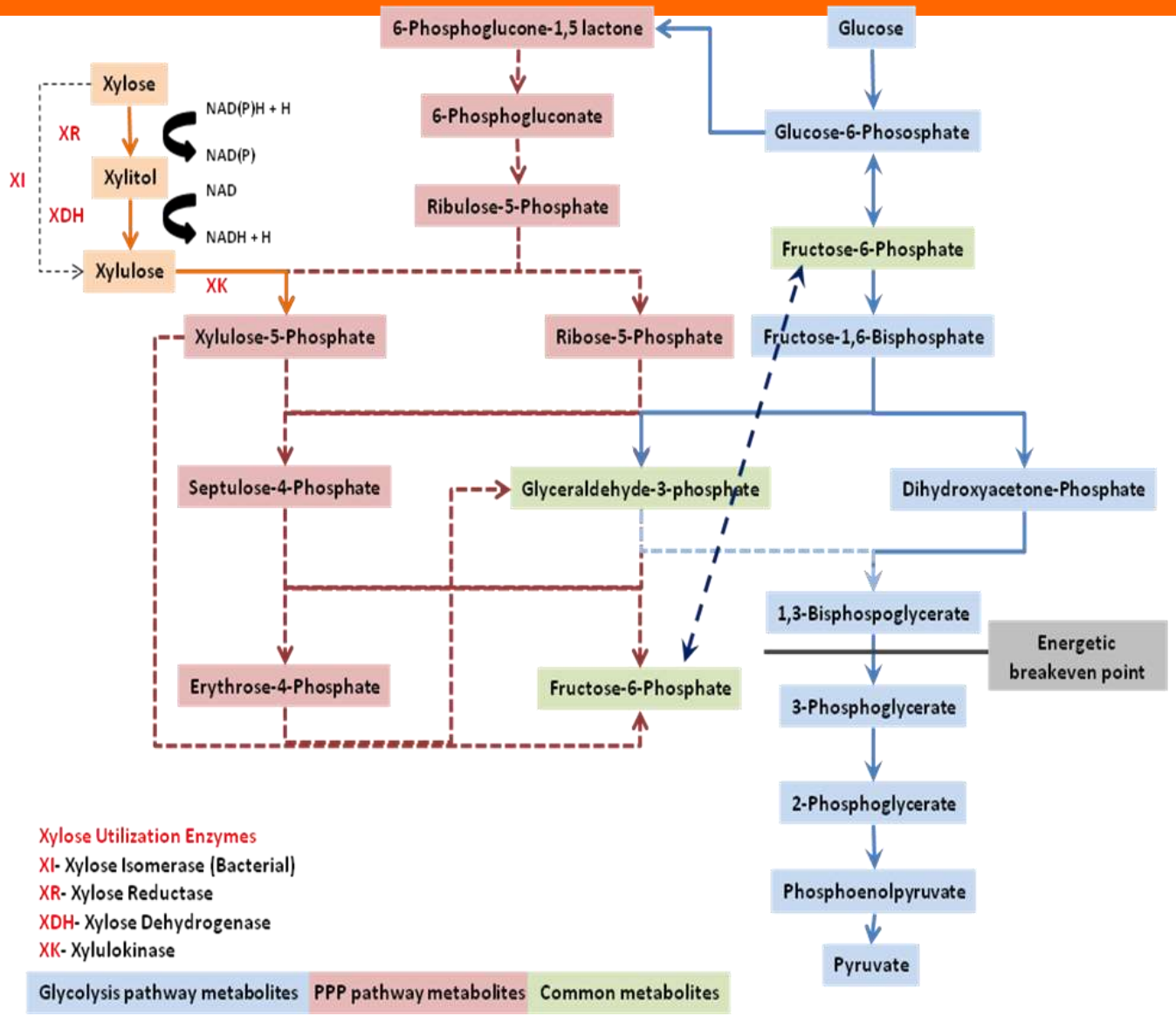
Ref: Avanasi et al. (2010)

Industrial Xylose Fermenting Yeasts

Strain	Hydrolysate	Detoxification	Setup	Ethanol productivity	Ethanol yield on total sugar
TMB 3400	Spruce	Nondetoxified	Fed-batch	0.25	0.43
TMB 3006	Spruce	Nondetoxified	Fed-batch	0.66	0.37
424A LNH-ST	Corn stover	Overliming	Batch	–	0.41
424A LNH-ST	Corn stover	Not known	Batch	–	0.44
Adapted TMB 3006	Northern spruce 70%	Nondetoxified	continuous, D 0.1	–	0.41 (on glucose)
MT8-1/Xyl/ BGL	wood chip hydrolysate	Overliming	Batch	0.42	0.41
F 12	Still bottoms fermentation residue (vinasse)	Not known	Batch	0.005–0.24	0.27[a]
TMB 3400	Corn stover, steam pretreatment	Nondetoxified	Batch SSF	–	0.32
TMB 3400	Corn stover, steam pretreatment	Nondetoxified	Fed-batch SSF	–	0.30

Ref: Hagerdal et al. (2007)

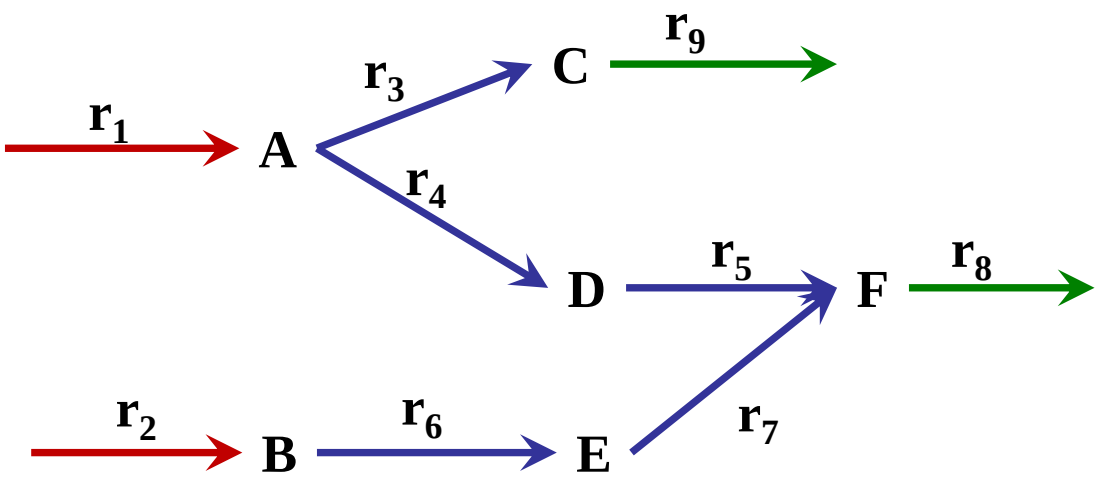
Metabolic Engineering of Yeast



Flux Balance Analysis (FBA)

