# Cellulases and other enzymes in the paper industry: A professional training course designed for the SCA group

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# *Cellulases and other enzymes in the paper industry*

*A professional training course designed for the SCA group*

The course material has been compiled using the author's own knowledge and a wide-range of published sources. The author has endeavored to cite sources when appropriate.

February 2015

# Cellulases and other enzymes in the paper industry

## Training course contents

**Day 1 - 18th February 2015**

1. Sugars and carbohydrates
2. The plant cell wall
3. Enzymes – the basics
4. Biomass-active enzymes

9:00 – 10:30 Section 1

BREAK

11:00-12:30 Section 2

12:30 LUNCH

13:30-15:00 Section 3

BREAK

15:30-17:00 Section 4

**Day 2 - 19th February 2015**

5. Enzymes in papermaking
6. Assaying enzyme activity
7. Sources and production of biomass-acting enzymes

9:00 – 10:30 Section 5

BREAK

11:00-12:30 Section 6

12:30 LUNCH

13:30-15:00 Section 7

BREAK

15:30-17:00 Course wrap-up

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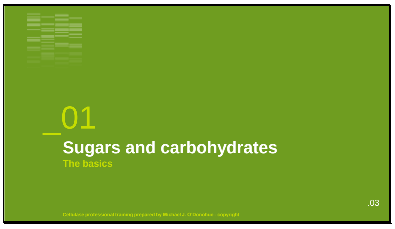


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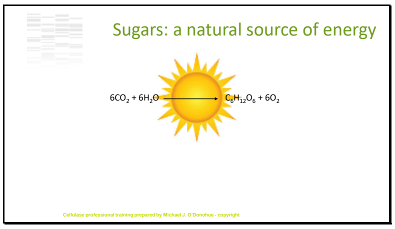


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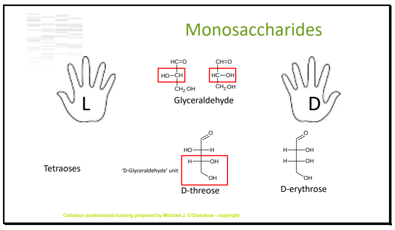

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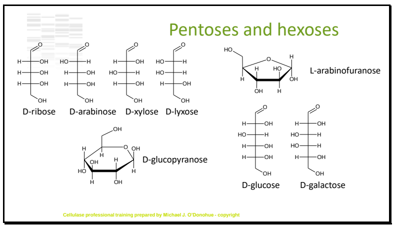


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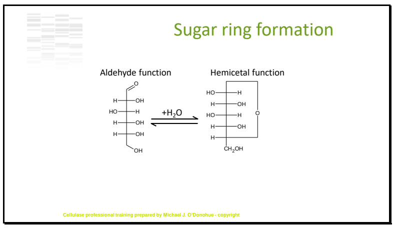


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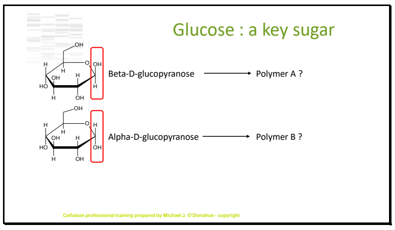

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_02

## The plant cell wall

From complex biomass to fibres





Slide 10

## The fundamental components

- The polysaccharides
  - Cellulose
  - Hemicelluloses
- Lignins



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_02.1

## Carbohydrate compounds

From complex biomass to fibres

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| Component | Softwood | Hardwood |
|---|---|---|
| | % of dry weight | |
| Cellulose | 33-42 | 38-51 |
| Glucomannan | 14-20 | 1-4 |
| Xylan | 5-11 | 14-30 |
| Other polysaccharides | 3-9 | 2-4 |
| Lignin | 27-32 | 21-31 |

Table adapted from Sjöström (Wood Chemistry: Fundamentals and Applications 1993).



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## Cellulose

- Most abundant natural polymer on Earth
- Approx average 40% dw of wood
- Linear homogeneous polymer composed of glucose units
  - Linked through beta-1,4 covalent bonds



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## Cellulose

- Degree of polymerization is 9000-10,000 units
  - Max of 15,000 observed
- High capacity to form H-bonds
  - Basis of the formation of microfibrils
  - In wood, approx 35 cellulose chains per microfibril (80 in cotton)
- Macroscopic organization in plant cell walls

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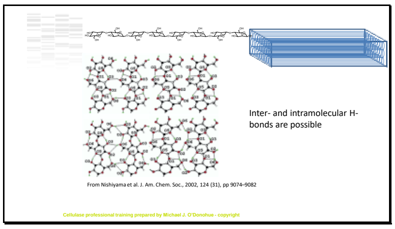


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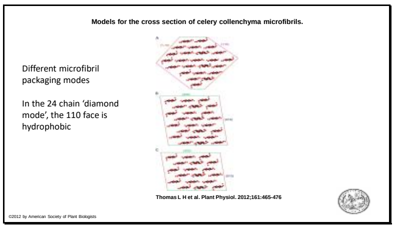


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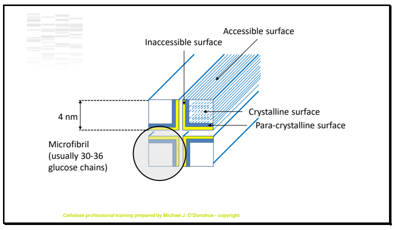

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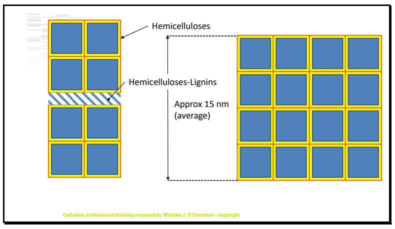


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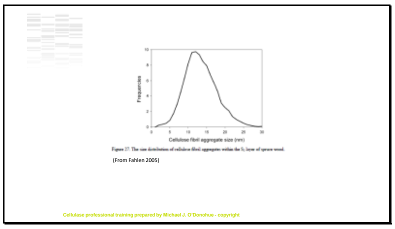


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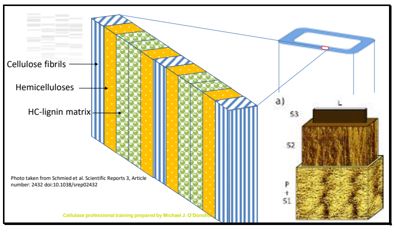

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## Ultrastructure

- Multilamellar structure
- Concentric arrangement *wrt* cell wall axis
  - Number of lamellae dependant on degree of swelling
  - Several hundred lamellae in a wall (each around 1000 angstrom thick) and separated by a distance of smaller dimensions



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## Hemicelluloses

- All polysaccharides that aren't cellulose
- Large differences between species
  - Gymnospores (pines, softwoods) have galactoglucomannans
  - Angiospores (including hardwood trees) mostly have xylans



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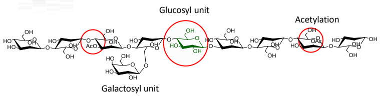
## Galactoglucomannan




$Ac=O_2C-CH_3$

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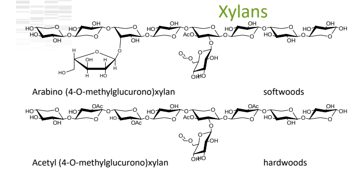
## Xylans



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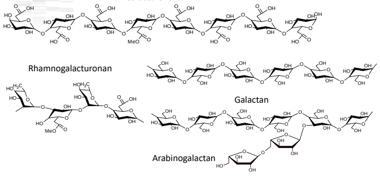
## Pectins



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## Lignins

- The major non-polysaccharide component of plant cell walls
  - A polyphenolic macromolecule with seemingly random structure
- Lignin contributes to cell wall reticulation and hydrophobisation

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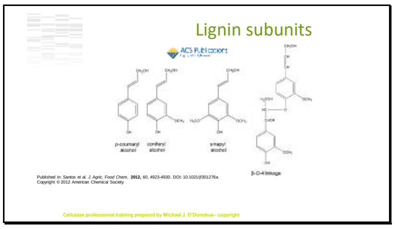


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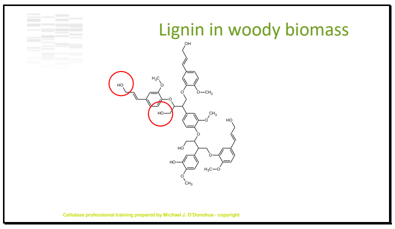


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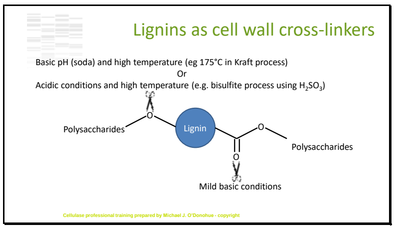

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## Cellulose fibres

- Virgin fibres – fibres that have been freshly extracted from biomass (wood or other)
- Fibre 'activation' and 'deactivation'
- Recycled fibres can contain stickies:
  - Organics such as styrene, butadiene, styrene acrylic latex binders, rubber, vinyl acrylates, polyisoprene,polybutadiene etc.
- Virgin fibres can also exhibit stickies, but these are due to pitch



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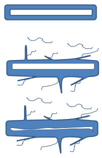
## What happens during pulping and papermaking?

