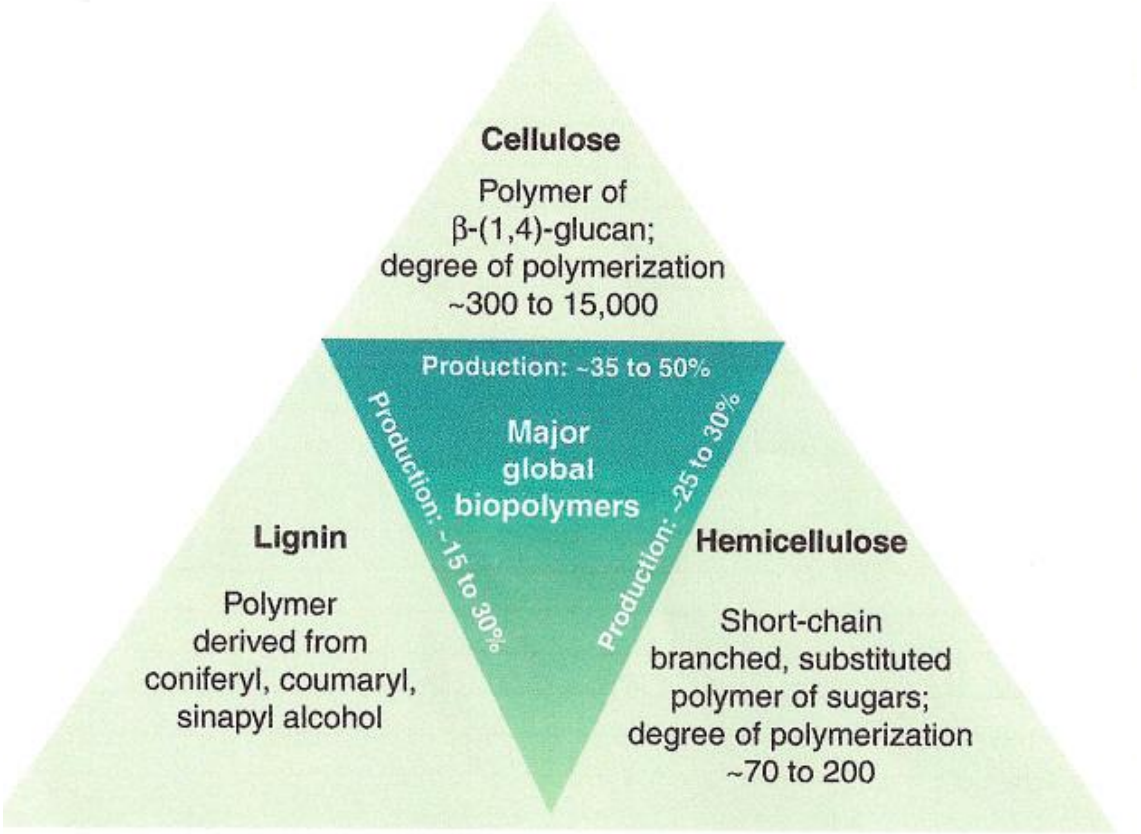
University of Brasília
Institute of Biology Science
Department of Cellular Biology
Laboratory of Enzymology

# The Role of Hydrolases on Degradation of Plant Material

PROF. EDIVALDO XIMENES

# Biomass

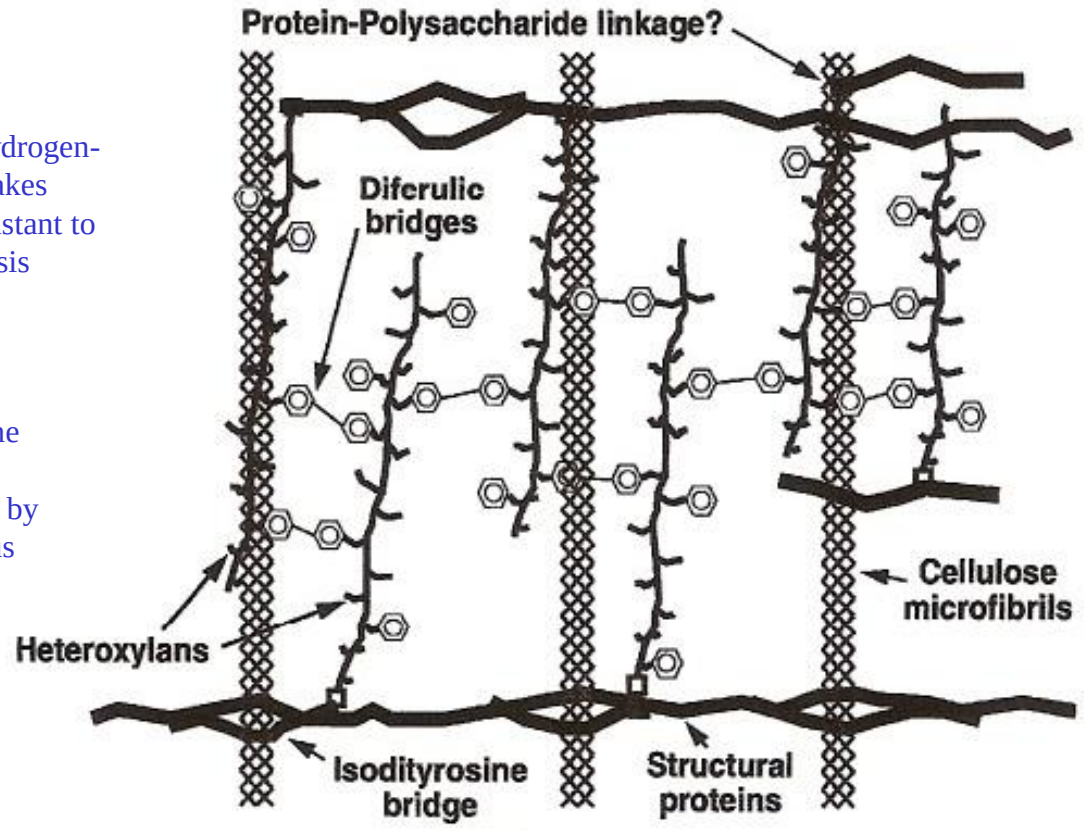



**Ragauskas *et al.*, 2006. Nature, 311: 484-489**

# Recalcitrance of Cell Wall Structure

The strong interchain hydrogen-bonding network makes crystalline cellulose resistant to enzymatic hydrolysis

Access to the crystalline cellulose cores of microfibrils is restricted by a coating of amorphous cellulose and hemicellulose



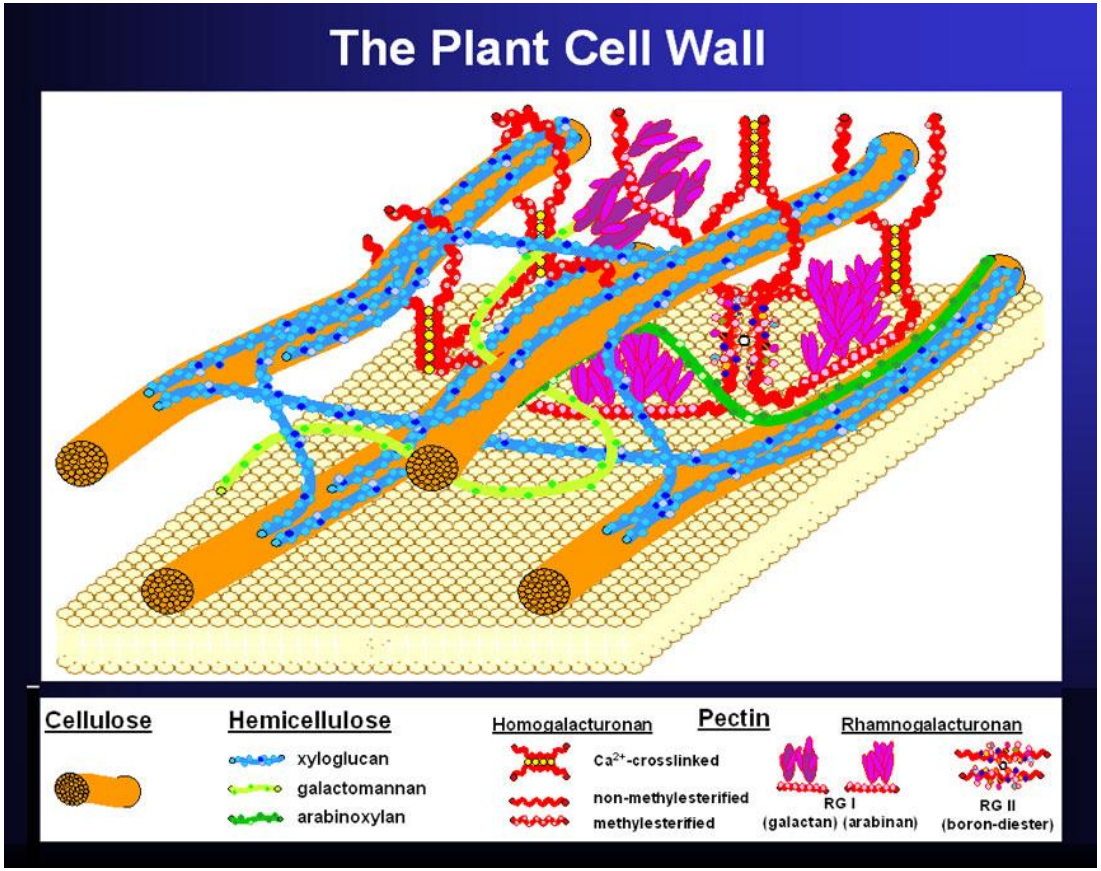

**Saha, 2003. J. Ind. Microbiol. Biotechnol., 30: 279-291**

The structural complexity and heterogeneity of cell-wall constituents such as microfibrils and matrix polymers contribute to the recalcitrance to enzyme action

The hydrophobic interactions between cellulose sheets makes crystalline cellulose resistant to enzymatic hydrolysis, because it contributes to the formation of a dense layer of water near the hydrated cellulose surface

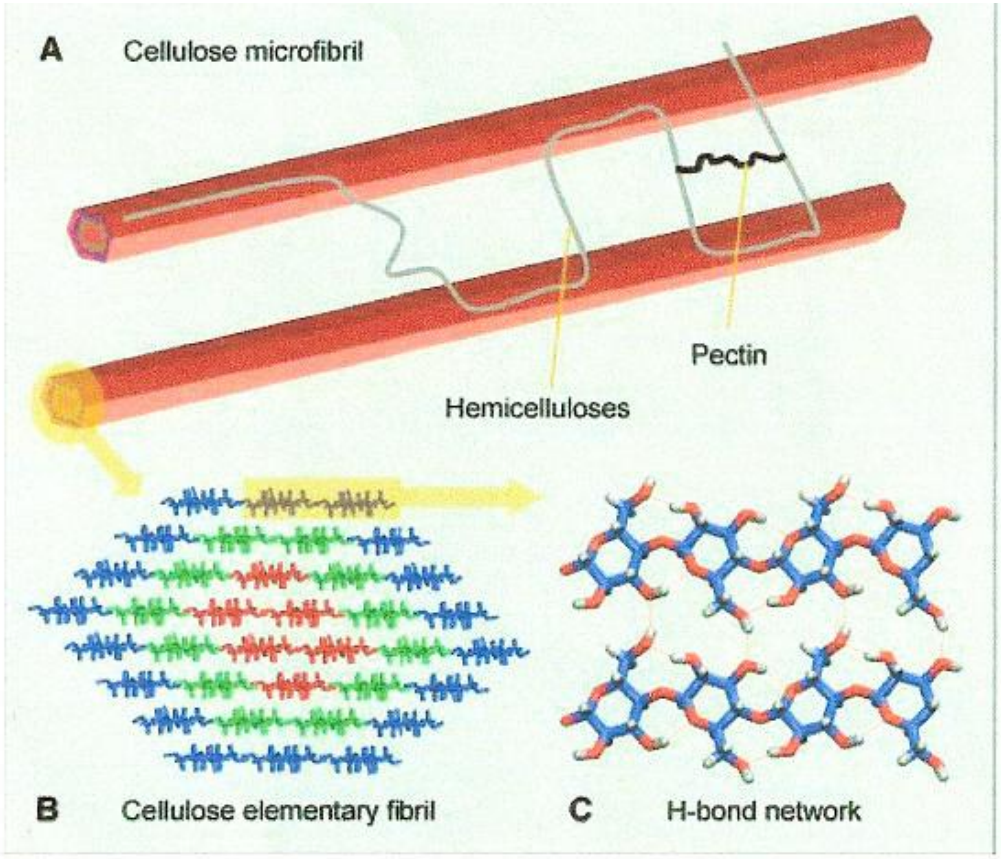
# Model of the plant cell wall polysaccharide networks