Mass Balances

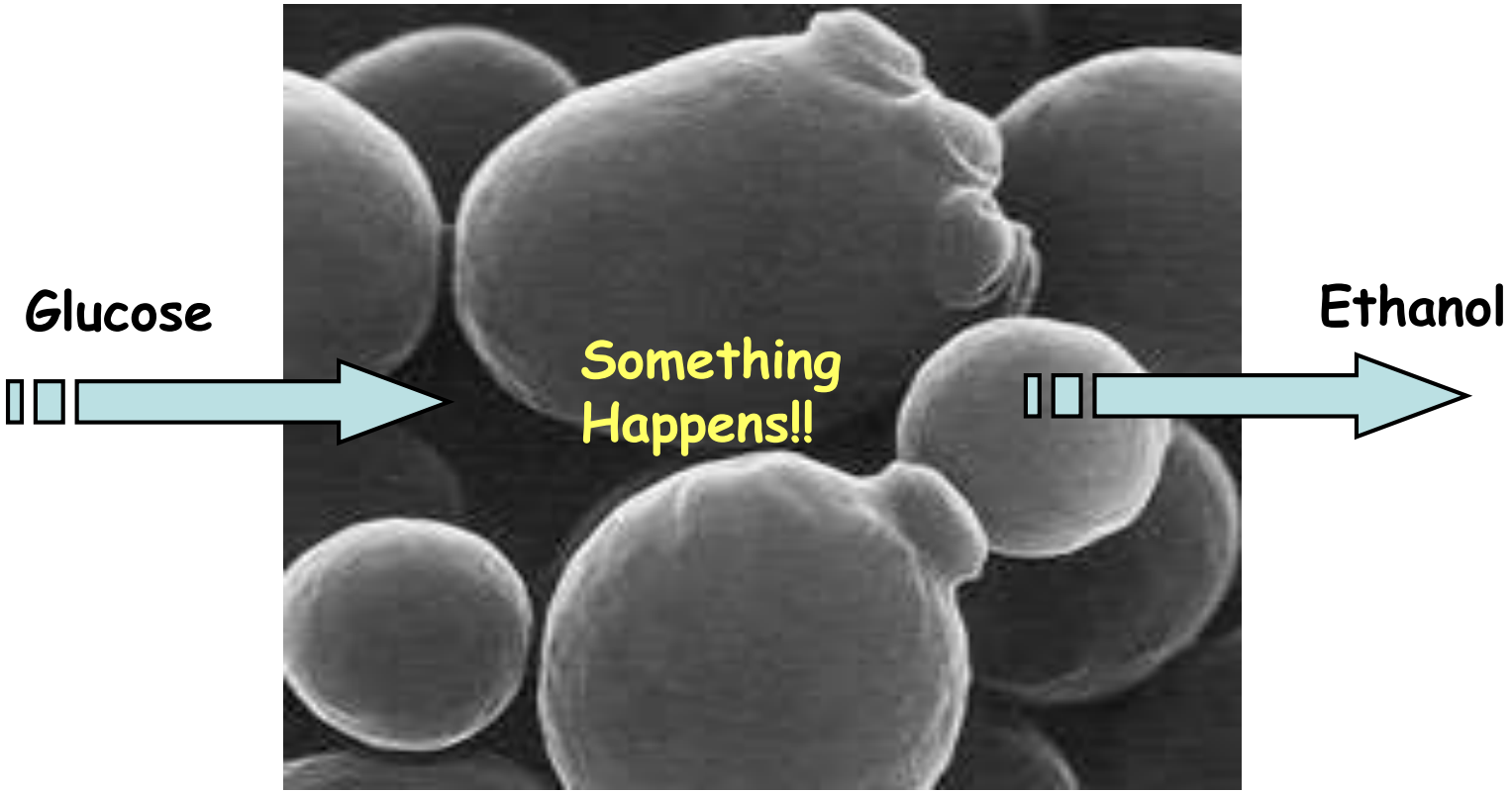
- $\dot{A} = r_1 - r_3 - r_4$
- $\dot{B} = r_2 - r_6$
- $\dot{C} = r_3 - r_9$
- $\dot{D} = r_4 - r_5$
- $\dot{E} = r_6 - r_7$
- $\dot{F} = r_5 + r_7 - r_8$



Stoichiometric Matrix

$$\begin{bmatrix} \dot{A} \\ \dot{B} \\ \dot{C} \\ \dot{D} \\ \dot{E} \\ \dot{F} \end{bmatrix} = \begin{bmatrix} 1 & 0 & -1 & -1 & 0 & 0 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 & 0 & -1 & 0 & 0 & 0 \\ 0 & 0 & -1 & 0 & 0 & 0 & 0 & 0 & -1 \\ 0 & 0 & 0 & 1 & -1 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 1 & -1 & 0 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 & 1 & -1 & 0 \\ r_1 & r_2 & r_3 & r_4 & r_5 & r_6 & r_7 & r_8 & r_9 \end{bmatrix} = 0 \text{ (at steady state)}$$

Yeast



Engineers' view of yeast

Dynamic Flux Balance (dFBA) Model

$$\text{Maximize objective function: } c^T v = \sum_{i=1}^{n} c_i v_i$$

$$\text{Subject to: } \frac{ds}{dt} = \dot{s} = NvX \neq 0$$

$$\dot{X} = \mu X$$

$$c_i = \text{constants}, i = 1, 2, \ldots n$$

$$v_{i,min} \leq v_i \leq v_{i,max}, i = 1, 2, \ldots n$$

$$|\dot{v}| \leq \dot{v}_{max}$$

$$Z(v, s) \leq 0, s, X \geq 0$$

$$s(t_0) = s_0 \; X(t_0) = X_0$$

- $s = [s_1 \; s_2 \ldots s_m]^T, v = [v_1 \; v_2 \ldots v_n]^T$, and $N \in Z^{m \times n}$ is the stoichiometric matrix
- X and Z are biomass and an additional nonlinear constraint vector, respectively.
- The metabolic network was a genome scale metabolic model of *S. cerevisiae* iND750 (750 genes and associated 1149 reactions) developed by Duarte et al. (2003)