- The surface area is increased enormously (especially during chemical pulping)
  - Lignin is removed (exposes surface)
  - Kraft pulping yields carbohydrate-rich surfaces
- Size and volume of pores increases
  - See pore size estimation





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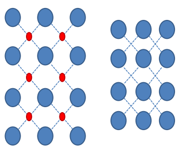
## Fibres and water

- Water is added
  - causes fibre swelling
  - Occurs at amorphous regions and increases fibre wall thickness in the direction of the fibre lumen
  - Water in pores increases fibre flexibility and sheet density
- Water is removed
  - hemicelluloses removal causes water loss
  - Pressing and drying remove water



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## Fibre length

- Scots pine without mechanical treatment (sulfite pulping)
  - Approx 2.5 mm
- Scots pine with mechanical treatment (sulfite pulping)
  - Approx 1.8 mm



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## _02.2

**Non-carbohydrate compounds**

**From complex biomass to fibres**



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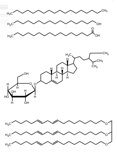
## Lipophilic extractives



Linear alkanes, fatty alcohols and fatty acids

Phytosterols (free and glycosylated)

Triglycerides

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_03

## Enzymes

The catalysts of living organisms

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## Primer on enzymes

- Enzymes are proteins
  - What does this imply?
- Enzymes participate in cellular metabolic processes with the ability to enhance the rate of reaction between biomolecules
- Enzymes can be isolated using various protein purification methods. The purity of an enzyme preparation is measured by determining it's specific activity
  - Specific activity is the amount of activity per quantity of proteinaceous material



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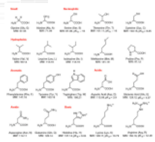
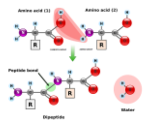
## Amino acids and peptide bonds





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## Enzymes as catalysts

- Enzymes are catalysts
  - What does this imply?
- Some enzymes can even reverse a reaction from the direction it would normally take, by reducing the activation energy (Ea) to the extent that the reaction favors the reverse direction.
- Similarly, enzymes can catalyze reactions that might not otherwise occur, by lowering the Ea to a more "affordable" level for the cell.
- Many enzymes catalyze reactions without help, but some require an additional non-protein component called a co-factor. Co-factors may be inorganic ions such as $Fe^{2+}$, $Mg^{2+}$, $Mn^{2+}$, or $Zn^{2+}$, or consist of organic or metalloorganic molecules knowns as co-enzymes.



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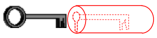
## The key and lock theory



Emil Fischer

What does this theory tell us?



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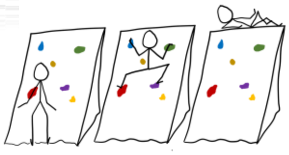
## Transition states



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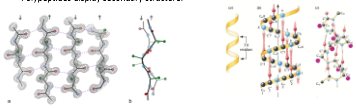
## More about enzymes

- Molecular weights of 10,000 daltons to over 1 million. How many amino acids in a 20 kDa protein ($M_{w\ average}$ amino acid = 110 da)?
- Polypeptides display secondary structure:





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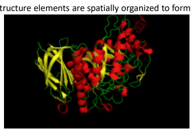
## More about enzymes

- Secondary structure elements are spatially organized to form 3D objects





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## Enyme-catalyzed reactions

- E + S ⟶ E + P
- Usually enzyme display saturation kinetics
  - The more substrate is fed to the enzyme the faster the reaction will proceed until a maximum rate is reached
  - The maximum rate or Vmax is characteristic of an enzyme-substrate couple
- Enzymes display an intrinsic capacity to perform cycles of specific reactions (**turnover number**)
  - This capacity is not the same for different enzymes
    - Catalase can perform about 4.0 x $10^7$ cycles $s^{-1}$
    - Often cellulases are slow (e.g. 20 $s^{-1}$)

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## Enyme-catalyzed reactions

- Turnover can only be measured when substrate availability is illimited
  - In this case turnover number (or $k_{cat}$) is $\frac{V_{max}}{[E]}$
- This calculation assumes that every enzyme molecule is active!!!



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## Enyme-catalyzed reactions

Product formed Enzyme

- Specific activity (µmol $min^{-1}mg^{-1}$) is a standardized activity measurement that tells you how much activity can be attributed to a given amount of enzyme
- If $V_{max}$ conditions are assumed, specific activity is directly related to turnover number (µmol $s^{-1}$ $\mu mol^{-1}$ enzyme)
  - Discrepancies reveal either purity problem or loss of activity
- Cellulases are often discribed by **FPU (filter paper units)**



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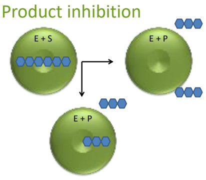
## Factors affecting enzyme performance

- Substrate accessibility
  - Classical enzyme kinetics works for soluble substrates (100% availability)
  - In biomass, not all substrate is available
- Substrate complexity/heterogeneity
  - Classical enzyme kinetics works for simple, pure substrates
  - In biomass, the substrate for any one enzyme is not pure and there can be more than one substrate
- Inhibition
  - Enzyme-catalyzed reactions can be inhibited by
    - Compounds that bind to the enzyme thus preventing activity
    - Product inhibition is a common phenomenon in biomass-active enzymes

## Slide 48

### Product inhibition

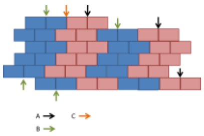
- Instead of being released the product binds to the enzyme
  - Usually in the active site
- This is a concentration depend phenomenon and often occurs at the end of reactions




## Slide 49

### Enzyme synergy

- The action of one enzyme can promote that of another
  - Especially true in the case of complex substrates
- Synergy is when the overall measured activity is greater than the sum of the measured activities




## Slide 50

### Enzyme operational stability

- All reactions accelerate as a function of temperature
  - Arrhenius relationship $k = Ae^{-\Delta G/RT}$
- However, enzymes denature at a given temperature
  - Thermoactivity versus thermostability

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## Enzyme operational stability

- Enzyme activity is dependant on pH
  - Most hydrolases rely on acid/base catalysis
  - The protonation state of the catalytic amino acids is vital (*more later*).



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## Formulation of enzymes

- Industrial enzymes are usually 'shelf-life' stabilized
  - Increase shelf life and protect against microbial growth
  - Exact formulations are not always known

**Safety, handling, and storage**
For best product performance, Cellic CTec3 should be stored at cool temperatures in closed containers protected from sunlight. The product has been formulated for optimal storage stability; however, enzymes gradually lose their activity over time. The recommended storage conditions are 0-10 °C (32-50 °F). Prolonged storage time and/or adverse conditions such as higher temperature may lead to a higher dosage requirement.



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## Formulation of enzymes

- Major tricks of the trade
  - Immobilization
  - Enzyme modification
  - Additives
    - Presence of appropriate ions (eg metal cations)
    - Salts (increased ionic strength – promotes protein-protein interactions)
    - Polyols (eg sorbitol) – these reduce water activity and thus microbial growth

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## Enzyme classification

- The universal nomenclature system for enzymes is proposed by the IUBMB
- Enzymes are classified according to the reactions they catalyze
  - Six classes are:
    - Oxidoreductases EC1
    - Transferases EC2
    - Hydrolases EC3
    - Lyases EC4
    - Isomerases EC5
    - Ligases EC6



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## The workings of IUBMB nomeclature

- EC 3.2.1.4 is a cellulase
  - EC3 = hydrolase
  - EC 3.2 = glycosylase (a hydrolase that acts on sugars)
  - EC3.2.1 glycosylase that hydrolases *O* and *S*-linked compounds
  - EC3.2.1.4 glycosylase that acts on cellulose
    - EC3.2.1.1 a glycosylase that acts on starch



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## The IUBMB system doesn't tell you…

- Where the enzyme comes from
  - Bacteria, yeast, fungi, mammals
- What it looks like
  - 3D structure
- Whether two enzymes are similar in structure and properties
- Whether it catalyzes reactions in the same way

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## Industrial enzymes are mixtures

- The usual term for enzyme mixtures is ‘*cocktail*’
- Most cocktails contain numerous enzyme activities with certain being dominant

Cellic CTec3 contains proficient **cellulase components** boosted by proprietary enzyme activities, including **advanced GH61 compounds**, improved **β-glucosidases** as well as a new array of **hemicellulase** activities, and together, they improve the conversion efficiency of Cellic CTec3 by at least 1.5 times over Cellic CTec2.