**(Picture by MSU-DOE Plant Research Laboratory Michigan State University).**



# Holocellulose

Cellulose fibril may contain three groups of glucan chains:
C1 (red) are six crystalline chains
C2 (green) are 12 subcrystalline chains with small degree of disorder
C3 (blue) are 18 surface chains that are subcrystalline with a large degree of disorder

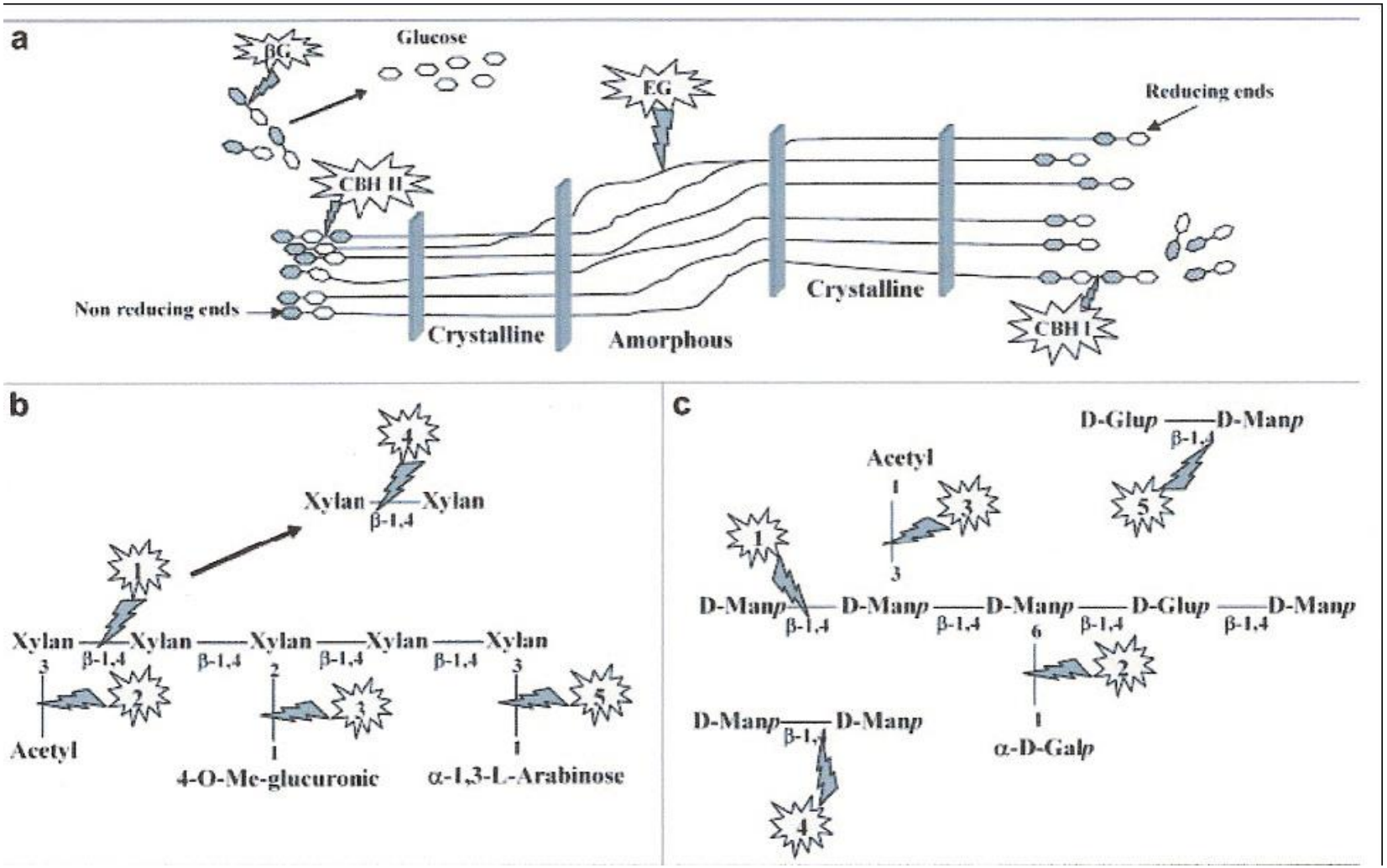



**Himmel *et al*., 2007. Nature, 315: 804-807**

Hemicelluloses are closely associated to the surface of the rigid cellulose crystalline forming the microfibirl network

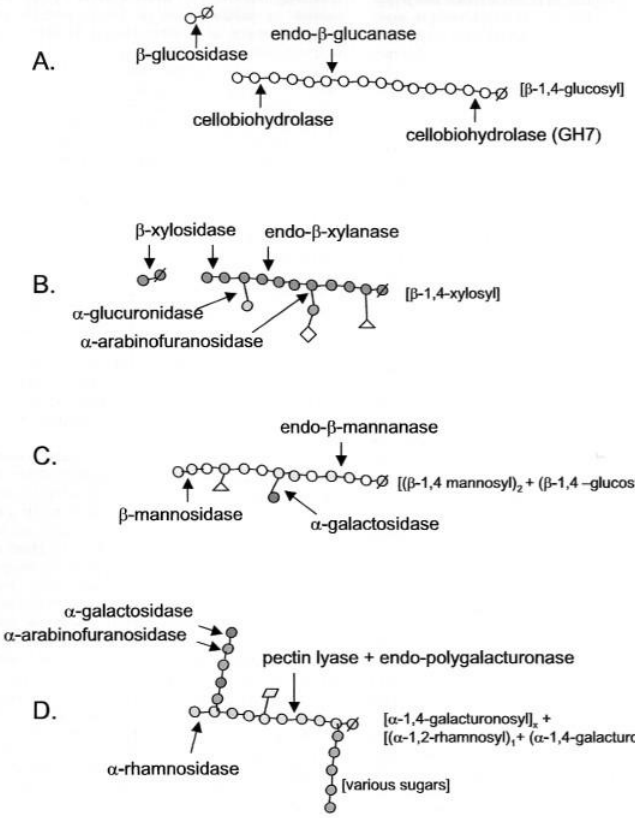
Pectins are cross-linked polysaccharides forming a hydrated gel that glues the cell-wall components together

# The holocellulose Enzymatic Desconstruction




**Pérez *et al*., 2002. Int. Microbiol., 5: 53-63.**

# Enzymatic Attack on Holocellulose Structure




Cellulase

Xylanase

Mannanase

Pectinase

**Figure 3**
Simplified structures and sites of enzymatic attack on polymers from lignocellulose. A cellulose chain fragment (A) is shown, along with hypothetical fragments of the hemicelluloses xylan (B), glucomannan (C), and pectin (D). Sites of attack of some of the major enzymes acting on the respective material are indicated by arrows. The glycosidic bond type of the main-chain is indicated in brackets to the right of each polymer fragment. Carbohydrates are indicated as circles, and the reducing end of each main chain is marked by a line through the circle. White = glucose, green = xylose, yellow = glucuronic acid, red = arabinose, light blue = mannose, dark blue = galactose, grey = galacturonic acid, and pink = undefined sugar residues. Acetate groups are shown as triangles, phenolic groups as diagonals, and methyl groups as rombs.

Turner *et al*., 2007. Microb. Cell Fact., 6: 1-23

# Enzymatic Breakdown of Holocellulose

Effective conversion of holocellulose to fermentable sugars requires:

1. Size reduction
2. Pretreatment/fracionation*
3. Enzymatic Hydrolysis
4. Non-linearity in the hydrolysis process due to variations in the acess to glycosidic linkages and terminal chains available in different regions of plant cell wall

* The characteristics of holocellulose substrates vary, depending on the pretreatment and origin

# Enzyme Characteristics for Conversion of Holocellulose

1. A higher catalytic efficiency in insoluble lignocellulosic substrates (DP e DS);
2. Increased stability at elevated temperature and at a certain pH;
3. Higher tolerance to end-product inhibition;

# An Overview of Substrate Modification

1. A reduction of substrate viscosity and/or an increase of reducing sugars;
2. A change of the topography surface and hydrolysis rates of holocellulose

# Enzyme action

1. Changes in holocellulose characteristics during enzymatic hydrolysis
2. A nonproductive binding of the enzyme on the surface of holocellulose
3. Dynamic interactions between CBM, catalytic domain and insoluble substrate in the plant cell wall
4. Enzyme diffusion, adsorption and catalysis on the surface of holocellulose
5. Heterogeneity of insoluble substrate

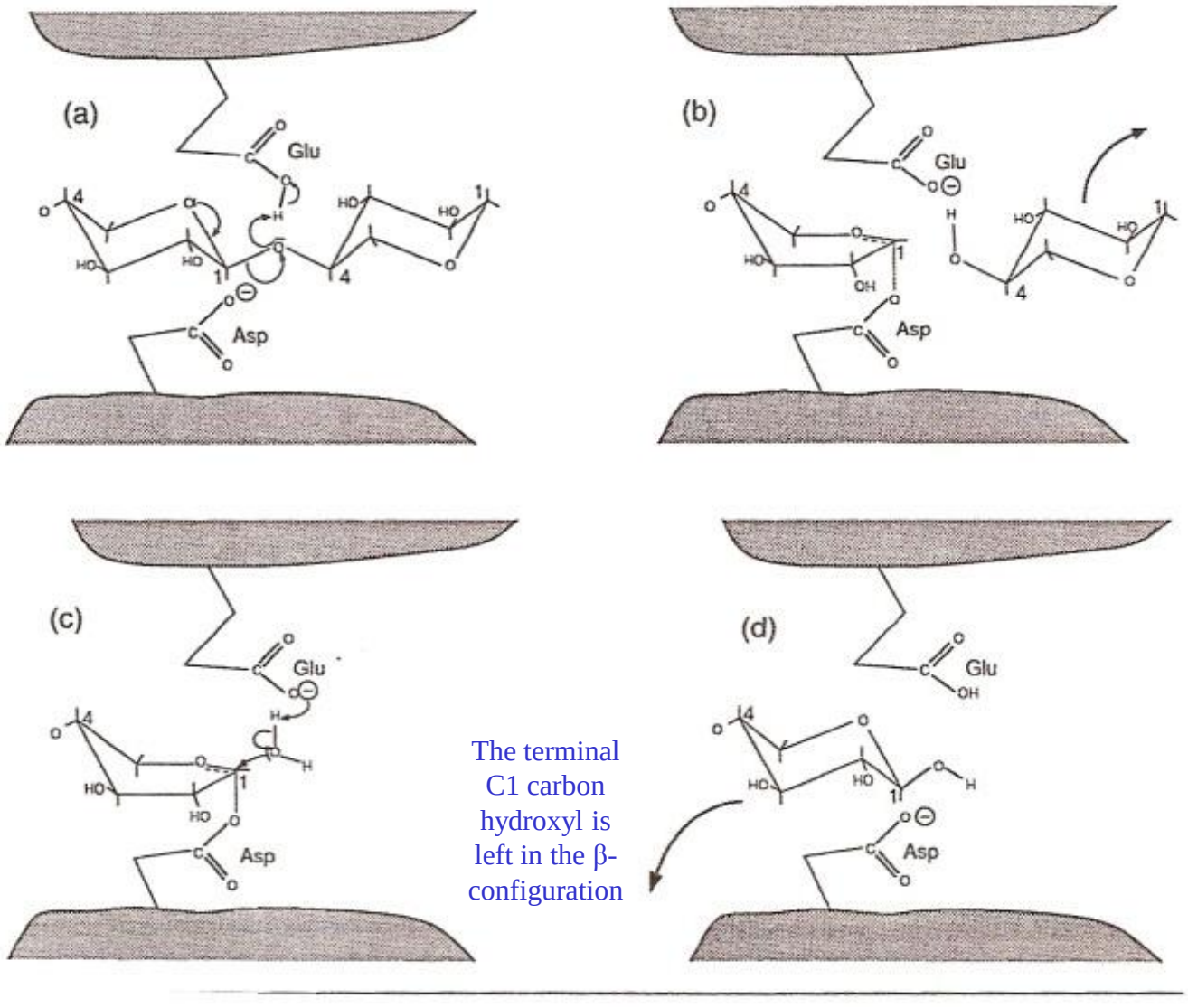
# Fungi or Bacteria?