Engineering Non Ethanologenic Bacterium

Comparison of performances

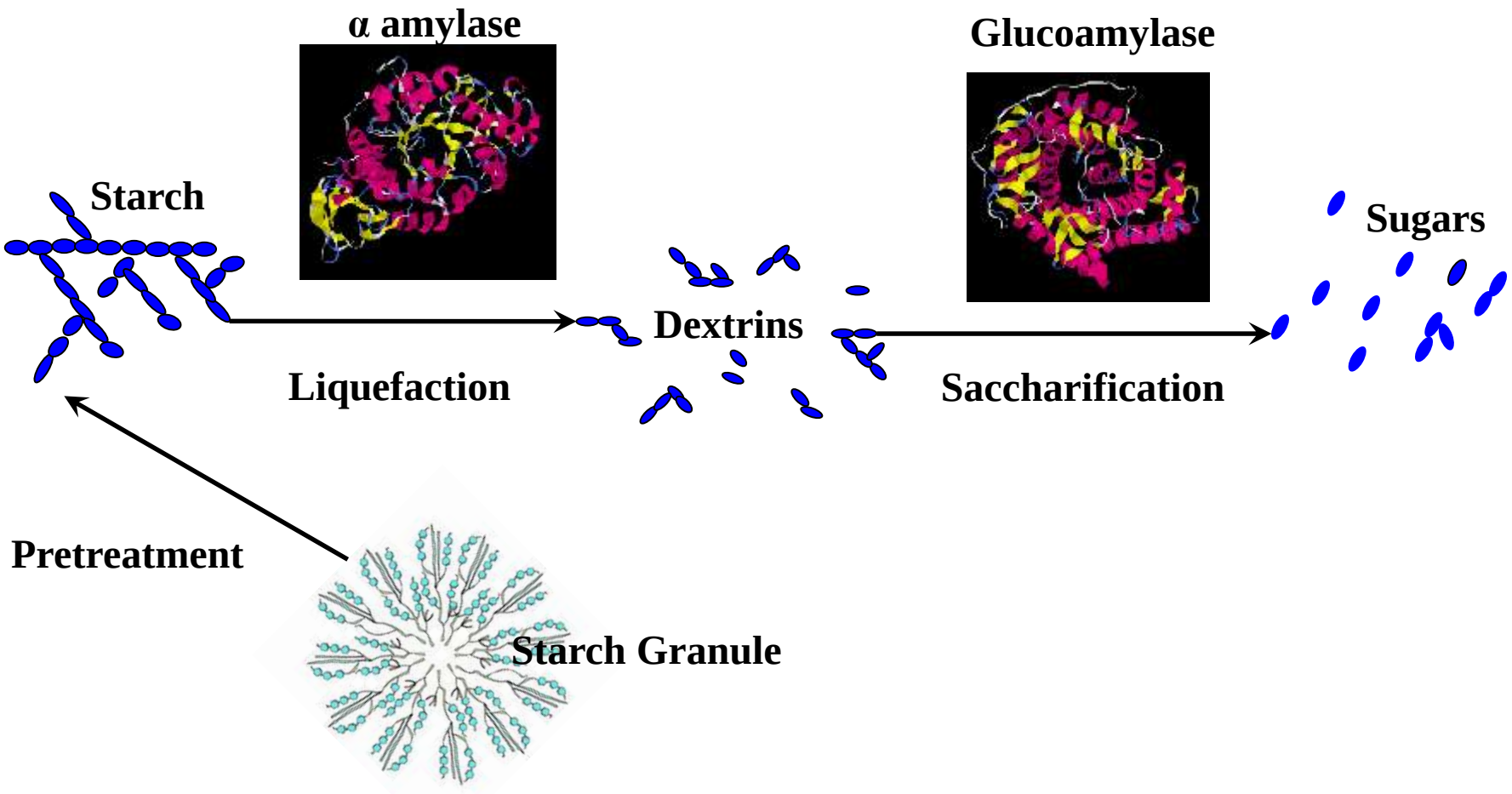
Host	Max. EtOH (g/l)	EtOH Yield (%)	EtOH Prod. (g/l/h)
E. coli	50-64	86-100	0.70-1.0
K. oxytoca	47	84-95	0.40-1.0
Z. mobilis	130 (68)	83-98	0.6-1.1
Saccharomyces	>150 (70)	64-88	0.5-0.6
P. stipitis	47	66-75	0.30

Courtesy: Dr. Bruce Dien, USDA

Lecture Seven

Enzymes in Ethanol Production

Enzymes



Ref: http://food.oregonstate.edu/learn/starch.html

Enzymes

Enzymes act as biological catalysts and help in lowering the activation energy. They, just like any other catalyst do not change the equilibrium.

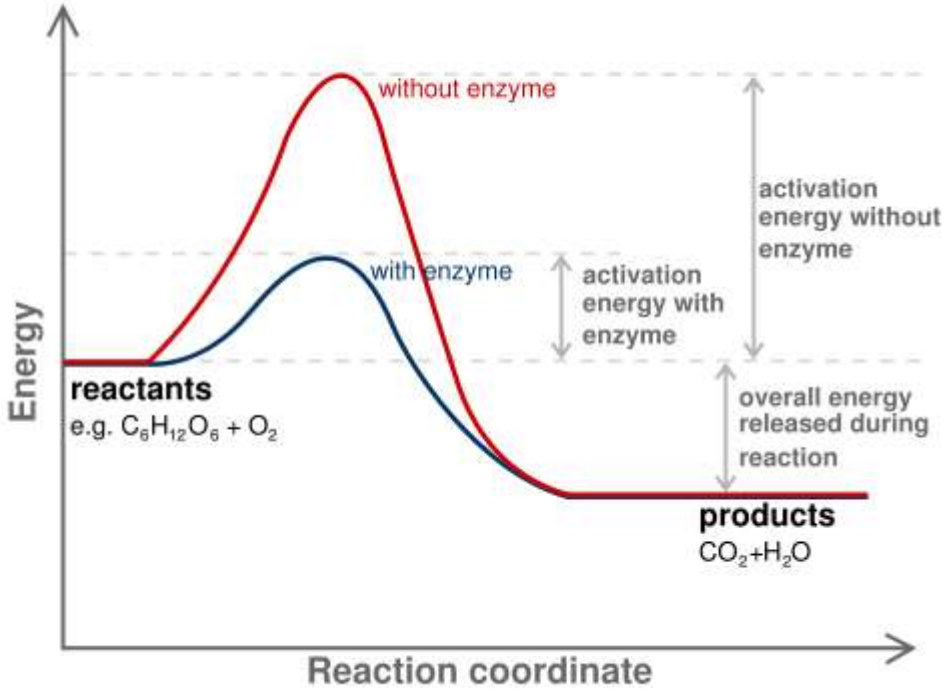
• Most enzymes are proteins. Tertiary and quaternary structure of protein provide the functionality of the enzyme.

• Most enzymes are very specific to

a reaction/functional group.

• Lock and Key ; Induced fit model

are models to explain enzyme action



Enzymes

Enzyme Kinetics: Enzyme reactions are most commonly described using Michaelis-Menton Equation (Important: assumes quasi steady state).

$$E + S \underset{K_{-1}}{\overset{K_1}{\leftrightarrow}} ES \xrightarrow{K_2} E + P$$

$$[ES] = \frac{k_1[E][S]}{k_{-1} + k_2} \qquad k_m = \frac{k_{-1} + k_2}{k_1}$$

$$\frac{dP}{dt} = k_2[ES] = V_{\max} \frac{[S]}{k_m + [S]}$$

Under what conditions is Michaelis-Menton equation inadequate to explain enzyme action? Limited/ restricted mobility of enzymes, two phase reactions, enzyme is not limiting, allosteric regulation

Enzymes

Enzyme inhibitors interfere in the enzyme action and reduce its activity.