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## _04

## Biomass-active enzymes

The basics

.058



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## Biomass-active enzymes

- Hydrolases
  - EC3
- Oxidoreductases
  - EC1
- Transferases
  - EC2
- Lyases
  - EC4.2

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## Carbohydrate hydrolases

- Hydrolysis of glycosidic bonds C-O-C
- Two principal reaction mechanisms
  - Retaining and inverting enzymes
- Two principal categories based on active site topology
  - Endo and exo-enzymes



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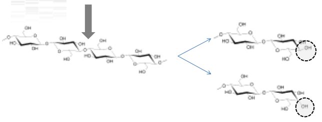
## Reaction mechanisms



Koshland, D. E. Stereochemistry and mechanism of enzymatic reactions. Biol. Rev. 28, 416-436 (1953).



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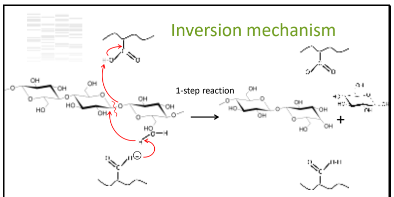
## Inversion mechanism



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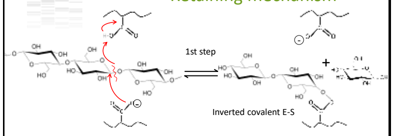
## Retaining mechanism



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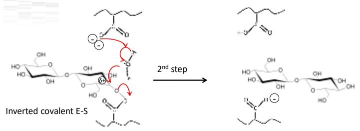
## Retaining mechanism



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## So what?

- Retaining enzymes form a *stable* E-S intermediate
  - This intermediate can react with water or another alcohol
  - Retaining enzymes can synthesize sugar compounds
    - A little like working in reverse
- Retaining enzymes can (not always) give surprising hydrolytic results

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## CAZYmes

- Carbohydrate-Active enZYmes
  - Enzymes that modify in some way sugars
- Enzymes that act on lignocellulose are CAZYmes



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## CAZy database

- The IUBMB classification orders enzymes according to the reactions they catalyze
- CAZY orders enzymes according to similarity



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## From sequence to structure

- Comparing a query sequence (peptide) against a database reveals 'hits'
- 'Hits' can be classed according to sequence similarity
- Fixing a cutoff creates a family of related sequences
- Family members display the same structure and the same reaction mechanism
- But not necessarily the same activity!

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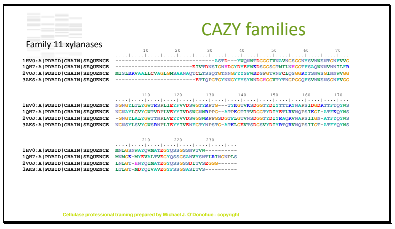


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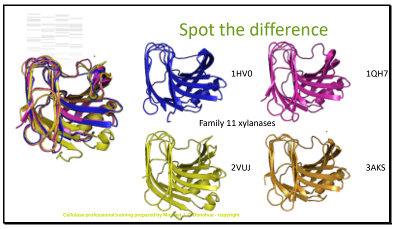


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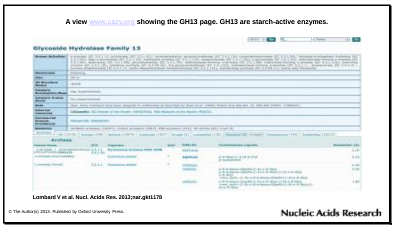

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## CAZy database organization

- Glycoside Hydrolases (GHs) 133 families – 14 clans – 209972 protein modules
  - hydrolysis and/or rearrangement of glycosidic bonds
- GlycosylTransferases (GTs) 97 families –164117 protein modules
  - formation of glycosidic bonds
- Polysaccharide Lyases (PLs) 23 families – 4959 protein modules
  - non-hydrolytic cleavage of glycosidic bonds
- Carbohydrate Esterases (CEs) 16 families – 23064 protein modules
  - hydrolysis of carbohydrate esters
- Auxiliary Activities (AAs) 13 families – 8340 protein modules
  - redox enzymes that act in conjunction with CAZymes



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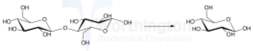
## Cellulases

Cellobiase or β-glucosidase (3.2.1.21) present in GH1, 3, 5, 9, 30, 116




Cellobiose β-D-Glucose



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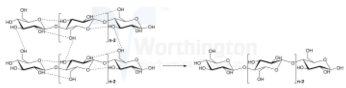
## Cellulases

Endocellulase (3.2.1.4) present in GH5, 6, 7, 8, 9, 12, 44, 45, 48, 51




Cellulose (crystal; n = $10^3$ - $10^4$ glucose residues) Cellulose (n = $10^3$ - $10^4$ glucose residues)

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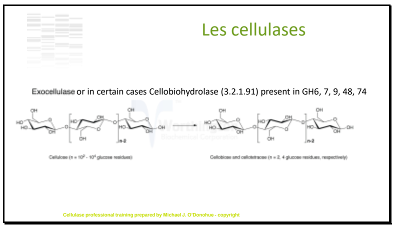


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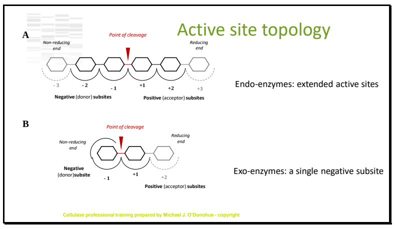


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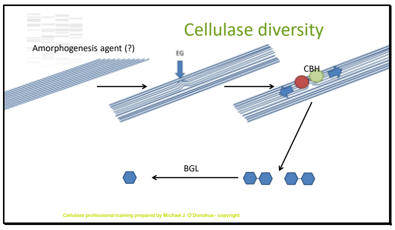

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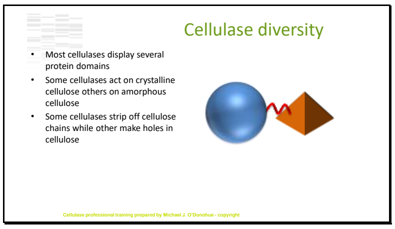


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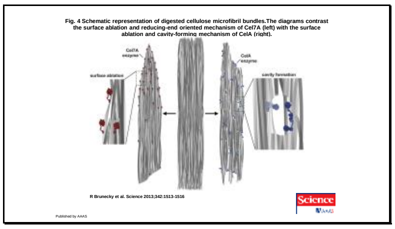


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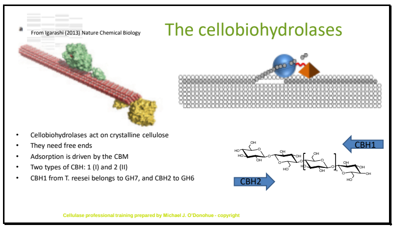

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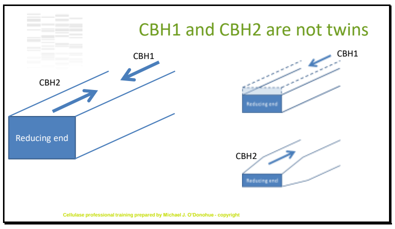


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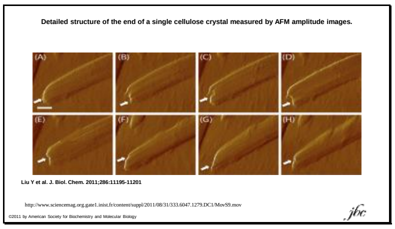


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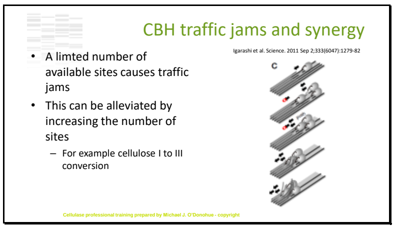

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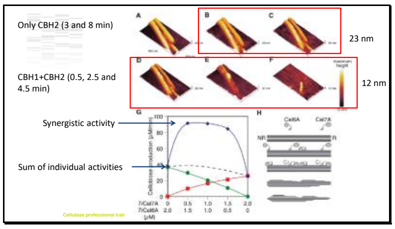


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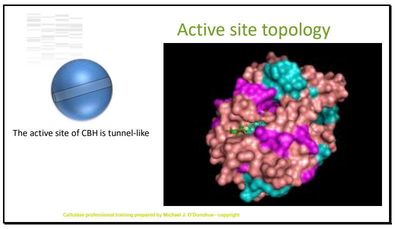


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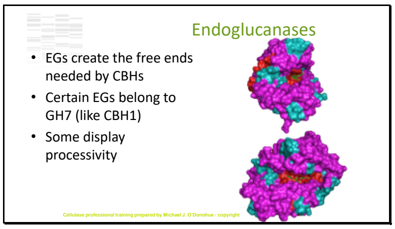

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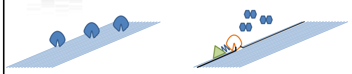
## Endoglucanases