- Fungi: produce a complex mixture of extracellular enzymes with high productivity and catalytic efficiency and low cost;
- Bacteria: produce an enzymatic complex associated to  cell wall

# Parameters for Holocellulose Hydrolysis

1) Mechanism of hydrolysis according to Koshland model
2) The role of $H_2O$
3) Steric hindrance
4) Synergistic action of enzyme systems:
5) Endo and Exo activities
6) Primary and secondary hydrolysis
7) Enzyme promiscuity

# Retention of Stereochemistry



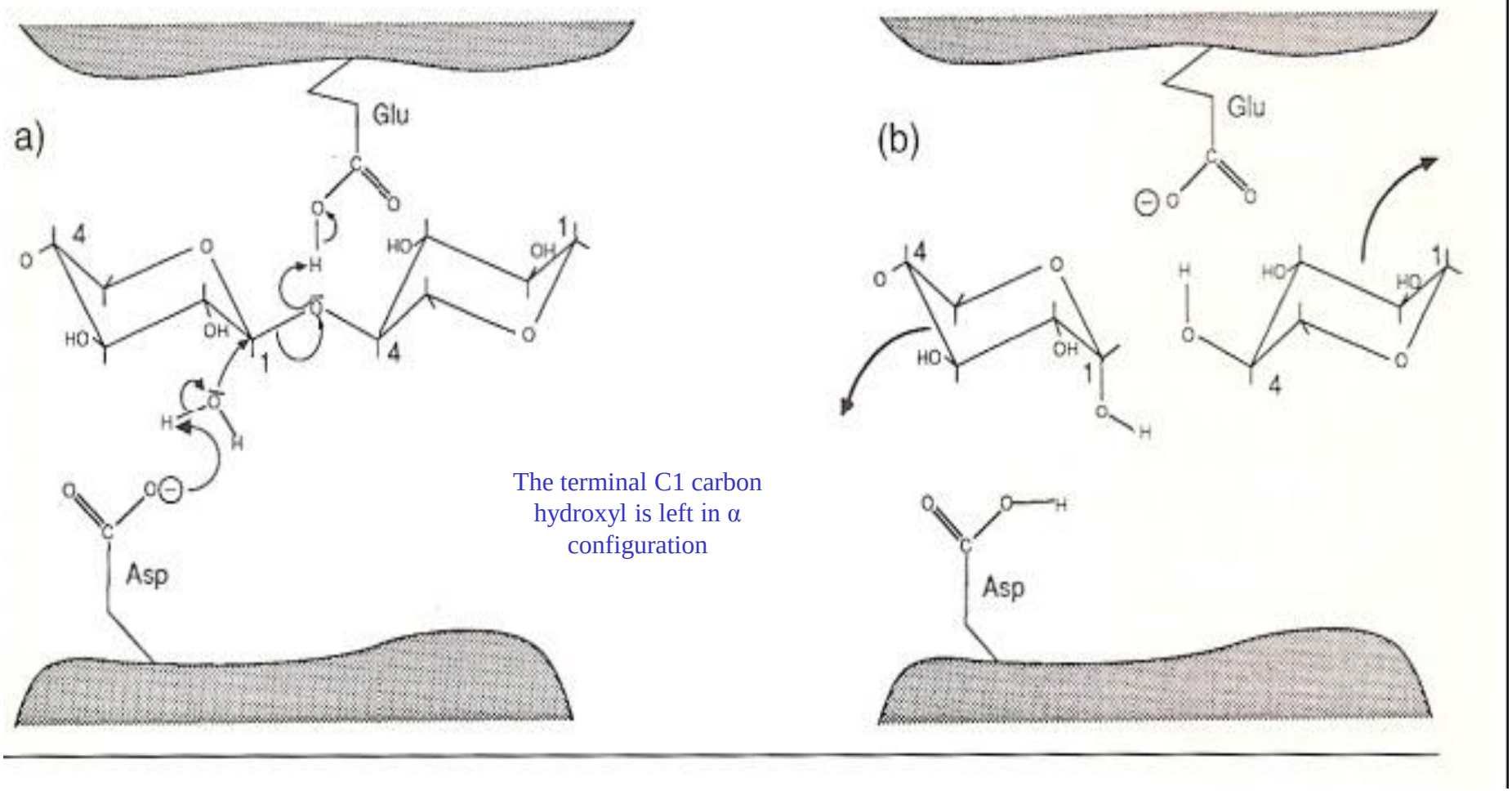

The terminal C1 carbon hydroxyl is left in the β-configuration

**Coughlan *et al.*, 1993. In: Hemicellulose and Hemicellulases, Portland Press, pp. 53-84**

# Retention Mechanism

- Hydrolysis of holocellulose by a double displacement reaction leads to retention of anomeric configuration
- The mechanism of reaction involves nucleophilic attack (donation of H+) by an unionized Glu or Asp residue on C-1 of the incipient reducing sugar
- The resulting glycosyl fragment diffuses away from the active centre
- The oxocarbonium ion intermediate (the residual fragment) is stabilized by covalent interaction with ionized Glu or Asp
- The reaction is completed by the addition (from water) of a hydroxyl group to the carbonium ion and a proton to the nucleophile

# Inversion of Stereochemistry




The terminal C1 carbon hydroxyl is left in α configuration

**Coughlan *et al.*, 1993. In: Hemicellulose and Hemicellulases, Portland Press, pp. 53-84**

# Inversion Mechanism

- Hydrolysis of holocellulose by a single displacement reaction leads to inversion of anomeric configuration
- The reaction involves the participation of a general acid (unionized Glu or Asp) and a general base (ionized Glu or Asp) in catalysis with attack by a nucleophile molecule of water

# $H_2O$

- Water molecule could invade the space under the nonreducing chain end and thus prevent it from reannealing into the cellulose crystal

# Enzymatic approach

- Degree of crystallinity of celulose;
- Type and distribution of lignocellulose;
- Inespecific adsorption of enzyme in holocellulose structure;
- A decrease in the amount of enzyme associated with holocellulose;
- Steric hindrance  and accessibility to enzymatic attack

# Synergism

- It is observed when the amount of product formed by two or more enzymes acting together exceeds the arithmetic sum of the products formed by the action of each individual enzyme

# Heterosynergy

- It is defined as the synergistic interaction between a side chain- and main chain-cleaving enzyme
- Uniproduct heterosynergy: the action of the main chain enzyme facilitates the release of substituent by the side chain enzyme or *vice versa*
- Byproduct heterosynergy: the extent of liberation of substituent and of hydrolysis of the main chain resulting from the actions of the combined enzymes exceeded the sum os those observed following the actions of the individual enzymes

# Homoesynergy

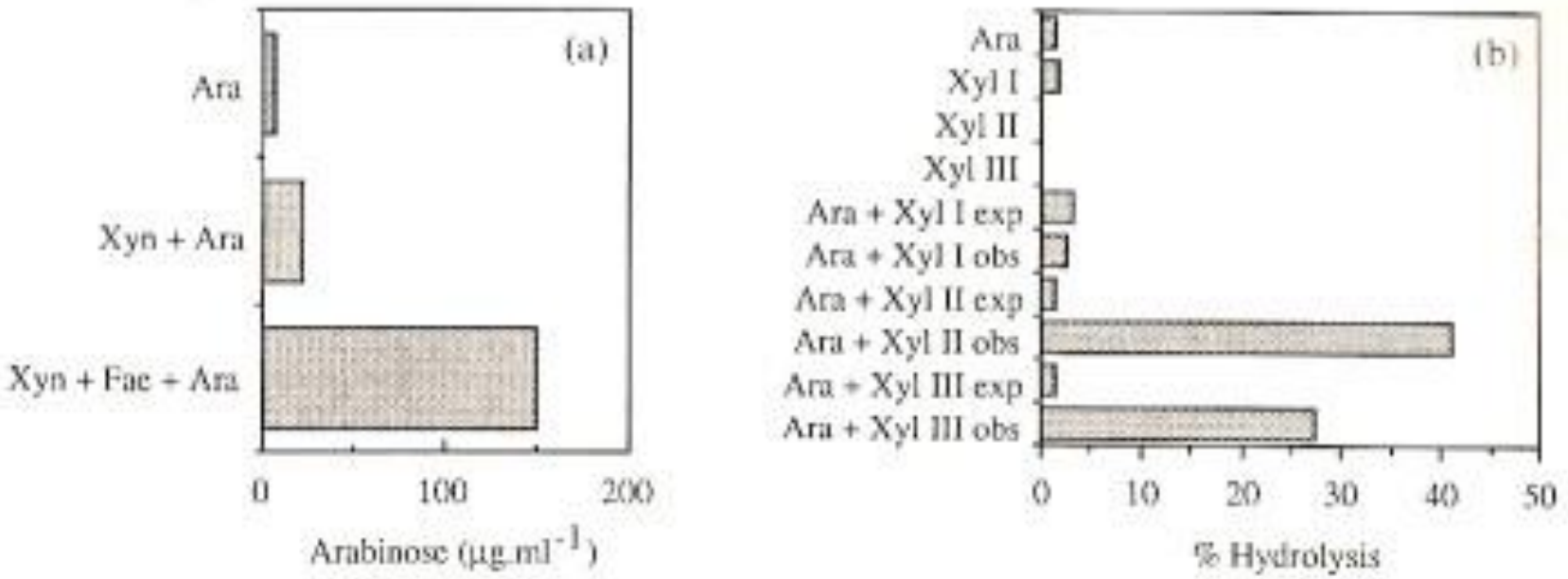
- The synergistic or co-operative interaction between two or more different types of side chain-cleaving enzyme or between two or more types of main chain-cleaving enzyme
- It is observed when mixtures of two or more main chain-cleaving enzymes (by endo- or exo-acting) of different specificities effect the release of greater amounts of product than the sum of the products released by the individual enzymes
- It is usually considered that the action of one enzyme provides the substrate for the other or allows the second enzyme access to its substrate

# Antisynergy

- The action of one type of enzyme preventing the action of a second
- Some enzymes cleave main chain linkages only in the vicinity of a particular type of substituent
- The prior removal of the substituent by the relevant side chain-cleaving enzyme would preclude action by the specific main chain enzyme
- Is it possible to occur *in vivo*?

# Example of Synergism

**Fig. 2.** **Heterosynergistic interactions in the hydrolysis of feruloylxylan (a) and arabino-xylan (b) by fungal enzymes**




Coughlan et al., 1993. In Hemicellulose and Hemicellulases, 53-84.

# Primary and Secondary Hydrolysis

- Primary hydrolysis occurs on the surface of solid
- Secondary hydrolysis occurs in the liquid phase
- Differences in substrate accessiblity, DP and chain end availability for different regions of holocellulose

# Enzyme Promiscuity

- "One that does things it is not expected to do"
- "Most enzyme active sites have great chemical potential, littered with potential catalytic groups" (Daniel Herschlag)
- Enzymes and their ability to catalyze a spectrum of reactions with different substrates and varying efficiency
- Enzymes exhibit both highly efficient native activities and less efficient but still biologically activities against a wide variety of nonnative substrates
- "It facilitates enzyme evolution because new catalytic functions can evolve from those that already exist weakly in existing enzymes" (Steve Reuland)
- Higher nonnative activity can confer a substantial fitness advantage
- Promiscuous activities share the main active site features with the native activity, including substrate positioning and mechanism

"Functional promiscuity can result from different conformations in the ensemble catalyzing different reactions, with the native activity catalyzed by the most stable (ground-state) conformation" (proposed by Wroe et al., 2007. HFSP J., 1: 79-87.