Irreversible inhibitors: Bind irreversibly to enzyme and inactivate it.

Reversible Inhibitors: Inhibition is reversible.

• Competitive Inhibitors: This type of inhibitor binds to the active site of the enzyme. Changes k_m and V_{max} remains the same

• Uncompetitive Inhibition: This type of inhibitor binds to Enzyme-Substrate complex and renders it inactive.

• Non competitive inhibition : They do not bind to the active site. Changes V_{max} and k_m remains the same.

• Mixed inhibitors: Consist of a combination of competitive and non competitive inhibitors.

Enzyme Classification

Six categories of enzymes

1. Oxidoreductases
2. Transferases
3. Hydrolases
4. Lyases
5. Isomerases
6. Ligases (synthases)

Enzymes

Amylases

• α-amylase (EC 3.2.1.1) 1,4-α-D-glucanohydrolase : This is an endo enzyme. Breaks down amylose and amylopectin by hydrolyzing α 1→4 bonds and yields dextrins. Optimum pH is ~6.0 and temperature 90°C.

• β-amylase (EC 3.2.1.2) 1,4-α-D-glucan maltohydrolase: This is an exoenzyme. It also breaks down amylose and amylopectin by hydrolyzing α 1→4 yielding a disaccharide, maltose. Optimum pH of 5.4

• γ-amylase (EC 3.2.1.3) Glucan 1,4-α-glucosidase/ glucoamylase/ amyloglucosidase: This exoenzyme hydrolyzes α 1→4 and α 1→6 (20 times slower) and releases glucose. Optimum pH is 4.5 and optimum temperature 60°C

Enzymes

Pullulanases

- EC 3.2.1.41is also known as α-dextrin endo-1,6-alpha-glucosidase (Debranching enzyme). This is an exo enzyme
- Type I act only on α-1→6 whereas type II can also act on α-1→4

Cellulose Degradation in Nature

Three Mechanisms

1. Free cellulose mechanisms

   ex. *Trichoderma reesei (aerobic fungi)*

2. Cellulosomes (cellulases with a carbohydrate binding module, CBM)

   ex. *Clostridium thermocellum (anaerobic bacteria)*

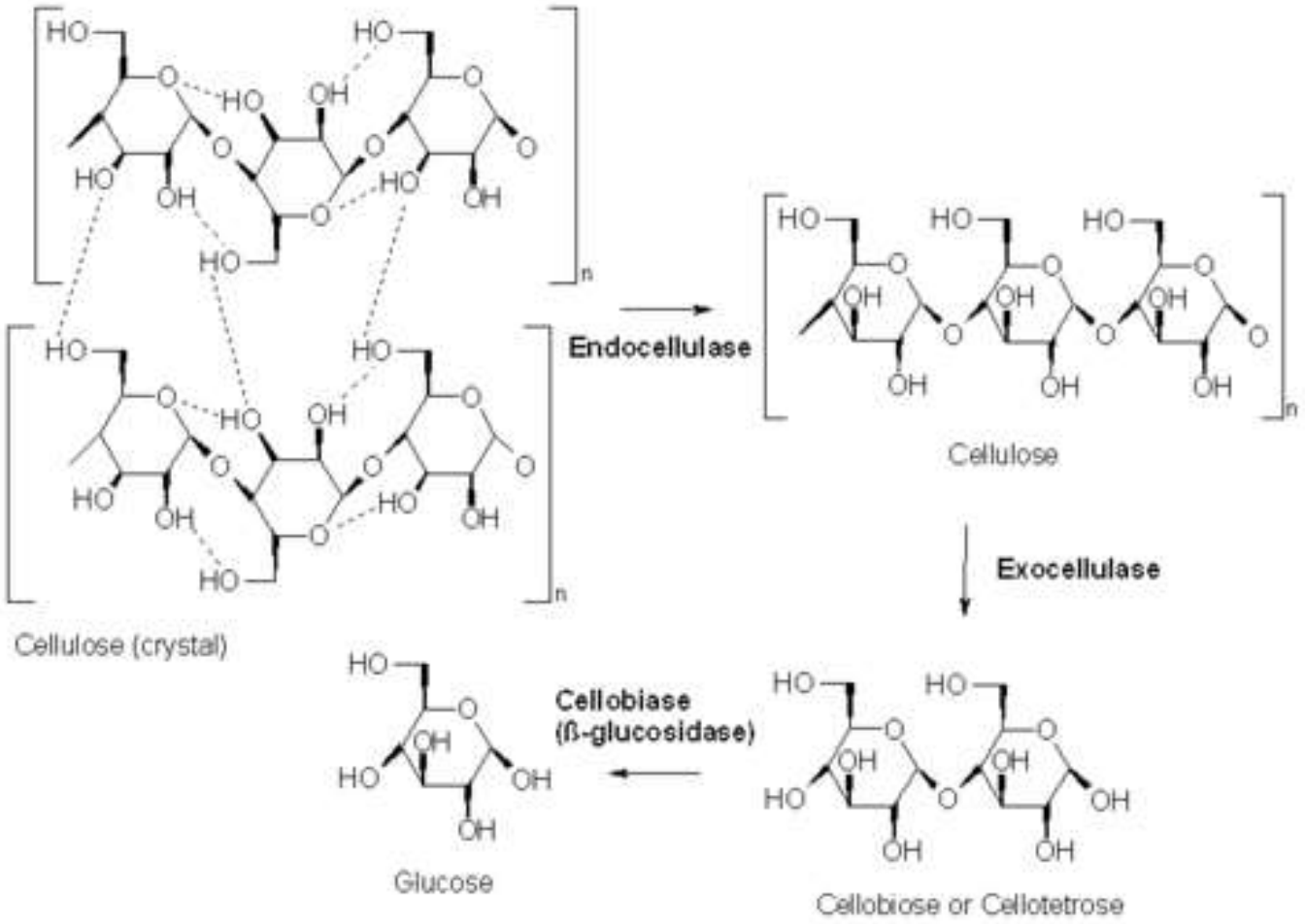
3. Cellululases without CBM

   ex. *Fibrobacter succinogens (anaerobic rumen bacteria)*

Ref: Wilson, D.B. 2011. Microbial diversity of cellulose hydrolysis.Curr. Opin. Microbiol. 14:259-263