- Random attack
- Rapid adsorption/desorption
- Many types (families) of Egs
  - Big and small
- Faster reduction of viscosity

- Some EGs are processive
- Slower adsorption/desorption
- Often bigger



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## Glucosidases


- Widespread and highly differentiated
- Commercial cocktails are sometimes BGL-deficient (T. reesei only produces one secreted BGL)
- Subject to product inhibition



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## Hemicellulases

- (recall) Hemicellulose is a generic term covering a wide range of enzymes
  - (galacto)glucomannans
  - (glucurono)arabinoxylans

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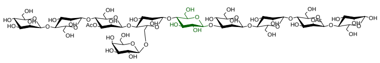
## GGM-specific enzymes





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## GGM-specific enzymes

- Endo activity
  - β-mannanase (1,4-β-D-mannan mannohydrolase, EC 3.2.178),
- Exo- activity
  - β-mannosidase (1,4-β-D-mannopyranoside hydrolase, EC3.2.1.25) β-glucosidase (1,4-β-D-glucoside glucohydrolase,EC 3.2.1.21) and α-galactosidase (1,6-α-D-galactoside galactohydrolase, EC 3.2.1.22)
- Auxiliary activity
  - Acetyl mannan esterase (EC 3.1.1.6)



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## GGM-specific enzymes

- Like cellulases, GGMases are often multimodular

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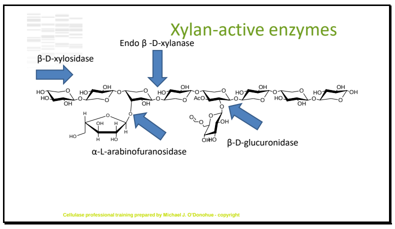


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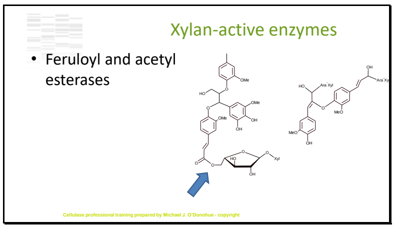


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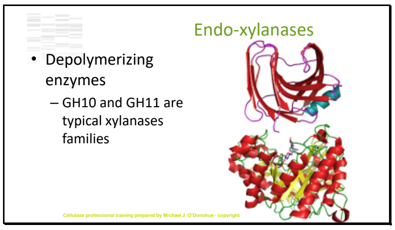

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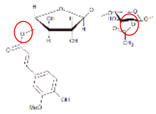
## Esterases

- Cleave ester bonds





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## _05

**Enzymes in papermaking**

What do they do?

.097

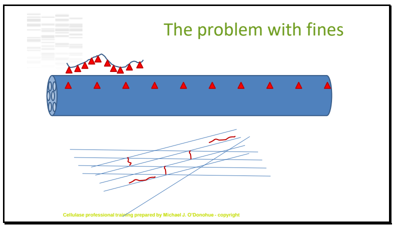


Slide 98

## Enzyme treatment of fines

- Pulp Dewatering and Refining
  - Pulps contain fine particles that impede dewatering
    - Even more so in recycled pulps

Slide 99

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## Enzyme treatment of fines

- Mixtures of hemicellulases and cellulases can be used to degrade carbohydrate fines
  - Drainage rate increased
- What is the danger of this process?

Slide 101

## Xylanases and papermaking

- Xylanases were among the first enzymes to be deployed in papermaking
  - In which part of the process?

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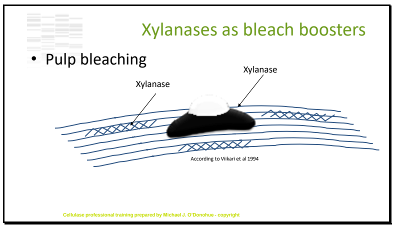


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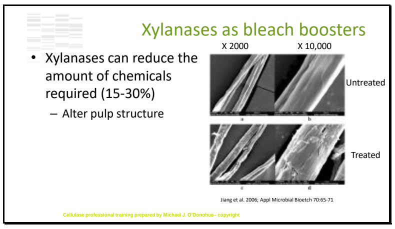


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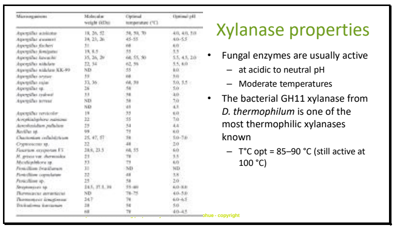

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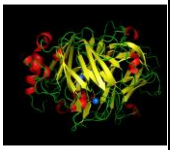
## Laccases

- Laccases are oxidative enzymes that act on lignin
  - They contain copper and are often called 'blue' enzymes





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## A quick recap on redox

- Oxidation is gain of oxygen (loss of electrons/more positive)
- Reduction is loss of oxygen (gain of electrons/more negative)

$$Fe_2O_3 + 3CO \xrightarrow[\text{The carbon compound is oxidized}]{\text{The iron compound is reduced}} 2Fe + 3CO_2$$



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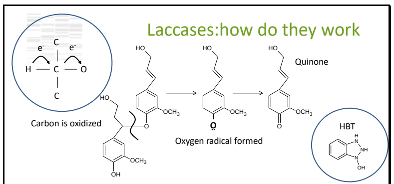
## Laccases:how do they work




The reaction occurs in a cascade and requires a « mediator »

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## Laccases:how do they work

- Mediator is consumed (or theoretically should be recycled)
- Cost and toxicity of mediator (and its by-products)
- Production of radical species
  - Damage to cellulose



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## Xylanases and laccases

- Sequential xylanase-laccase treatment has been shown to be better than xylanase alone
  - Xylanase before laccase boosts the laccase effect
  - What is that called ?



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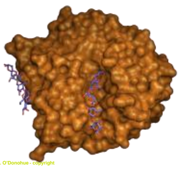
## GGMases and pulping

- Many mannanases belong to family GH5, but also GH26
- GGMases can be used for prebleaching of softwood
  - Cellulase-free preparations avoid fiber loss



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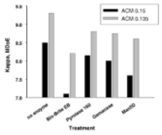
## Enzymatic pre-bleaching

- Example of Mannanase from GH5
  - An enzyme that hydrolyzes β-1,4 links between mannosyl residues. Produces mannobiose
  - Experiment performed on softwood kraft pulp
  - The enzyme lowers the kappa number but xylanase preparation is best




Benech et al (2007) Enz Microb Technol 41 (2007) 740–747



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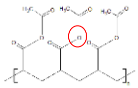
## Other enzymatic treatments in papermaking

- Stickies
  - Esterases and lipases




PVAc



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## Pitch degraders

- Pitch : a natural 'sticky'
- Fungal control using an albino strain of *Ophiostoma piliferum* (Cartapip 97/Cesco White Lightning™)
- Spray onto wood prior to pulping (during storage)
- Fungus penetrates cell walls and feeds on pitch
- Added effect – biocontrol avoids staining by other species

http://www.parrac.co.nz/

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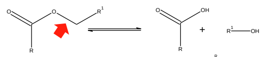
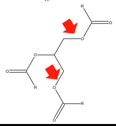
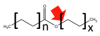
## Pitch degraders



- Lipases
  - e.g. lipase from *Candida rugosa* lip2







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## De-inking enzymes

- Ink is a surface-applied treatment
  - Remove surface fibers and other surface material
- Cellulose fibers
  - Surface fibers can be removed using cellulases
- Starch
  - Starch can be removed using amylases



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## Machine cleaning

- Biofilms
  - A gel-like structure (slime)
  - Formed by EPS
    - A variety of structures
  - Contain complex microbial communities
- No simple enzyme solutions
  - Pectin-active cocktails have been used

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## Summary

| | |
|---|---|
| Amylases | Starch modification<br>Deinking<br>Drainage improvement<br>Cleaning |
| Xylanases | Bleach boosting<br>Energy saving |
| Cellulases | Deinking<br>Drainage improvement<br>Energy saving<br>Tissue and fiber modification |
| Lipases and esterases | Pitch and stickies control<br>Deinking<br>Cleaning |


From Novozymes enzyme brochure

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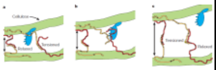
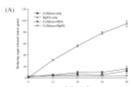
## Post-scriptum: non-enzymatic proteins

- Expansins and swollenins
  - Cell-wall loosening proteins
  - Produced during plant growth, but also by fungi and bacteria
  - In vivo expansins provoke cell enlargement
  - Some expansins are allergens and are related to pollen penetration
  - It is not known for expansins are enzymes
    - Related to GH45



Cosgrove. 2000. Nature 407:321-326


Butterngsook et al. 2014. Biores Technol 159:64-71



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## Post-scriptum: non-enzymatic proteins