**Box 1. Types of enzyme promiscuity**

- **Enzyme condition promiscuity**

Shown by enzymes with catalytic activity in various reaction conditions different from their natural ones, such as anhydrous media, extreme temperature or pH.

- **Enzyme substrate promiscuity**

Shown by enzymes with relaxed or broad substrate specificity.

- **Enzyme catalytic promiscuity**

Shown by enzymes catalyzing distinctly different chemical transformations with different transition states. Enzyme catalytic promiscuity can be either:

(i) **accidental** – a side reaction catalyzed by the wild-type enzyme;
(ii) **induced** – a new reaction established by one or several mutations rerouting the reaction catalyzed by the wild-type enzyme.

A mutation that increases the stability of a nonnative conformation increases its occupancy into the ensemble and the activity corresponding to this conformation

Conformational changes enable the same enzyme to accomodate different substrates

Hult and Berglund, 2007. Trends in Biotechnology, 25: 231-238

# Robustness and Plasticity
# "Great Facilitators"

Robusteness of enzyme native function:

activity is not decreased by a large amount

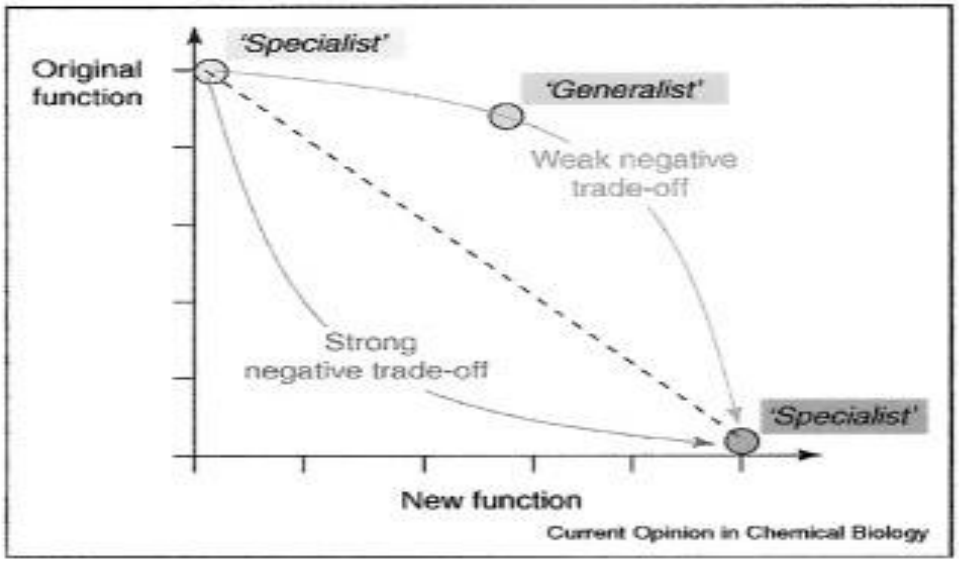
for the native activity

Plasticity toward enzyme promiscuous functions:

activity is substantially improved for other

promiscuous activities

When a microrganism is faced with new challenges, an enzyme can improve its activity towards a new substrate or new reaction while mantaining a high level of native function




Possible routes to new function acquisition. Under selection, a weak, promiscuous activity of a protein with an existing function (blue circle) gradually evolves. By the end of this process, which typically requires many generations of mutation and selection, the 'new' function has traded off with the original one (green circle). However, the dynamics of this process may vary. The gain–loss of the new *versus* old function, and the conversion of one 'specialist' protein into another, may trade-off linearly (dashed line), or follow either concave or convex routes. Results of numerous directed evolution experiments indicate that the convex route ('weak negative trade-offs') is the more likely one — large increases in the promiscuous function under selection ('new function') are accompanied by significantly smaller decreases in the original function (Table 1). By virtue of gaining a 'new' function without losing the original one (and often gaining other new functions not selected for), the intermediates of these routes are 'generalists', and their evolution can therefore proceed *prior* to gene duplication. By contrast, the concave route implies that gene duplication is a necessary prerequisite, because acquisition of even low levels of the 'new' function is accompanied by large losses of the original one. This route is observed in the laboratory, in particular under a dual selection, for gain of a new function and loss of the old one.

Khersonsky et al., 2006.
Curr. Op. Chem. Biol.,
10: 498-508

# An example of xylanase with relaxed specificity

TABLE 2

Substrate specificities of two xylanases from *Penicillium capsulatum*

| Substrate | Main chain linkage | XynA | XynB |
|---|---|---|---|
| | | relative activity[a] | |
| Oat spelts xylan (soluble) | β-1,4 | 100.0 | 100.0 |
| Oat spelts xylan (insoluble) | β-1,4 | 37.3 | 37.6 |
| Wheat straw xylan (soluble) | β-1,4 | 67.9 | 95.6 |
| Wheat straw xylan (insoluble) | β-1,4 | 95.7 | 180.3 |
| *Rhodymenia palmata* xylan | β-1,4 (82%); β-1,3 (18%) | 124.4 | 123.0 |
| Cellulose (filter paper) | β-1,4 | 0 | 0 |
| CM-cellulose | β-1,4 | 0 | 23.3 |
| Barley β-glucan | β-1,4 (75%); β-1,3 (25%) | 4.9 | 89.4 |
| Pneumococcal RS III | alternating β-1,4 and β-1,3 | 0 | 1.0 |
| Laminarin | β-1,3 | 0 | 18.7 |
| Lichenan | β-1,4 (65%); β-1,3 (35%) | 0 | 5.0 |
| Polygalacturonate | α-1,4 | 0 | 0 |

[a] The samples of XynA and XynB used had 10.4 and 5.3 IU·ml$^{-1}$, respectively, as measured with soluble oat spelts xylan as substrate. These values were arbitrarily assigned as representing 100% activity in each case.

Filho et al., 1993. J. Ind. Biotechnol., 11: 171-180

# What to expect?

- This greatly increases the chances of successfully achieving a novel function without disrupting the old one.
- An enzyme evolving a new function must mantain a high level of fitness throughout its evolution otherwise it will be constrained by selection.
- Extracellular enzymes can be exposed to reactions conditions and substrates in the cell wall structure that will challenge their specificity and might force them to handle substrates and catalyze reactions thye were not initially designed for, is it possible?

# Outstanding Question!!

- Does enzyme promiscuity actually play a role in natural evolution?
- “ When a need for new enzymatic function arises, nature recruits existing enzymes that promiscuily bind the new substrate, or catalyze the new reaction, and then tinkers with their active site to fit the new substrate and reaction”
- Consequence from above: new family members have diverged from existing ones, yielding the large and functionally diverse enzyme families

# Strategies for Improving the Properties of Individual Holocellulose-Degrading Enzymes

1. Rational Design (based on knowledge on the enzyme structure and mechanism of catalysis)
2. Directed Evolution (the improved enzymes are selected after random mutagenesis and/or molecular recombination)
3. The action of enzymes on insoluble substrates, yielding an improved hydrolysis rate or higher holocellulose digestibility