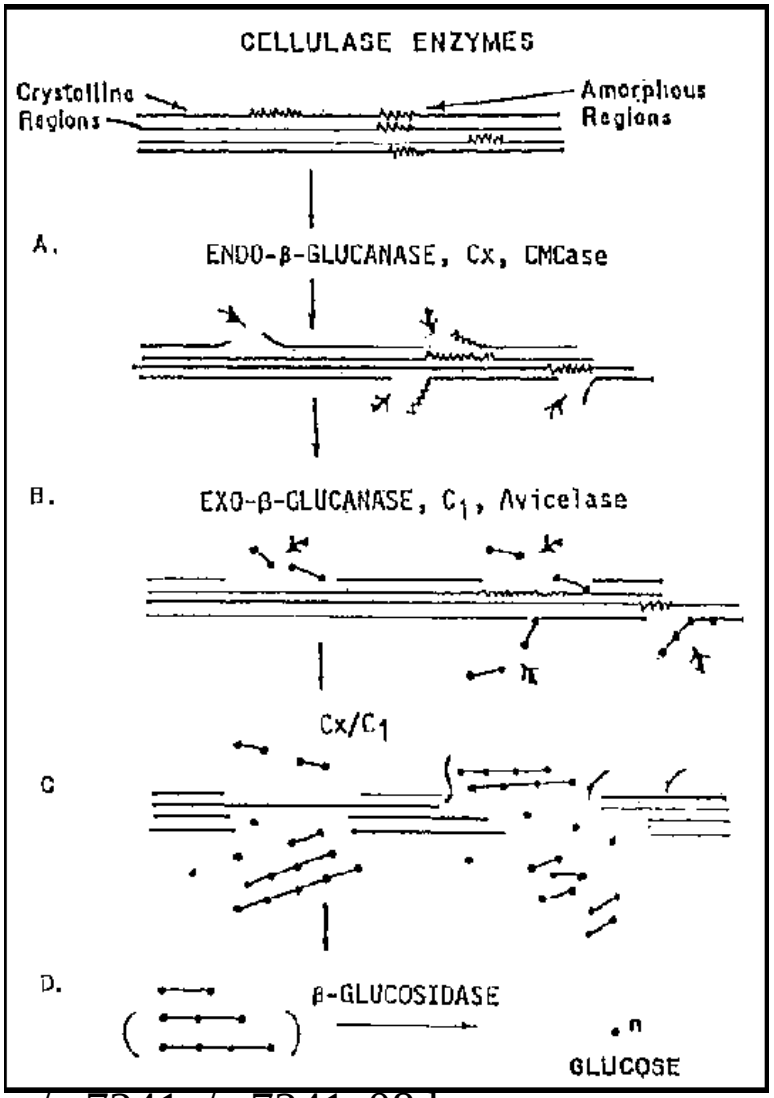
Enzymes

Cellulases



Ref: http://en.wikipedia.org/wiki/Cellulase

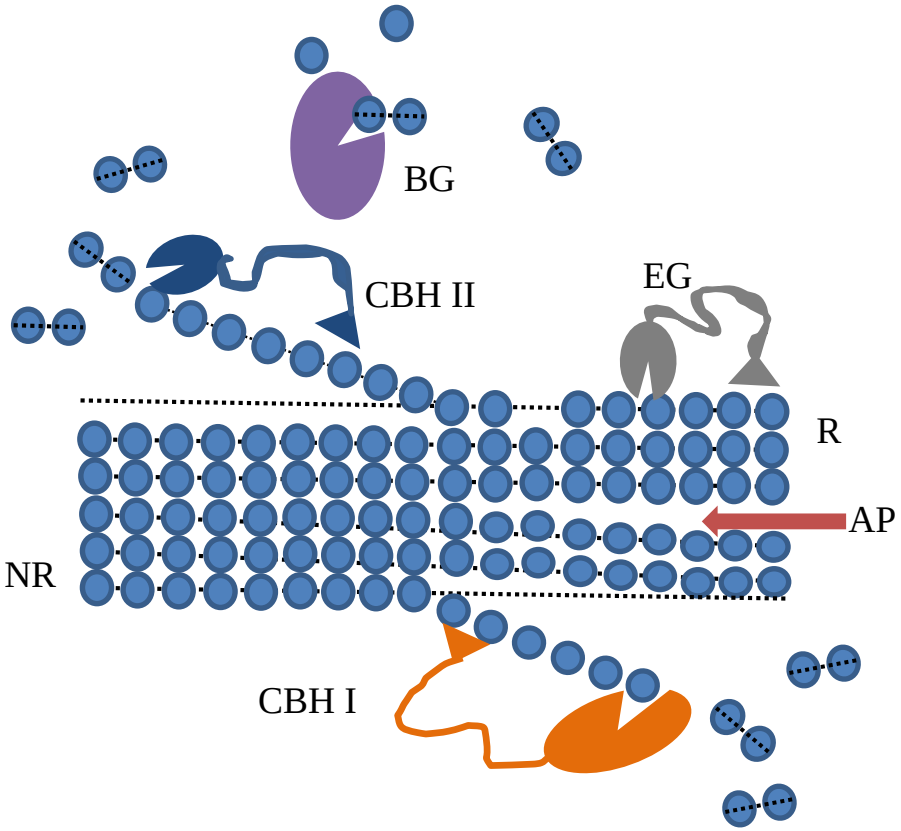
Enzymes



Ref:http://www.fao.org/docrep/w7241e/w7241e08.htm

Cellulase Enzymes

Endoglucanases (EG) act on internal chains to create additional chains. Cellobiohydrolases (CBHI and CBHII) cleave the -1,4 bonds creating cellobiose units. CBH I act from the reducing ends (R) while CBH II act from the non-reducing ends (NR). Betaglucosidase (BG) acts of the cellobiose/cellodextrins to produce glucose. Accessory proteins (AP) facilitate the hydrolysis through a currently unknown mechanism.



Enzymes

Cellulases

• Endo Cellulases: Facilitate hydrolysis by exposing cellulose chains and disrupting the crystalline structure

• Exo cellulases: They further hydrolyze cellulose and yield cellobiose (a disaccharide)

• Cellobiase: These enzymes hydrolyze cellobiose to glucose.