- Carbohydrate binding modules
  - Proteins that are linked to many GHs
  - Several possible functions:
    - Increase enzyme concentration on the surface of the substrate/proximity effect (the phase transfer)
    - substrate targeting/selectivity
    - disruption of crystalline substrate
  - Cellulose-specific CBMs
    - interaction with crystalline cellulose (type A CBMs)
    - interaction with non-crystalline cellulose (cellooligosaccharides

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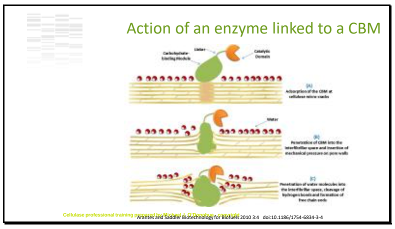


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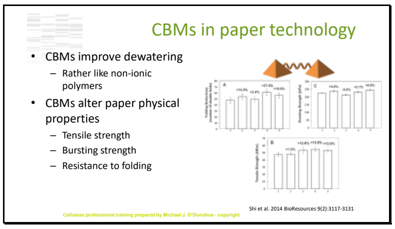


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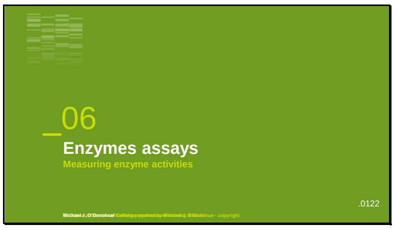

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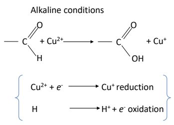
## Reducing sugar assays

- Sugar ring opening provides a free aldehyde
- Aldehydes are oxidized in the presence of appropriate compounds
- Many assay methods have been developed based on the use of Cu II ions
  - e.g. Nelson-Somogyi method
- Other methods using ferricyanide or dinitrosalicylic acid are also used
- Reducing sugar assays are generic
  - Most monosacharides
  - Sugar mixtures



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## The Nelson-Somogyi method

- Cu II is reduced to Cu I
- Cu I species is formed which then reacts with an arsenomolybdate reagent
  - An intensely blue-coloured species is formed (measured at 520 nm/610 nm).



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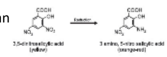
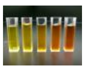
## The DNS method

- The sugar aldehyde reduces a nitro group to an amide
  - Causes a colour shift from yellow to red (assay at 540 nm)
  - The most widely used assay
    - Industry and academia



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## Limitations of reducing sugar methods

- DNS method uses highly alkaline conditions
  - Can cause hydrolysis of certain bonds (e.g. β-1,3 bonds in glucans).
  - Partially degrades cellobiose to glucose
    - RS cellobiose measured as 1.5 glucose equivalents!
  - Severe overestimation for xylose and mannose-based molecules
    - 3-6 fold for glucuronxylan
    - Up to 13-fold for arabinoxylan



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## Comparing NS and DNS

- DNS is not proportional
  - NS is more so
- DNS 10 x times less sensitive (lower limit approx 0.5 mM glucose) than Nelson-Somogyi assay

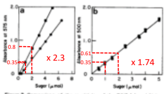


Figure 3 Comparison of the sensitivity of the DNS (a) and Nelson and Somogyi (b) methods for assaying D-glucose (●) and cellobiose (■)



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## Limitations of reducing sugar methods

- Assay response depends of product size (true for DNS and NS)
  - The standard curve is usually glucose
  - *Solution: Adapt standard to expected product*

| Molecule | Abs 520 nm (NS assay) |
|---|---|
| Glucose | 0.2 |
| Cellobiose | 0.25 |
| Cellobiose | 0.7 |
| Cellobiose | 0.3 |
| Cellobiose | 0.32 |
| Cellobiose | 0.33 |

McCleary et al (2012)

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## Assays using synthetic substrates

- A range of substrates for endo and exo-enzymes
- Useful for evaluating pure enzymes or specific components of cocktails
  - Be careful of interference effects!



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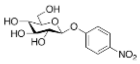
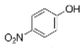
## Evaluating exo-enzyme activity

- *p*-nitrophenyl substrates are widely used
- Small substrates are quite specific for exo-acting enzymes
- In cocktails, a xylosidase might degrade pNP-glucoside and xylanase might degrade pNP-xylotetraose




Colourless




Yellow (at alkaline pH)



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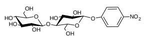
## Evaluating exo-enzyme activity

- *p*Np-cellobiose
  - Same principal but only works for one type of CBH ★ and some EGs
- Other pNP disaccharides (and longer) also commercially exist



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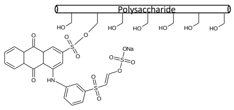
## Remazol Brilliant Blue dyed polysaccharides

- Very universal being applicable to a wide range of soluble polysaccharides
- May are available from Megazyme or Sigma
- Approx 1 dye molecule per 15-20 sugar units
- RBB-dyed substrates are water soluble



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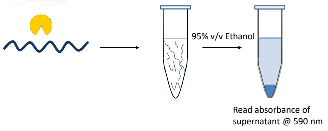
## Using RRB or AZO-dyed molecules



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## Exercise

- You have an unkown enzyme cocktail. You want to know how many units of EG are in there.
- Using the knowledge we have gained, how can we proceed?

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## Answer

- 1. Obtain a commercial sample of pure EG
- 2. Measure its activity using a reducing sugar assay
  - Will provide reducing sugar equivalents released per min per mg protein
  - Perform a test with RBB substrate using different amounts of enzyme and an excess of RBB-substrate



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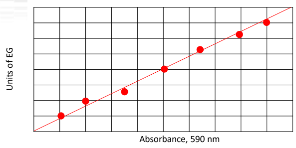




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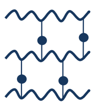
## Insoluble chromogenic substrates

- AZCL or Azo-crosslinked
- Can be purchased in tablet form
  - For easy use and automation
  - Cellulose is always modified (hydroxyethylation or carboxymethylation)



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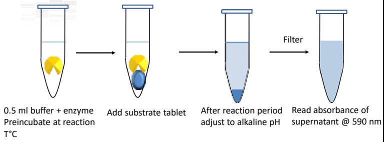
## Using RRB or AZO-dyed molecules



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## Overview

| Assay | Target enzyme activity | Comments |
|---|---|---|
| Reducing sugar assay (Nelson-Somogyi and DNS) | Exo and Endo, but mainly endoenzymes | • Quite tedious<br>• Nelson-Somogyi more accurate than DNS<br>• DNS most used<br>• Yields results of a primary nature |
| pNP-oligosaccharides | Mainly exo, but also endo-enzymes | • Quite straightforward<br>• Lends to automation |
| Dyed substrates | Mostly endo-but also exo-enzymes, | Quite straightforward<br>Automation not easy<br>Results are of a secondary nature |



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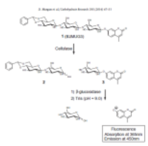
## A fluorescence-based assay

- Reducing sugar assays are tedious
- pNP-cellooligosaccharides can be hydrolyzed by cellobiohydrolase and glucosidase
- Dyed substrates are not so easy to use in automated assays (but can be done)



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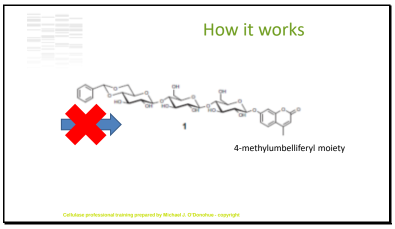


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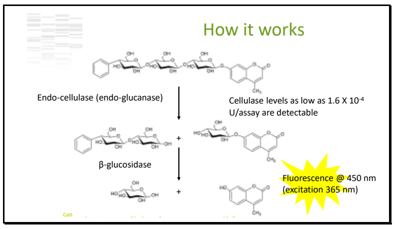


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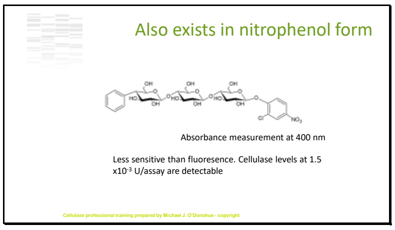

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## The dilemma of complex substrates

- Raw paper pulp is complex
  - Amorphous cellulose
  - Crystalline cellulose
  - Short glucan chains (short fibres)
  - Hemicellulose etc
- Enzyme hydrolsysis is limited by accessibility
- Substrate is insoluble
- Hydrolysis does not obey classical kinetics familiar to the enzymologist



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## The filter paper assay

- Recommended by the IUPAC commission
- Highly used method
- Can evaluate overall cellulose-degrading activity
- Uses the DNS reducing sugar method
- Subject to much error and is prone to reproducibility problems



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## Key features

- See Ghose 1987 (IUPAC)
- Whatman No 1 paper
  - Usually cut into 1 x 6 cm strips (50 mg)
- Assay designed so that only 4% (2mg) of substrate is converted into glucose
- No sophiscated equipment needed
- Suffers from the fact that β-glucosidase is often lacking in cocktails ★

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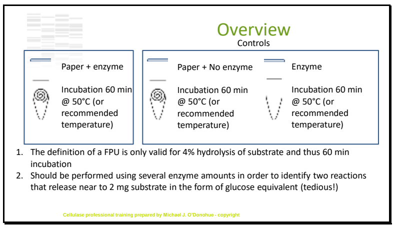


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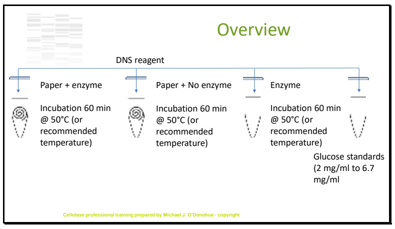


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## Microplate variation

- Microplate versions of the DNS assay have been developed
  - Simply need to maintain filter paper, enzyme, buffer, DNS reagent ratios
  - Filter paper can be cut into circles using a paper punch
  - A PCR 96-well microplate heating block can be used (must be equipped with a heated lid to avoid evaporation) and microplate should be thermosealed.
- β-glucosidase can be added to reduce cellooligosaccharide products down to glucose.