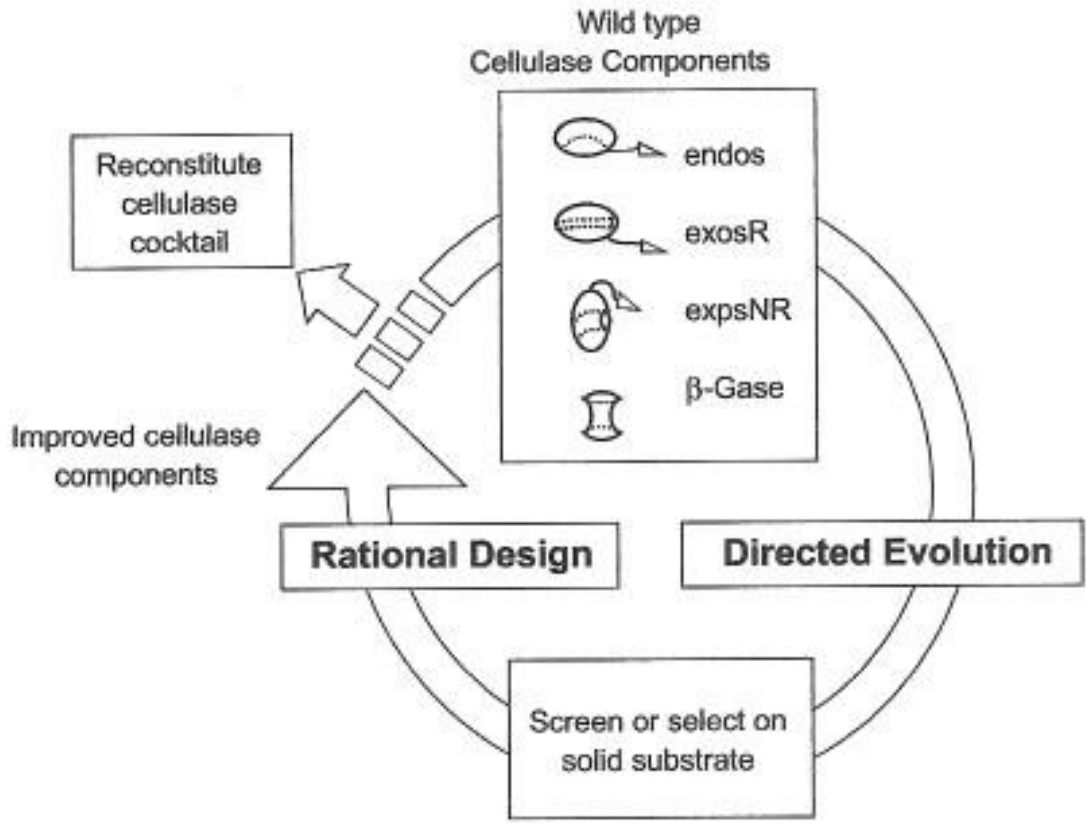


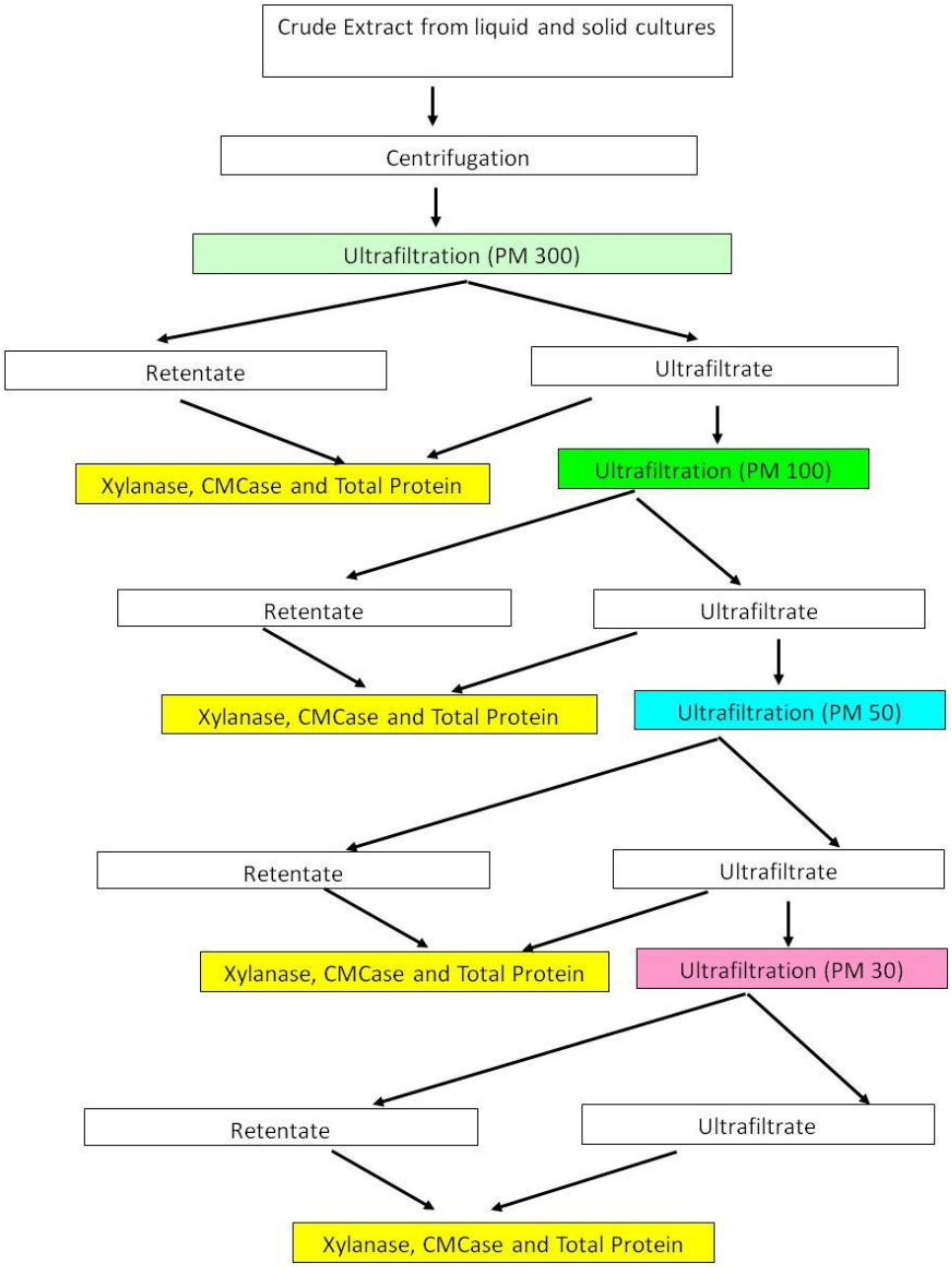

Fig. 1. Scheme of cellulase engineering for non-complexed cellulases. Endos, endoglucanases; exosR, exoglucanases acting on reducing ends; exosNR, exoglucanases acting on non-reducing ends; β-Gase, β-glucosidase.

Zhang et al., 2006. Biotechnol. Adv., 24: 452-481

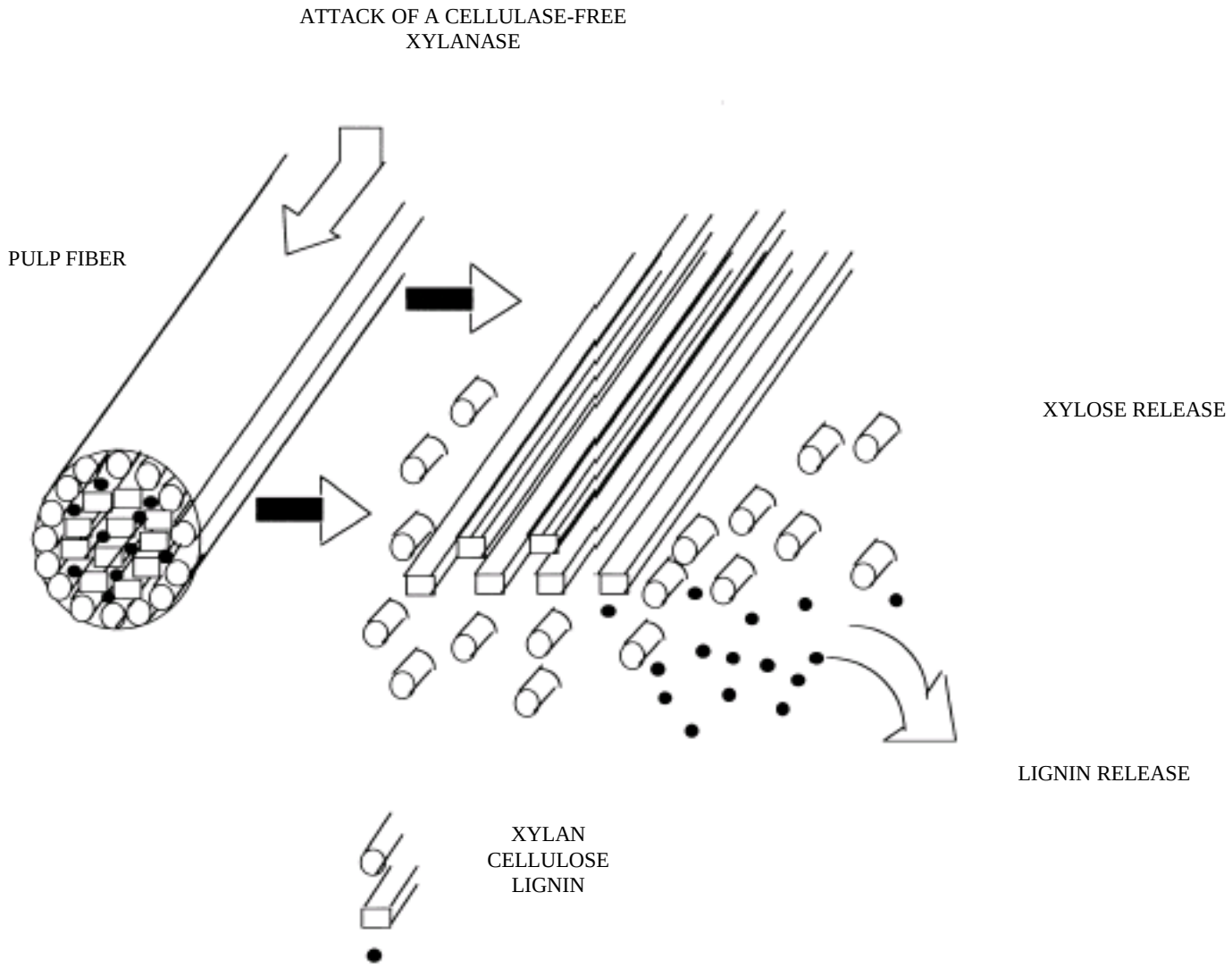
# Ultrafiltration

- It is is a technique for separating dissolved molecules in solution on the basis of size which means that molecules larger than the membrane pore size rating will be retained at the surface of the membrane.
- The ability of holocellulose-degrading enzymes to pass through ultrafiltration membranes with low-molecular weight cut off values;
- Compact structure of holocellulose-degrading enzyme;

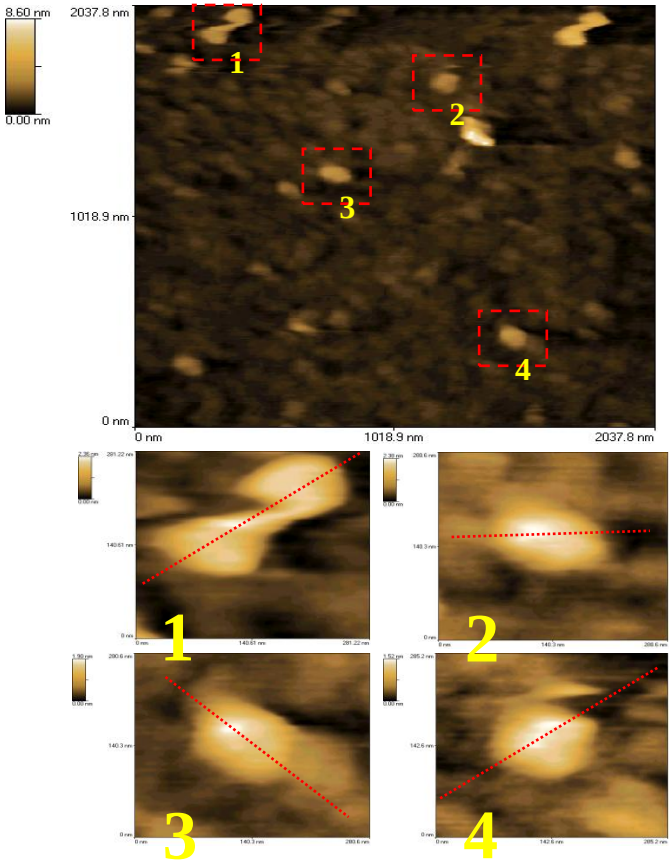
Purification Scheme by Ultrafiltration



# XYLANASE ACTION IN CELLULOSE PULP



# A Conformational Plasticity of Xylanase



# Glycosilation

- An important enzymatic strategy to survive during extracellular holocellulose breakdown
- A thermal tolerance strategy

# A β-Glucosidase from *Humicola grisea*


FIGURA 32 - Eletroforese em gel de poliacrilamida (gradiente de 4,5 - 12%), sob condições desnaturantes, do extrato do meio de cultura e frações I e II de beta glucosidase extracelular. Gel corado pelo Coomassie Blue G-250. Quantidades de proteínas submetidas a eletroforese: fração I extracelular (IE): 10 ug; fração II extracelular (IIE): 8,4 ug; extrato do meio de cultura (EMC): 25 ug. Marcadores de peso molecular (PM): anidrase carbônica (30.000 daltons), ovalbumina (46.000 daltons), albumina bovina (68.000 daltons), fosforilase b (97.400 daltons), beta-galactosidase (116.000 daltons) e miosina (205.000 daltons).

# β-Glucosidase

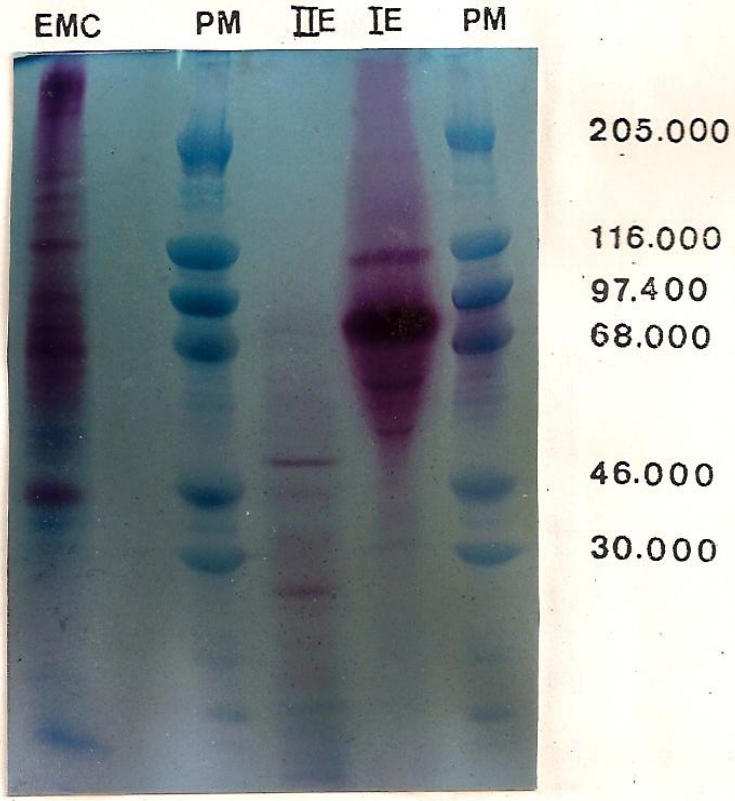
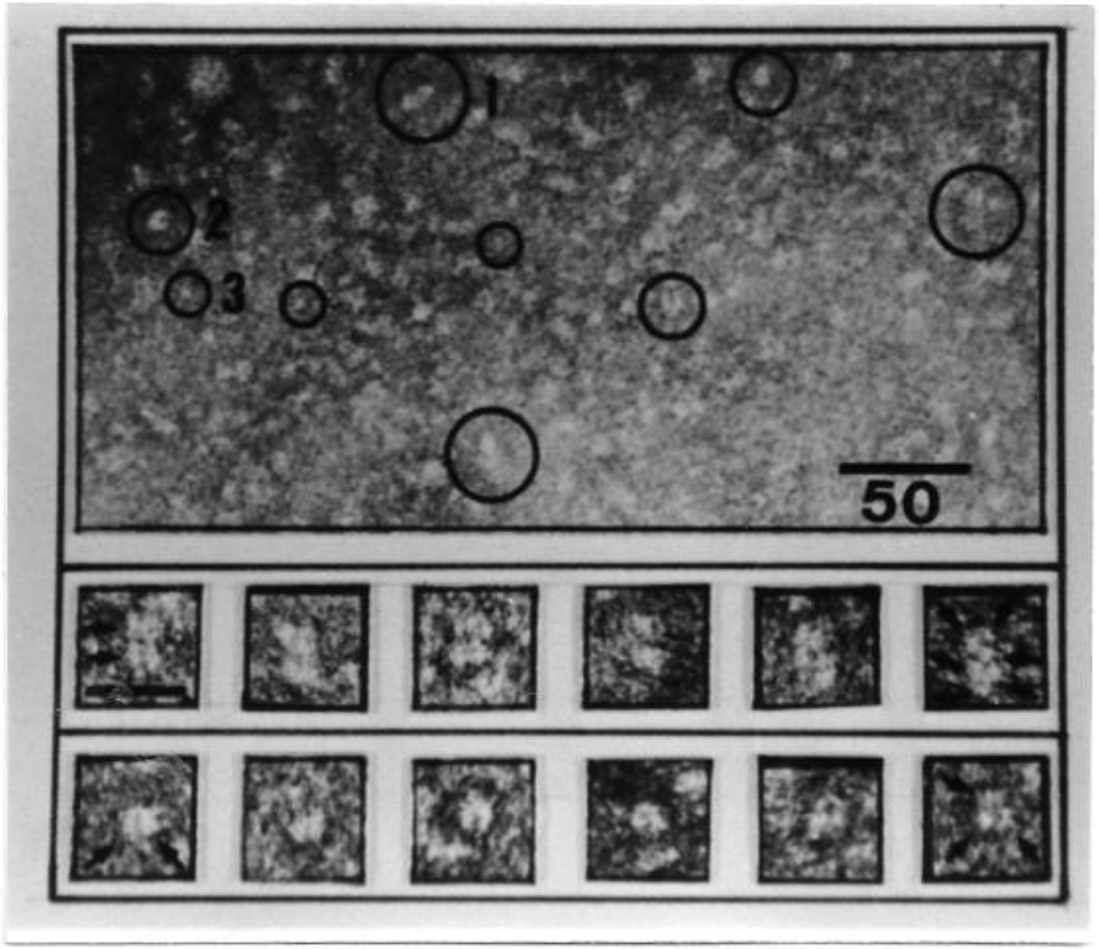