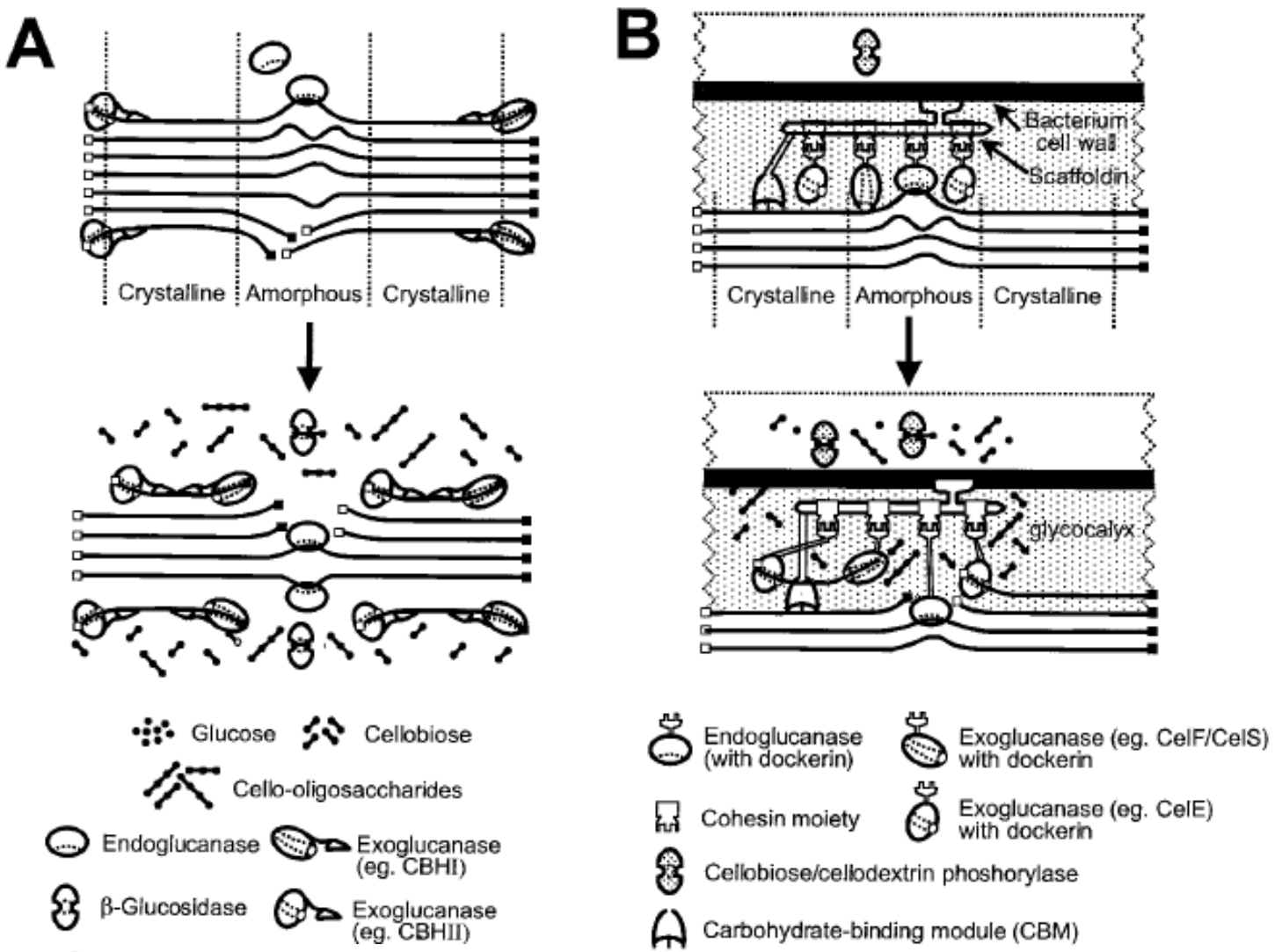
• Oxidative cellulases: “Depolymerize cellulose by radical reactions”

• Cellulose phosphorylases: “Depolymerize cellulose using phosphates instead of water”

• Progressive and non-progressive cellulases

Ref: Wilson, D.B. (2009, 2011); Gowen and Fong (2010)

Enzymes



Ref: Lynd et al. 2002

Enzymes

Cellulase producing fungi in nature

Fungi	Fungi
Acremonium cellulolyticus	*Talaromyces emersonii*
Aspergillus acculeatus	*Thielavia terrestris*
Penicillium funmiculosum	*Trichoderma koningii*
Phanerochaete	*Trichoderma reesei*
chrysosporium	*Trichoderma viride*
Schizophyllum commune	*Aspergillus fumigatus*
Sclerotium rolfsii	*Aspergillus niger*
Sporotrichum cellulophilum	*Fusarium solani*

Ref:http://www.fao.org/docrep/w7241e/w7241e08.htm

Thank you

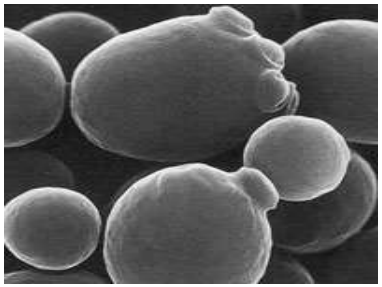
Lecture Eight

Fermentation Technology: Analytical Techniques

Summary of Analytical Techniques

What are some of the important parameters in fermentations process?

- Particle size distribution of feedstock.
- Moisture content of feedstock.
- Presence of toxin producing fungi (ex. *Aspergillus niger*)
- Temperature and pH



- Sugar composition of feedstock (Starch, cellulose and hemicellulose).
- Enzyme activity and stability.
- Yeast cell numbers, viability, vitality.
- Sugars, alcohols (primarily ethanol and glycerol), organic acids
- Protein and lipid content of feedstock and coproducts.
- Dextrose Equivalent