See Xiao et al 2004 Biotechnol Bioeng 88(7)

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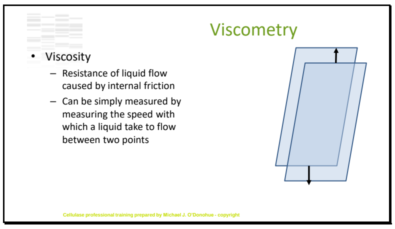


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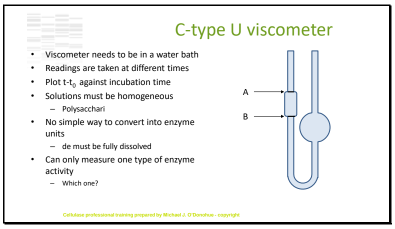


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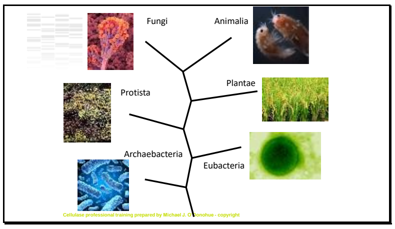


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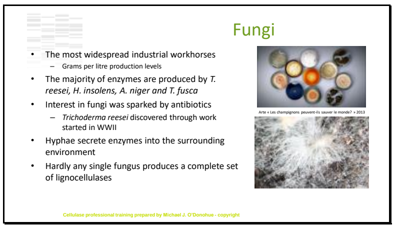


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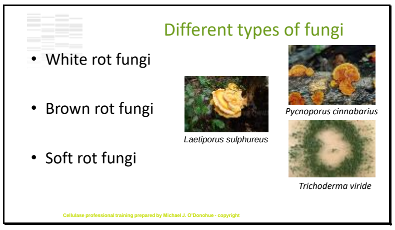

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## Fungal enzyme production

- Submerged fermentation
  - Most usual method
  - Employs artificial liquid media
    - Requires the addition of woody or other lignocellulosic biomass

Arte « Les champignons peuvent-ils sauver le monde? » 2013



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## Solid state fermentation

- SSF is carried out in the apparent absence of free water
  - Highly moisturized atmosphere
- Up to 10-fold lower cost
  - less dewatering
  - less energy (for dewatering, sterilization)
  - Higher productivity
- The fungus grows directly on the solid substrate



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## Bacteria

- Gram +ve and Gram –ve
- Aerobic and anaerobic
- Acidophiles and alkaliphiles
- Mesophiles, thermophiles and psychrophiles

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## Bacteria are everywhere

| Source of microorganisms | Isolated microorganism (s) | Enzyme (s) |
|---|---|---|
| Droppings of elephant | C. thermocellum CT2 | Cellulosome |
| Agriculture soil | Cellulomonas sp. TSU-03 | Cellulosome |
| Hot water spring | Anoxybacillus flavithermus, Geobacillus thermodenitrificans,Geobacillus stearothermophilus | Cellulosome |
| Salt pans | Halomonas caseinilytica, Halomonas muralis | Cellulosome |
| Soil | Cellulomonas sp. YJ5 | Endoglucanase |
| Gut of silk worm | Bacillus circulans, Proteus vulgaris, Klebsiella Ineumonia, Escherichia coli, Citrobacter freundii, Serratia liquefaciens, Enterobacter sp. Pseudomonas fluorescens, P. aeruginosa, Aeromonas sp. Erwinia sp. | Exoglucanase, endoglucanase |
| Vinegar waste | Acetobacter pasteurianus, Acetobacter oboediens, Gluconacetobacter xylinus, Gluconacetobacter hansenii, Gluconacetobacter europaeus, Gluconacetobacter intermedius, Gluconacetobacter entani | Cellulosome |
| Persimmon vinegar | Gluconacetobacter sp. RKY5, Gluconacetobacter intermedius TF2 | Cellulosome |
| Ripe olives | Cellulomonas flavigena | Cellulosomes |



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## Novel cellulase paradigms

- Cellulosome-bound enzymes
- Highly modular free enzymes



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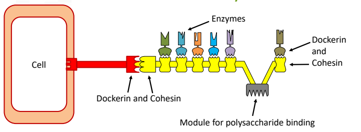
## Cellulosome:the enzymatic Swiss army knife



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## Multi-functional ‘free’ enzymes

- Composed of two or more catalytic modules on the same polypeptide chain.
- Some cellulolytic hyperthermophiles contain numerous multifunctional enzymes with multiple CBMs.
- This‘natural’ strategy may serve as a concept for artificial chimeric multifunctional enzymes.


Himmel et al. Biofuels (2010) 1(2)



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## Bacterial enzymes

- More difficult to produce
  - Lower quantities of protein
  - Fungi are world champions!



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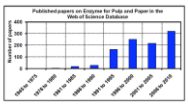
## Next generation enzymes for papermaking

- The progression from lab to market is slow
  - The number of new enzymes is high
  - The potential is mindblowing
  - 50% of enzymes are now from genetic engineering

Demuner et al. 2011. J. Technol.Manag Innov. 6(3)



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## Next generation enzymes for papermaking

" Enzymes have long been part of the papermaking process, but next gen enzymes use less energy, increase efficiency and operate at wider pH ranges" Mark Emalfarb CEO Dyadic International



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## What are Next Gen enzymes?

- NG enzymes are:
  - Recombinant enzymes made by GMO's
  - from the vast natural biodiversity
  - identified through high throughput screening
  - perhaps the result of engineering to adapt properties



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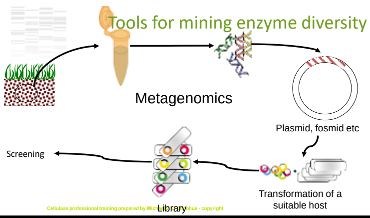
## Tools for mining enzyme diversity



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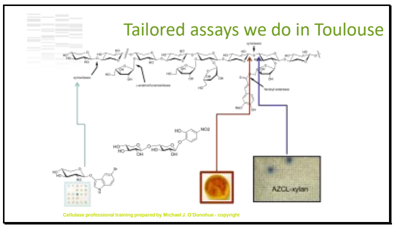


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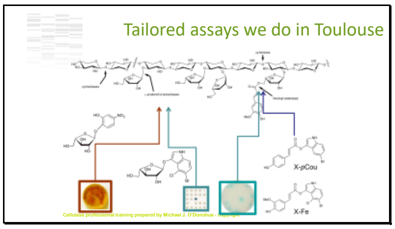

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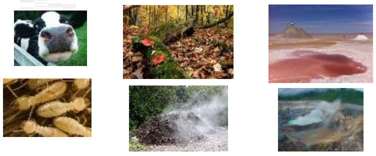
## Good places to explore biodiversity





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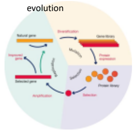
## Tools for enzyme engineering

*In silico* planning and directed engineering

*In vitro* molecular evolution





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## Technical and commercial barriers

- Early integration of all user constraints
  – the laboratory to application process often fails because of poor initial definition of tech. specifications
- Ascertain how the enzyme will be produced
  – The recombinant production of new enzymes in commercial conditions is not easy

# Nelson–Somogyi reducing-sugar assay (slightly modified by McCleary et al.)

## 1. Preparation of reagents

Reagent A: Dissolve 25 g of anhydrous sodium carbonate, 25 g of sodium potassium tartrate, and 200 g of sodium sulfate in 800 ml of distilled water. Dilute to 1 l and filter if necessary (Nelson, 1944; Somogyi, 1952). Store at room temperature.

Reagent B: Dissolve 30 g of copper sulfate pentahydrate in 200 ml of distilled water containing four drops concentrated sulfuric acid. Store at room temperature.