FIGURA 33 - Gel, descrito na figura 32, submetido a coloração pelo reagente de Schiff.

# An Enzymatic Complex from *Penicillium capsulatum*




**Connelly *et al*., 1991. Enzyme Microb. Technol., 13: 470-477.**

# Properties

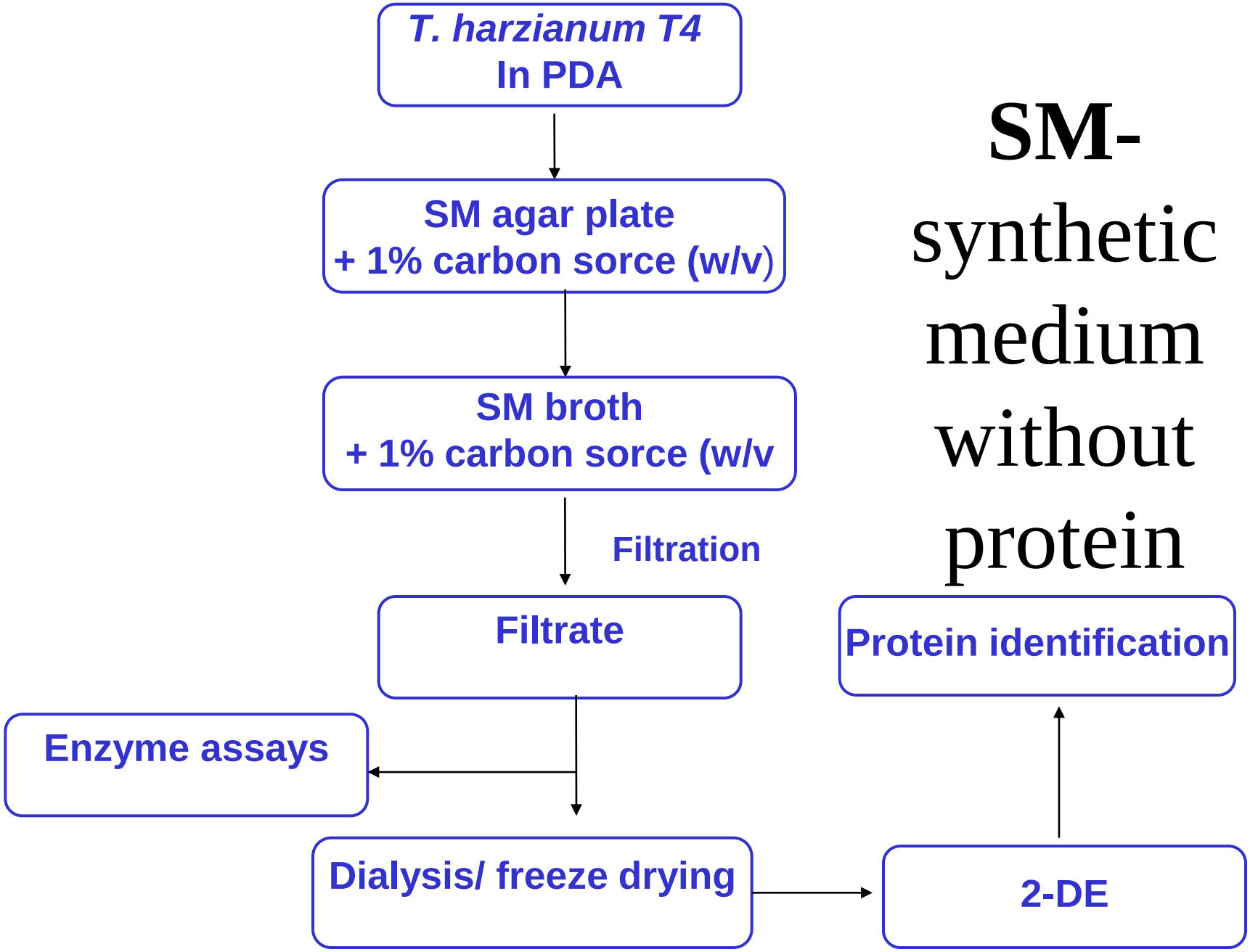
- Dimer with subunit of 135 kDa
- Each subunit is composed of three enzymes: β-glucosidase, β-laminarinase and β-glucanase
- Each subunit is a single protein with three domains, each displyaing one of the above activities

# Mechanism of Action

- Endoaction and Exoaction
- The products of the endoacting β-glucanase and β-laminarinase are imediately acted upon by the exoacting β-glucosidase component to yield glucose
- Oligomeric products released from glucan or laminarin by the β-glucanase or β-laminarinase component of the complex are cleaved at a faster rate by the exoacting glucosidase
- The function of the complex in vivo is to assure the rapid conversion of β-glucans or laminarin to a product, i.e. glucose, that is readily assimilable by the fungus
- An enzyme complex with the ability to effect complete conversion of polysaccharides to their monomeric constituent may also have considerable industrial application