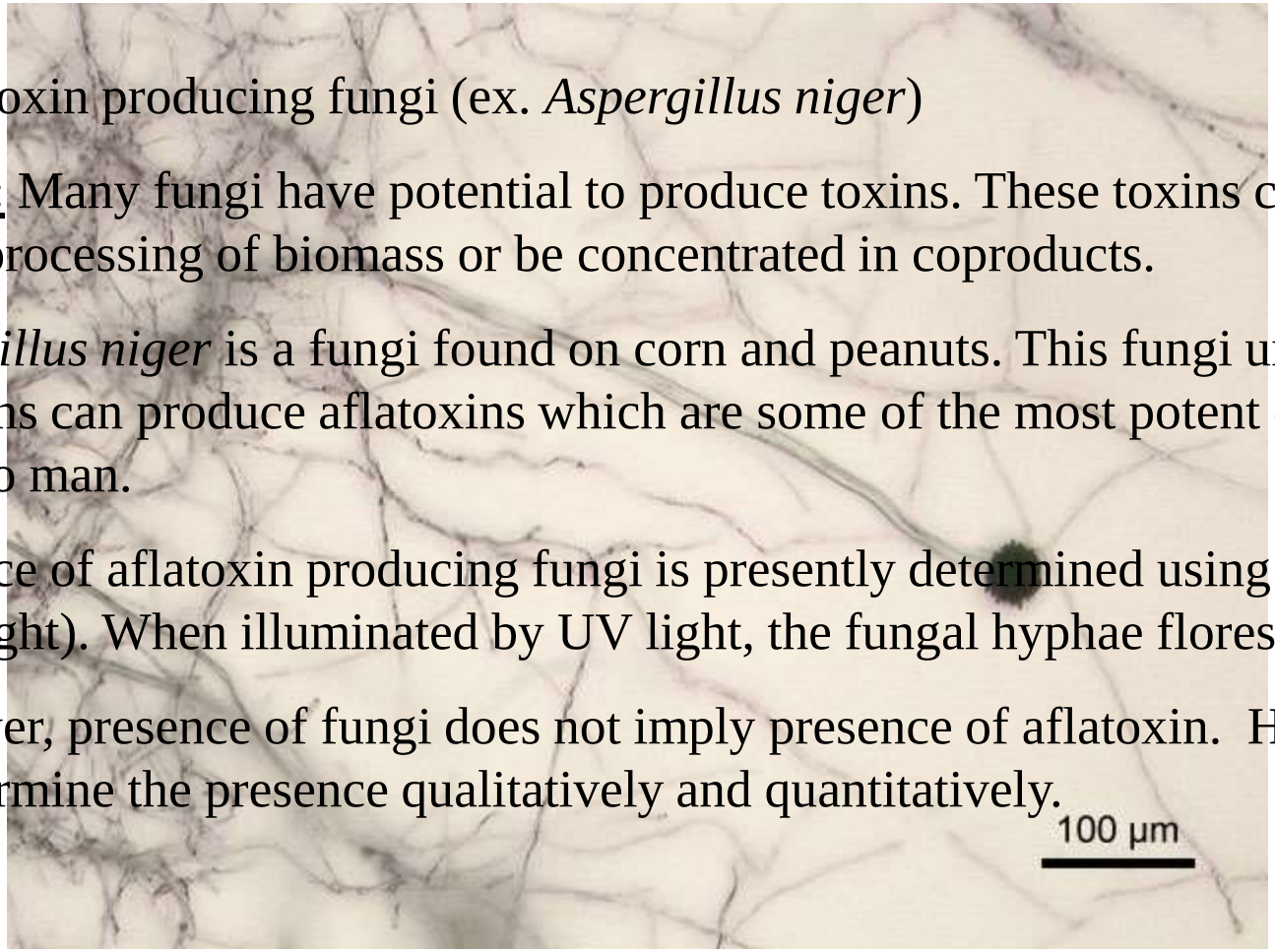
Summary of Analytical Techniques

Particle size distribution of feedstock.

Importance: Particle size distribution determines the available surface area for all the unit operations involving chemical reactions.

- Particle size analysis can be performed using sieve analysis.
- This test measures the weight distribution of different size particles.
- Sieves of varying opening sizes separate particles based on their average diameter.
- Mechanical sieve shakers (for large samples, >200g) and sonic sieve shakers (for small samples <10g) are commonly used.

Summary of Analytical Techniques



Presence of toxin producing fungi (ex. *Aspergillus niger*)

Importance: Many fungi have potential to produce toxins. These toxins could interfere with the processing of biomass or be concentrated in coproducts.

- *Aspergillus niger* is a fungi found on corn and peanuts. This fungi under suitable conditions can produce aflatoxins which are some of the most potent carcinogens known to man.
- Presence of aflatoxin producing fungi is presently determined using UV light (black light). When illuminated by UV light, the fungal hyphae floresce.
- However, presence of fungi does not imply presence of aflatoxin. HPLC methods can determine the presence qualitatively and quantitatively.

Ref. Image:http://en.wikipedia.org/wiki/Aspergillus_niger

Summary of Analytical Techniques

Temperature and pH

Importance: Effectiveness of all unit operations in ethanol production is dependent on these two critical parameters. Enzyme activity and stability, yeast viability and vitality are all determined by temperature and pH of the medium.

- Temperature is measured using thermometers, thermocouples or thermistors. Sources of inaccuracy include electrical noise, faulty connections and poor signal conditioning.

- pH can be measured using pH electrodes (both liquid filled and solid state based sensors). Sources of inaccurate reading are many, including degradation of electrodes over time (causes a drift in the reading), extreme pH, mechanical damage and poor signal conditioning.

Summary of Analytical Techniques

Moisture content of feedstock.

Importance: Moisture content determines susceptibility of feedstock to microbial and enzymatic reactions. It is an important parameter in maintaining proper water balance in a plant.

- Convection oven method is most commonly used.
- For biomass samples such as corn, straw etc containing relatively low moisture and volatile components, 105°C for 24hr followed by 2 hr at 135°C in a convection oven is the most common method.
- For high moisture content and high volatile content samples, liable for heat damage, a lower temperature procedure under vacuum is preferred.
- Infrared based instruments are used for rapid moisture determination.
- Chemical methods are not generally used in ethanol production process.

Ref: Laboratory Analytical Procedure 0012, NREL.

Summary of Analytical Techniques

Sugar composition of feedstock (Starch, cellulose and hemicellulose).

Importance: Knowledge of starch, cellulose and hemicellulose in feedstock is used to determine maximum theoretical yields.

- Starch content is measured by enzymatic hydrolysis procedures. Procedure involves complete hydrolysis (digestion) of starch using acids and enzymes. Resulting sugars are quantitatively measured.

- Cellulose and hemicellulose contents are determined by two step acid hydrolysis process. The procedure will degrade polymeric forms into sugar monomers which are measured by HPLC methods.

Ref: Laboratory Analytical Procedures, NREL.

Summary of Analytical Techniques

Protein and lipid content of feedstock and coproducts.

Importance: Protein and lipid content of feedstock could influence feedstock processing steps. Coproduct protein and lipid content is important in determining its value.

- Protein content is indirectly determined by measuring the nitrogen content of a sample.
- In Kjeldahl method sulfuric acid is used to decompose organic nitrogen to ammonium sulfate. Ammonium sulfate is converted to ammonia by addition of NaOH. Released ammonia is quantified by back titration.
- Other common method is based on quantitative determination of nitrogen gas released during complete burning of sample.
- Lipid content is measured using solvent extraction processes using petroleum ether (most common), hexane or other nonpolar solvents.

Summary of Analytical Techniques

Enzyme activity and stability.

Importance: Enzyme activity and stability determine the amount of enzyme to be added and the possible rate of reaction.

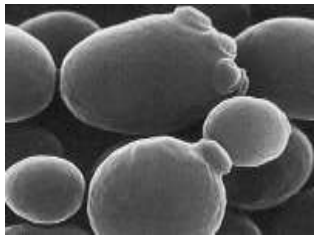
- Standard enzyme assays are used to determine the enzyme activity and stability.
- In these methods a precisely weighed pure substrate is added to known amount of enzyme. The concentration of substrate and products is determined at regular intervals. This information is used to determine the rate of reaction and the enzyme activity is calculated.
- Enzyme stability is determined by measuring the residual enzyme activity after treating the enzyme under different pH and temperature combinations.

Summary of Analytical Techniques

Yeast cell count, viability, vitality.

Importance: Yeast cell count, their viability and vitality are important in determining the rate of fermentation, completion of fermentation and the yields achieved.

- Yeast cell count is determined by cell heamocytometer.



- Yeast viability is determined by methylene blue test. When methylene blue dye is added, the viable yeast cells remain colorless while non viable/dead cells are colored blue.

- Vitality can be inferred from the rate of fermentations and budding index. High values of these parameters indicate high vitality.

Summary of Analytical Techniques

Dextrose Equivalent (DE): Dextrose equivalent is calculated as percentage of dextrose (glucose) expressed on a dry basis.