Reagent C: Dissolve 50 g of ammonium molybdate in 900 ml of distilled water and add 42 ml of concentrated sulfuric acid. Separately dissolve 6 g of sodium arsenate heptahydrate in 50 ml of water and add to the above solution. Dilute the whole to 1 l. If necessary, warm the solution to 55°C to obtain complete dissolution. Store at room temperature.

Reagent D: Add 1 ml of Reagent B to 25 ml of Reagent A and mix. Store at room temperature.

Reagent E: Dilute an aliquot of solution C fivefold (e.g. 50 to 250 ml) with distilled water before use (stable at 4°C for approximately 1 week). Store at room temperature.

## 2. Protocol

(This protocol is designed to text enzyme activity using pure substrates, such a β-glucan or cello-oligosaccharides)

To do this experiment you need a boiling water bath, glass tubes with screw caps (or polypropylene microtubes with caplocks), an automatic microdispenser (very useful if you do a lot of assays) and a UV/VIS spectrophotometer or a microplate reader (microplate versions of the Nelson-Somogyi protocol have been developed). A benchtop microcentrifuge is also required.

1. Transfer 0.5 ml of polysaccharide substrate solution (10 mg/ml) [or 0.2 ml of oligosaccharide substrate solution (10 mM)] in 100 mM buffer (of the required pH) to the bottom of five glass test tubes (16-120 mm) and preincubate at 40°C for 5 min.
2. Preincubate enzyme solution (approximately 5 ml) in the same buffer at 40°C for 5 min.
3. Add 0.2 ml of enzyme solution to each of the test tubes containing substrate, mix well and incubate at 40° C for 3, 6, 9, and 12 min.
4. Terminate the reactions by adding 0.5 ml of Nelson–Somogyi solution D, mixing vigorously stirring on a vortex mixer.
5. Prepare the zero time incubation by adding Nelson–Somogyi solution D to a substrate solution to which no enzyme has been added. Upon addition of the enzyme, stir the tube contents vigorously.
6. Prepare a series of D-glucose standard solutions (e.g. 0 – 100 mg/ml) in buffer. Add 0.5 ml of each standard solution to the bottom of glass test tubes (prepare triplicates) and then add 0.5 ml of Nelson–Somogyi solution D
7. Incubate all of the well-sealed glass tubes solutions in a boiling water bath for 20 min
8. Remove the tubes and add 3.0 ml of Nelson–Somogyi solution E with vigorous stirring. Allow the tubes to stand at room temperature for 10 min and mix the contents again. If a turbidity forms, centrifuge the tubes at 3000 rpm for 10 min in a bench centrifuge. Measure the

absorbance of the reaction solutions and standards against the substrate blank in a spectrophotometer set at 520 nm.

9. Plot the measured $Abs_{520\ nm}$ of the glucose standard solutions against glucose concentration (mg/ml or moles/l). This will provide a standard curve.
10. Using the standard curve, determine the concentration of glucose equivalents for each of the experimental time points. Plot concentration of glucose equivalents against time in order to derive a reaction rate (glucose equivalents per unit of time).

# Filter paper assay method

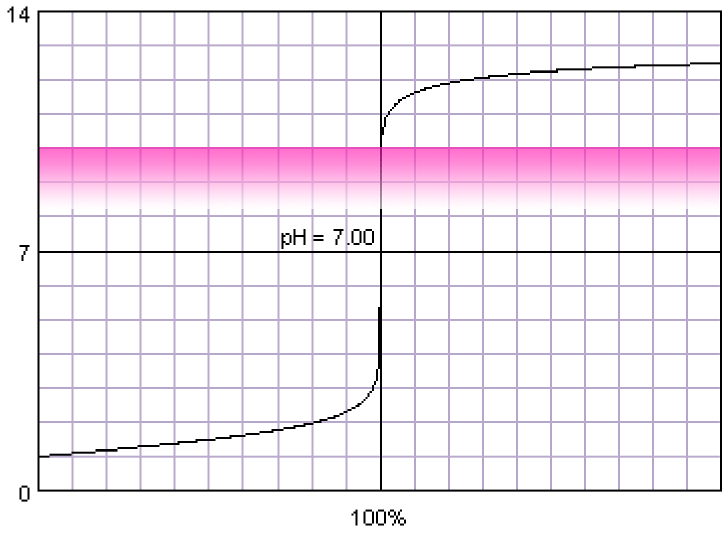
This protocol is based on the method by Ghose 1987 (Pure & Appl. Chem., Vol. 59, No. 2, pp. 257-268, 1987), but is actually drawn from the NREL protocol authored by Adney and Baker in 1996 (NREL/TP-510-42628, revised version 2008).

## 1. Preparation of reagents

### DNS reagent

Mix 1416 ml distilled water with 3,5 Dinitrosalicylic acid (10.6 g) and sodium hydroxide (19.8 g). Sir ina beaker until the solid reagents have dissolved, then add 306 g Rochelle salts (sodium potassium tartrate), phenol (7.6 ml) and sodium metabisulfite[1].

Titrate 3 ml of the DNS reagent with 0.1 N HCl until the phenolphthalein endpoint is reached. This is visually ascertained by observing the transition from red to colourless. It should take 5–6 ml of HCl before this occurs. Add NaOH if required (2 g of NaOH added = 1 ml of 0.1 M HCl used for 3 ml of the DNS reagent). The DNS should be stored at 4°C wrapped in foil. It is advisable to make it up freshly as is convenient. The maximum lifetime of the reagent is 1 month.




### Citrate buffer

The buffer will actually depend on the enzyme that you are studying and the manufacturer's instructions. For *Trichoderma* enzymes, 50 mM citrate buffer at pH 4.8 is appropriate.

Dissolve citric acid (210 g) in 750 ml of distilled water and add NaOH (50-60 g) until pH 4.5 is achieved. Add water to achieve almost 1 litre and check the pH. If ok (i.e. is pH 4.5) complete to 1 litre. When the stock buffer solution (1 M) is diluted to 50 mM for use, the pH should be 4.8. Check this and adjust if necessary.

---

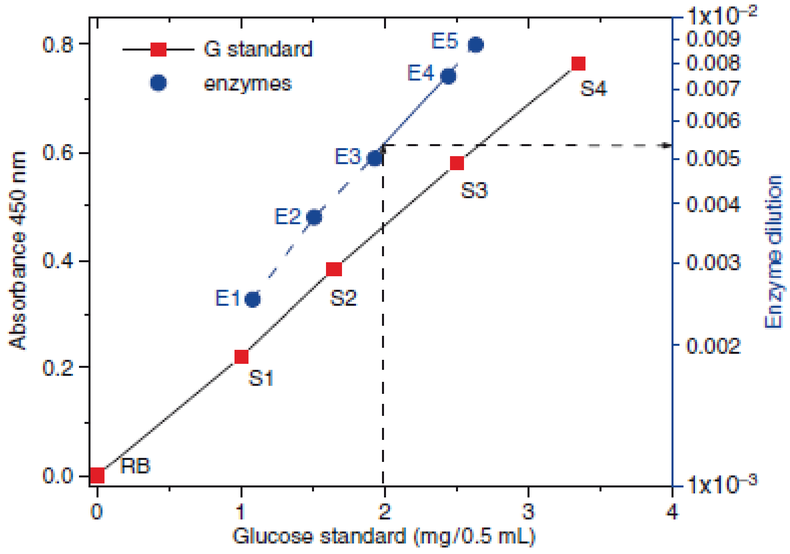
[1] Many of the reagents are corrosive and should be handled with care. Phenol is solid at room temperature and so it must first be molten in a water bath and 50°C. Phenol can cause skin burns and has the tendency to penetrate ordinary rubber gloves. Therefore, utmost caution needs to be applied when handling phenol. Also, after use, the stock of phenol is best conserved in the freezer (-20°C).

## 2. Protocol

To do this experiment you need two water baths: a boiling water bath and another one at 50°C. You also need glass tubes (13 x 100 mm) with screw caps (or polypropylene microtubes with caplocks), an automatic microdispenser (very useful if you do a lot of assays) and a UV/VIS spectrophotometer or a microplate reader (microplate versions of the DNS protocol have been developed).