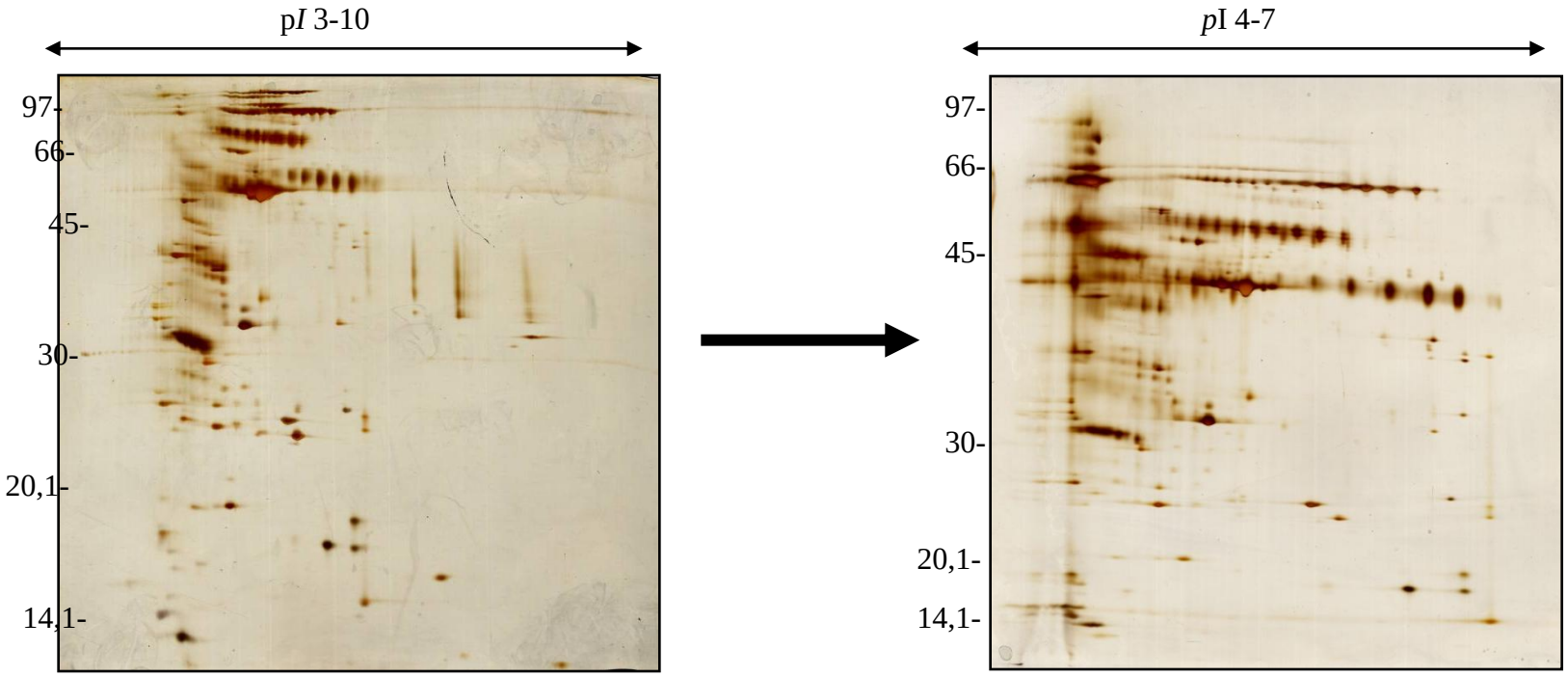
## Secretome or Exoproteome

The population of gene products that are secreted from the cell

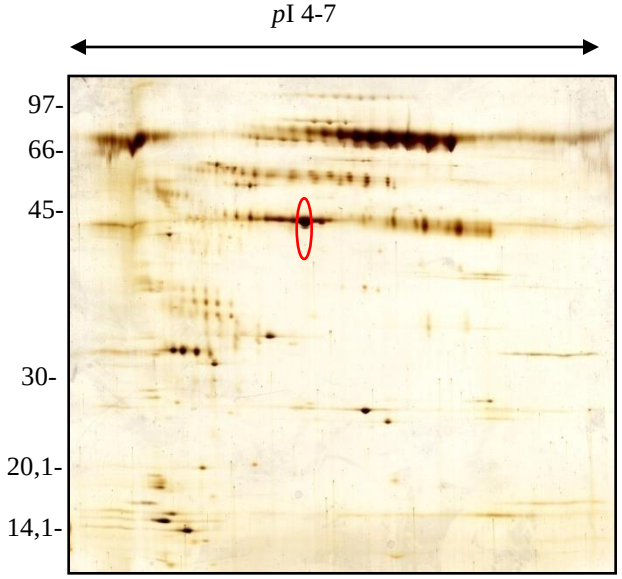



**SM-** synthetic medium without protein

# Narrow range pH gradient (sugar cane as the carbon source)



# Protein identification MS/MS




**Mascot Search Results**

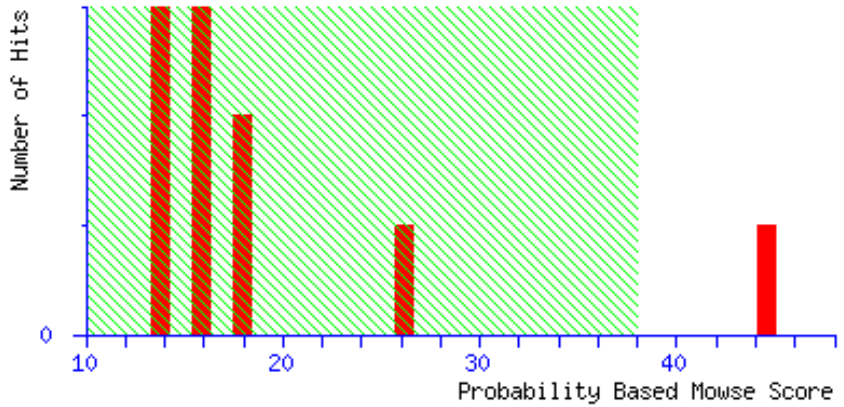
**Protein View**

Match to: **Q6RKQ1_AURPU** Score: **45**

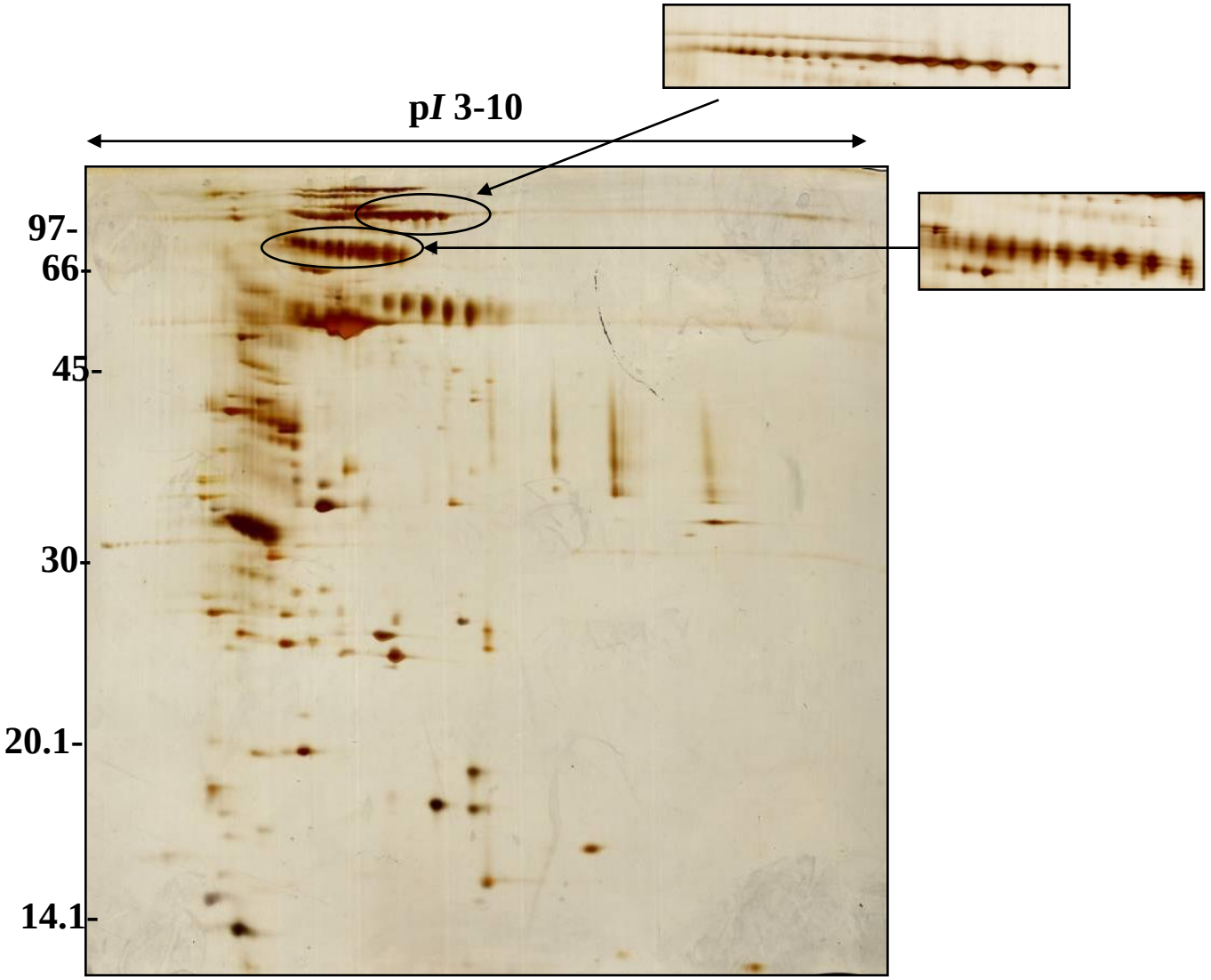
**Alpha arabinofuranosidase (EC 3.2.1.55).- Aureobasidium pullulans.** Found in search of DATA.TXT Nominal mass ($M_r$): **52410**; Calculated pI value: **5.35**

NCBI BLAST search of Q6RKQ1_AURPU against nr Unformatted sequence string for pasting into other applications Taxonomy: Aureobasidium pullulans

Links to retrieve other entries containing this sequence from NCBI Entrez: AAR87863 from Aureobasidium pullulans



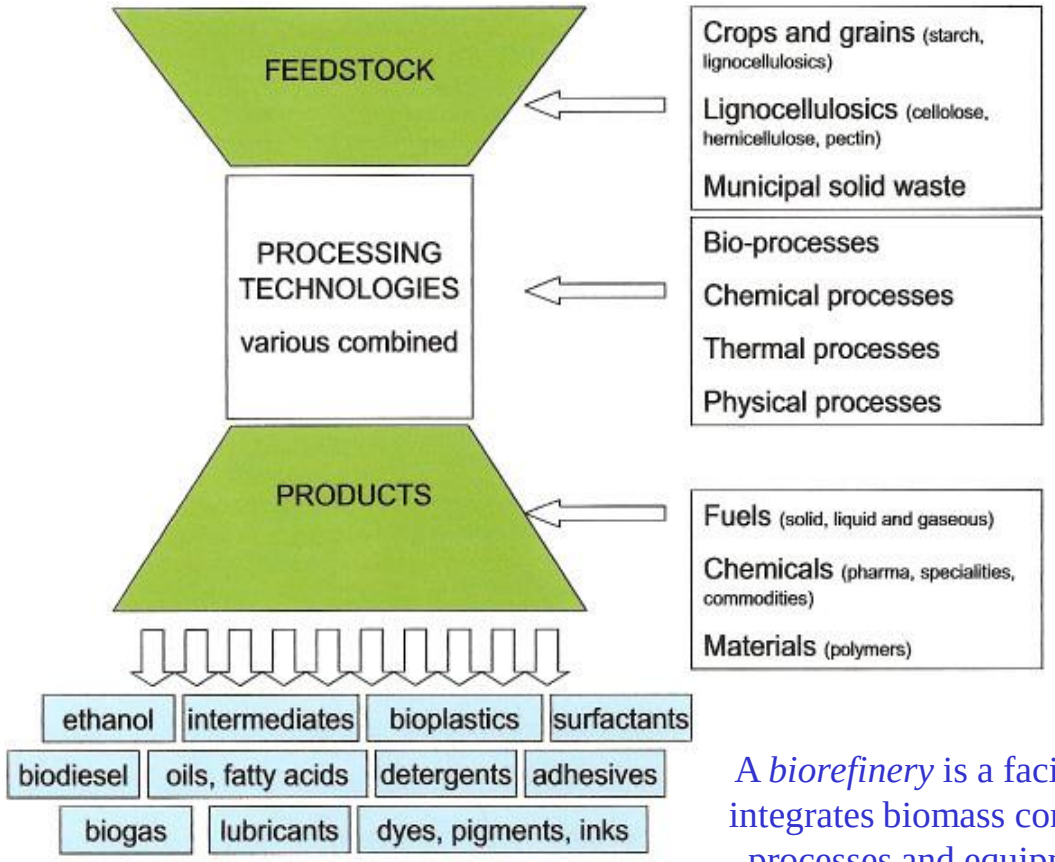
# *Narrow pH gradient (4-7) 1% sugarcane bagasse*



# Conclusions

- *T. harzianum* secretome displayed different 2-DE profiles in response to pure and carbon sources
- Protein identification was not achieved by peptide mass fingerprinting
- Protein spots are presently being identified by MS/MS in order to correlate enzyme activity with secretome composition

# Biorefinery
## A Strategic Brazilian Project

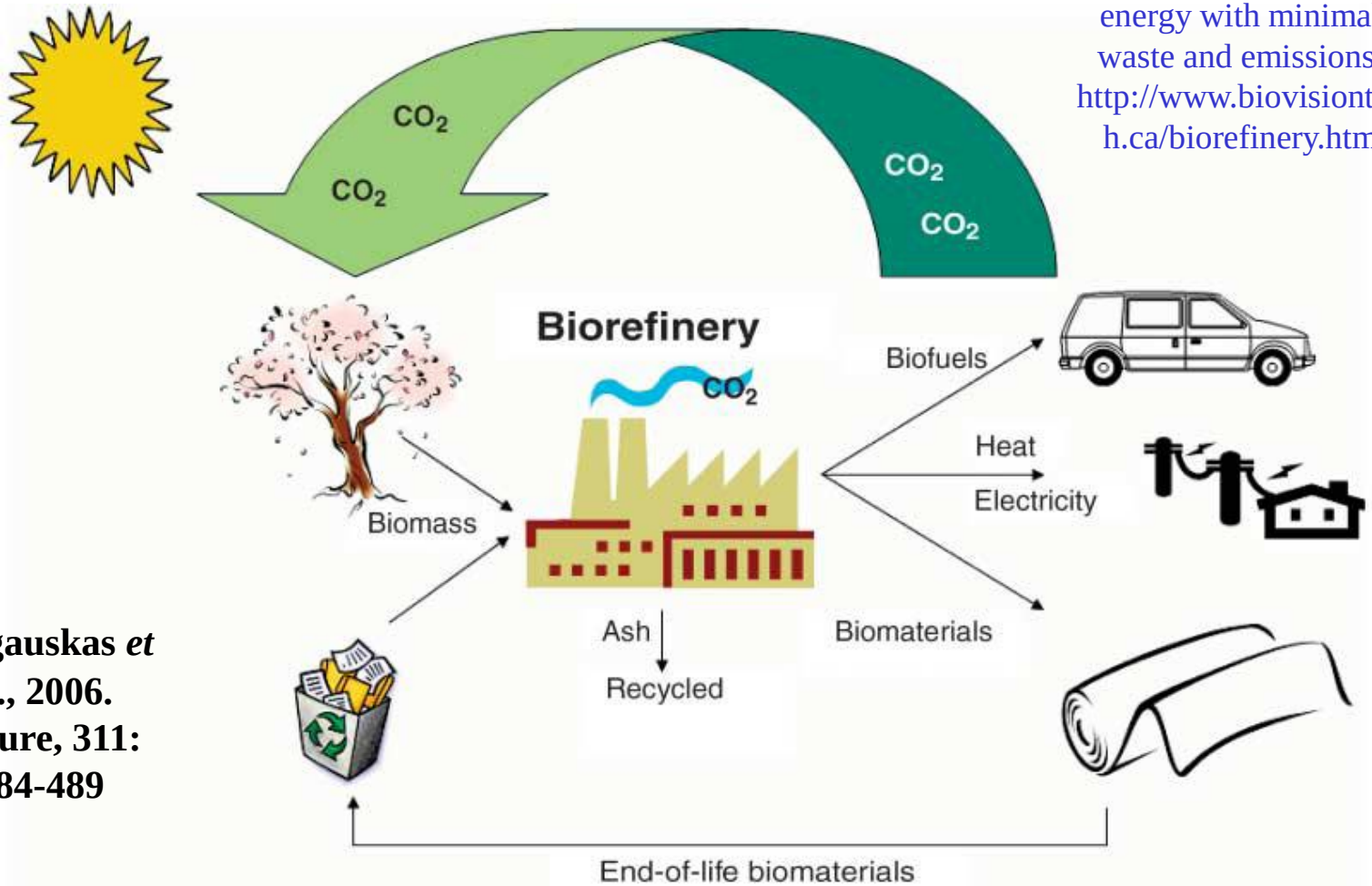



A *biorefinery* is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass *www.nrel.gov/biomass/**biorefinery**.html*