Importance: It determines the total amount of *reducing sugars* and is indicator of the extent of hydrolysis.

- Dextrose Equivalent of a sample is determined by measuring the reducing sugar content in a sample (Method: http://www.starch.dk/isi/methods/27DE.htm)

Sugar	DE
Glucose	100
Maltose	50
Maltotriose	33.33
Starch	~0

Ref: http://class.fst.ohio-state.edu/fst621/Additive%20classes/Sweet/cornsy.htm

Summary of Analytical Techniques

Sugars, alcohols (primarily ethanol and glycerol), organic acids.

Importance: Determination of substrates (sugars) and products (alcohols, organic acids) is one of the very important measurements that aids in understanding the hydrolysis and fermentation of feedstock.

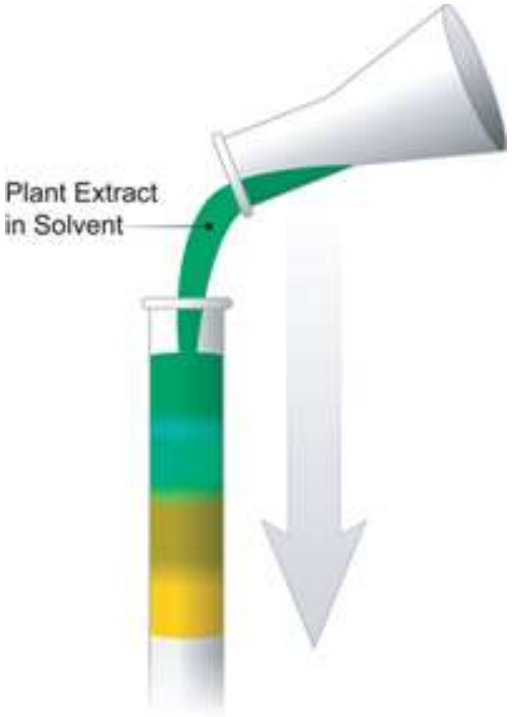
- Most common method is HPLC (High performance liquid chromatography) method. HPLC methods is used for analytical purposes and takes about 30 min for each analysis.

- Rapid detection and quantification methods are based on immobilized enzymes. These methods can measure usually only few components in <5 min.

Summary of Analytical Techniques

High Performance Liquid Chromatography (HPLC) is a method to *separate* different compounds based on their interaction with stationary and mobile phase.

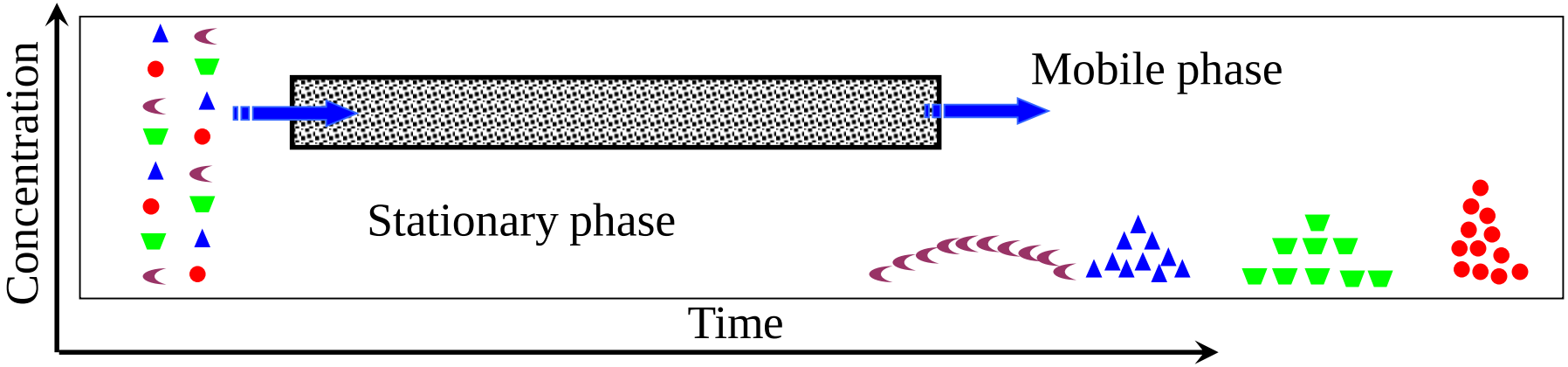
First studied by Russian botanist Mikhail S. Tswett

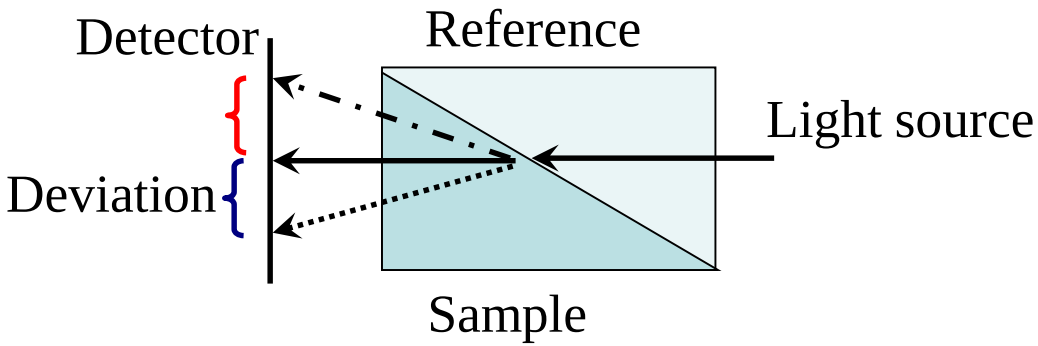


Ref: http://www.waters.com/waters/nav.htm?cid=10048919&locale=en_US

Summary of Analytical Techniques

High Performance Liquid Chromatography (HPLC) is a method to *separate* different compounds based on their interaction with column and mobile phase.





Refractive index detector: principle of operation

Summary of Analytical Techniques

High Performance Liquid Chromatography (HPLC) is a method to *separate* different compounds based on their interaction with column and mobile phase.

- There are two phases in HPLC: stationary phase and mobile phase.
- In HPLC, compounds are separated based on their *relative* polarity.
- Normal phase HPLC uses a polar stationary phase and *relatively* non polar mobile phase.
- Reverse phase HPLC (most common) uses non polar stationary phase and polar mobile phase.
- Isocratic and gradient elution are two methods of analysis.
- Internal diameter, particle size, pore size and pump pressure are some of the parameters that determine the retention time, sensitivity and accuracy of analysis.

Ref: http://en.wikipedia.org/wiki/HPLC
http://www.waters.com/waters/nav.htm?cid=10048919&locale=en_US
http://www.pharm.uky.edu/ASRG/HPLC/HPLCMYTRY.html
http://www.forumsci.co.il/HPLC/program.html

Thank you