1. Cut the Whatman N°1 paper into strips (1 x 6 cm). These should weigh 50 mg each. Roll the strips and place them in glass tubes (13 x 100 mm) using tweezers.
2. Add 1 ml of 50 mM citrate buffer to each tube. The buffer should submerge the strip.
3. Prepare a series of enzymes dilutions. If the enzyme preparation is supposed to be clear, it might be a good idea to centrifuge or filter the preparation first. If filtering be careful not to use filters that remove proteins! The enzyme dilutions should be made with the aim of having a reaction that releases 2 mg of glucose. To begin with you may not know exactly how much enzyme will do this. In that there will be some trial and error testing before a definitive test can be done. In any case, even when you know approximately how much enzyme to add, several dilutions should be made in order to get as close to 2 mg as possible in at least one reaction.
4. Equilibrate the filter paper-containing tubes at 50°C for 15 min. Equilibrate the enzyme solution at 50°C for 15 min.
5. Add 0.5 ml of enzyme dilution to the tubes containing the filter paper and incubate at 50°C for exactly 60 min.
6. Simultaneously controls (without enzyme and without filter paper) and blank (1.5 ml of citrate buffer) should be incubated at 50°C for 60min. For the controls without filter paper, the enzyme concentrations should match those of the different dilutions.
7. During the 60 min period, using a stock glucose solution (10 mg/ml) prepare several dilutions: 2, 3.3, 5 and 6.7 mg/ml).
8. After the 60-min incubation period, immediately add 3 ml of DNS reagent to every single tube, including the glucose standards.
9. Place all tubes into a gently boiling[2] water bath for 5 min and then immediately transfer to an ice bucket.
10. Allow the insoluble pulp to settle and transfer 0.5 ml of supernatant into a 1.5 ml microtube. Centrifuge at 10,000 x g for 3 min and transfer 0.2 ml of supernatant into a polystyrene spectrophotometer cuvette (3 ml). Add 2.5 ml of distilled water and mix by inversion (close the cuvette with parafilm).
11. Read the absorbance at 540 nm using the blank as the reference. For the glucose standards, plot a curve of absorbance (y-axis) versus glucose concentration (x-axis). The $R^2$ value should be close to 1.
12. For the actual experimental results, correct data using the results from the controls and then determine the quantity of glucose equivalents present using the standard curve.
13. Assuming that two experiments yielded almost 2 mg of glucose equivalents the preparation of a graph such as the one below will allow the identification of the enzyme dilution that would have produced exactly 2 mg of glucose equivalents (this is done by drawing a straight line between the two experimental points that are closest to 2 mg).

[2] It has been shown that it is better to use a gently boiling water bath than a vigorously boiling one. In the former, non-specific alkaline lysis of glucosidic is lowered and so the results are more accurate.




RB is the blank. S1-S4 are the glucose standards. E1-E4 are the corrected data from the experiments. The solid straight line is drawn between the two 'best points'. This line is used to determine how much enzyme would have yielded exactly 2 mg.

14. To calculate the number of FPU (filter paper units)

$$\mathrm{FPU} = \frac{0.37}{concentration\ of\ enzyme\ releasing\ 2\ mg\ glucose}\ units/ml$$

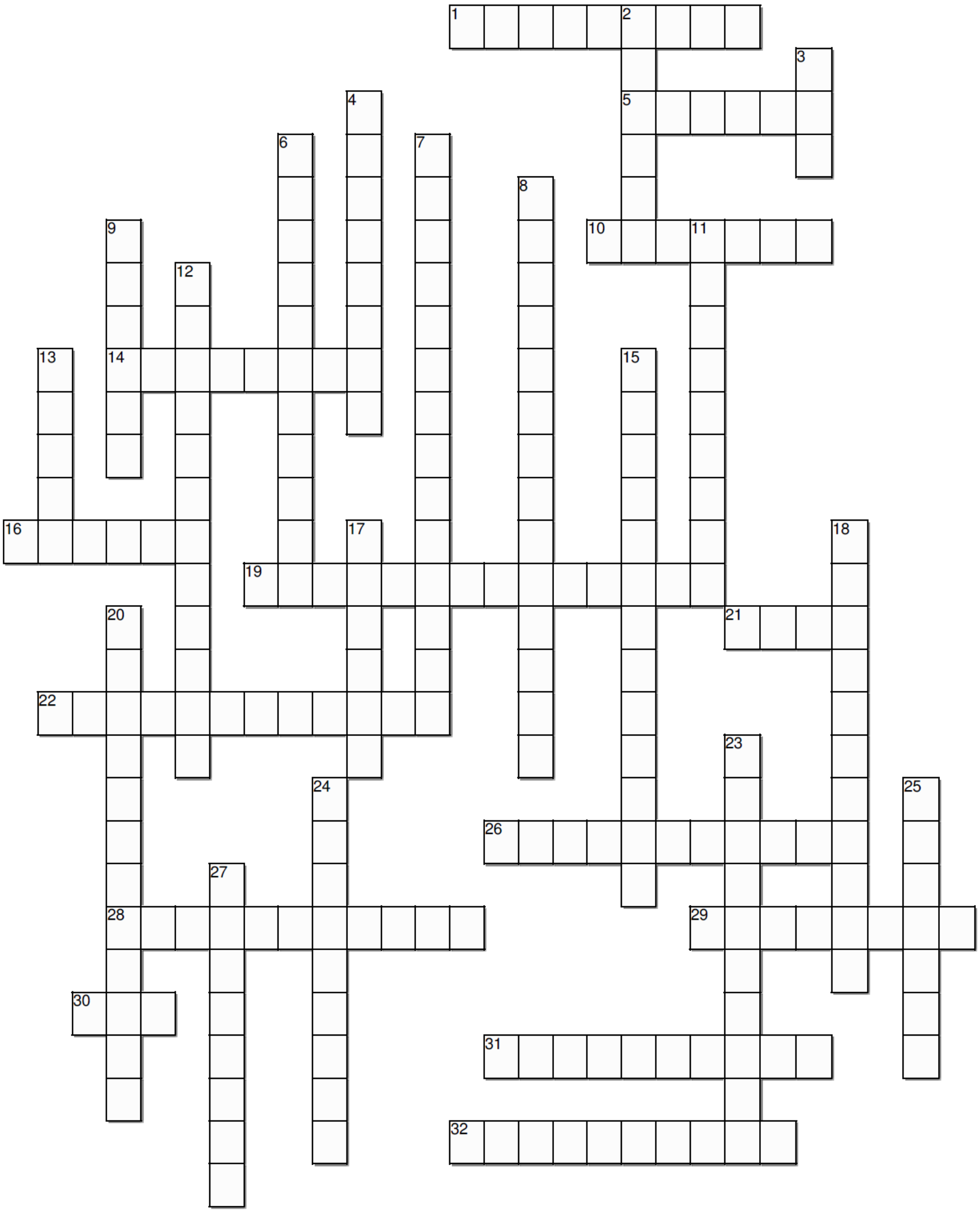
## Variations on the DNS method

The DNS assay is probably the most used and studied assay. In recent years, many researchers have sought to miniaturize and automatize the DNS assay. In this respect, the reader might like to consult Wood et al. 2012.[3] According to the conclusions of these authors, the best sample: DNS reagent ratio is 1:20 sample (for samples concentrations in the range 0–100 g $L^{-1}$ reducing sugars). Also, these authors conclude that solutions in microtubes should be heated for a shorter time length for colour development. They recommend just 1 min at 100 °C in a thermocycler. Finally, the authors have shown that the optimal wavelength for the analysis is 540 nm for samples containing 0–25 g $L^{-1}$ of reducing sugars and 580 nm for samples containing 25–100 g $L^{-1}$ reducing sugars. If a microplate reader with wavelength scanning ability is used, it is perfectly reasonable to measure at both wavelengths and then adapt according to the estimate of sugar concentration.

[3] Ian P. Wood, Adam Elliston, Peter Ryden, Ian Bancroft, Ian N. Roberts, Keith W. Waldron, Rapid quantification of reducing sugars in biomass hydrolysates: Improving the speed and precision of the dinitrosalicylic acid assay, Biomass and Bioenergy, Volume 44, September 2012, Pages 117-121. http://dx.doi.org/10.1016/j.biombioe.2012.05.003.

# Enzymes crossword

Complete the crossword below



## Across

**1.** One of two common mechanisms in glycoside hydrolases
**5.** A famous English admiral and the name of a reducing sugar assay
**10.** The world's most important sugar
**14.** Five carbon sugars
**16.** The F in FPU
**19.** Are removed in the bleaching process
**21.** A well known database hosted in Marseille
**22.** Glucan chains are organized into these
**26.** When cellulose structure is ordered
**28.** Building blocks of proteins
**29.** The term used to describe substrate to product cycling
**30.** Their uniforms were degraded by fungi
**31.** Everything that is EC3
**32.** Two glucosyl moieties linked by a beta-1,4 bond

## Down

**2.** The active sites of cellobiohydrolases are ------like
**3.** A colorimetric reducing sugar assay
**4.** A five-carbon sugar ring
**6.** The ultimate enzyme in cellulose hydrolysis
**7.** A natural sugar-making process
**8.** The composite material that makes up plant cell walls
**9.** The divalent metal in the Nelson Somogyi assay
**11.** Common name for enzyme mixtures
**12.** What happens to enzymes at a critical temperature
**13.** Most industrial enzymes are produced by these.
**15.** A factor that affects enzyme performance, especially true for cellulases
**17.** A non-sugar cell wall component
**18.** A lipophilic extractive
**20.** The effect of temperature on reaction rate
**23.** The generic term for enzymes that degrade cellulose
**24.** Most family GH11 are these
**25.** When two enzymes boost each other
**27.** Carbon number one in sugars