**Turner *et al*., 2007. Microb. Cell Fact., 6: 1-23**

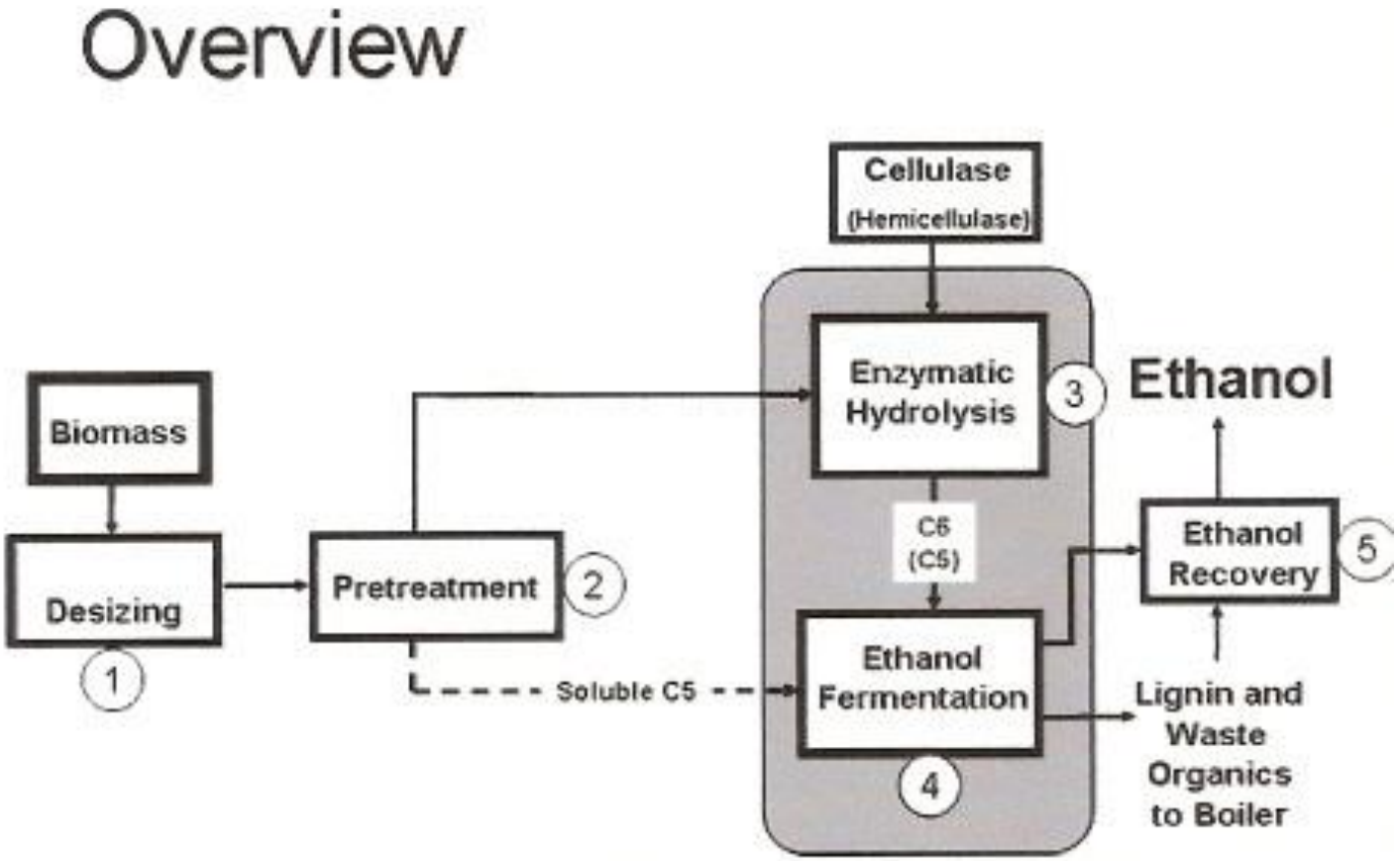
# Biorefinery

A "biorefinery" is a relatively new term referring to the conversion of biomass feedstock into a host of valuable chemicals and energy with minimal waste and emissions. http://www.biovisiontech.ca/biorefinery.htm




**Ragauskas *et al.*, 2006. Nature, 311: 484-489**

# Biomass Conversion




**Merino and Cherry, 2007. Adv. Biochem. Engin./Biotechnol., 108: 95-120.**

# Strategies to make the biorefinery processing more economical

1. Incresing commercial enzyme volumetric productivity
2. Producing enzymes using cheper substrates
3. Producing enzyme preparations with greater stability for specific processes
4. Producing enzymes with higher specific activity on solid substrates (Ex: cellulose breakdown in the solid phase by Endo- and Exo-glucanase is rate-limitng step)
5. Improvement in enzyme performance
6. Reduction in enzyme production cost
7. Increase in sugar yields

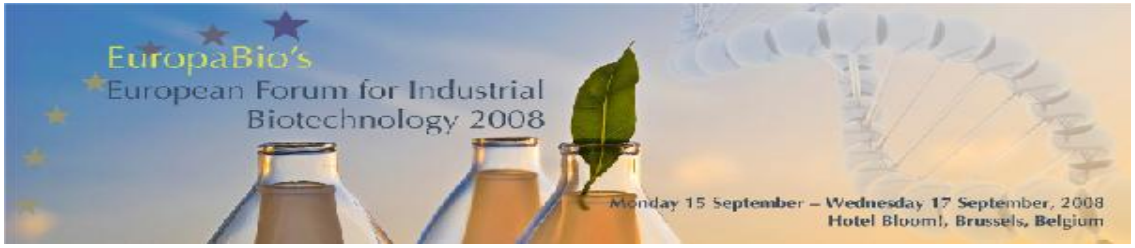
# Benefits for Development of Technologies for Converting Agricultural and Foresty Residues to Fermentable Sugars

1. Improved strategic security;
2. Decreased trade deficits;
3. Healthier rural economies;
4. Improved environmental quality;
5. Technology Exports
6. A sustainable energy resource supply

Zhang *et al*., 2006. Biotechnol. Adv., 24: 452-481.

# Biorefinery Euroview

- The **BIOREFINERY EUROVIEW** project aims at **preparing for future EU research and technological development activities**, including monitoring, assessment activities in the field of biorefineries, and the implications for agriculture and forestry policy.
- http://iarpolefr.nexenservices.com/biorefinery/public/index.html



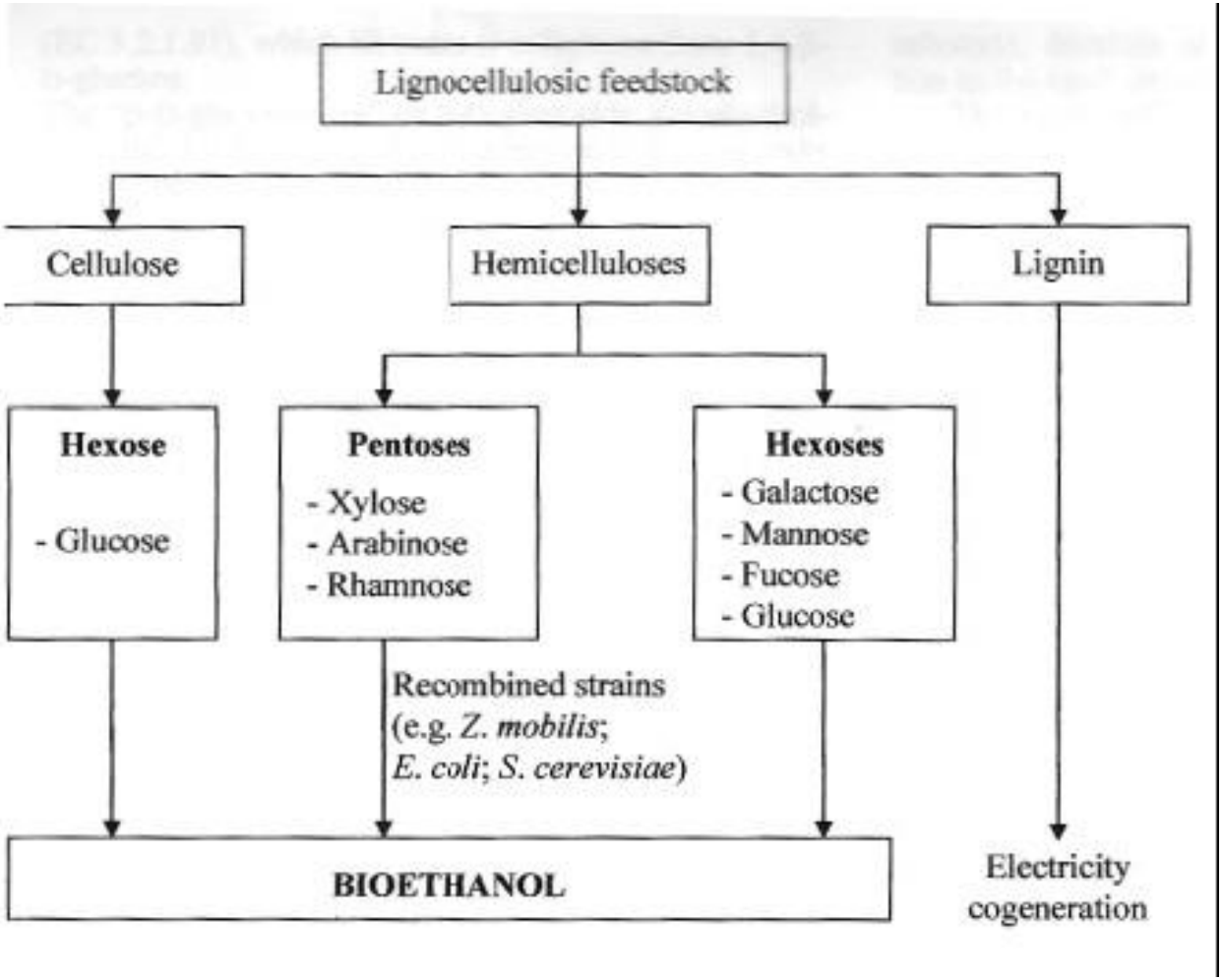

- This inaugural event will feature two workshops and a forum with approximately 30 leading speakers who will assess the prospects for industrial biotechnology in Europe, through presentations, question-and-answer sessions and panel discussions. Bringing together a senior and international group of biotechnology producers, chemicals and plastics suppliers, biomass and biorefineries, end users from a wide variety of industries and academia, EFIB2008 will provide the **perfect meeting place for science, industry, policymakers and investors of industrial biotechnology**. As more companies are recognising the potential of industrial biotechnology and developing a strong interest in bioproducts, new opportunities are opening for organisations in the know.

**Table 1.** Types of lignocellulosic materials and their current uses.

| Lignocellulosic material | Residues | Competing use |
|---|---|---|
| *Grain harvesting*<br>Wheat, rice, oats barley and corn | Straw, cobs, stalks, husks, | Animal feed, burnt as fuel, compost, soil conditioner |
| *Processed grains*<br>Corn, wheat, rice, soybean | Waste water, bran, | Animal feed |
| Fruit and vegetable harvesting | Seeds, peels, husks, stones, rejected whole fruit and juice | Animal and fish feed, some seeds for oil extraction |
| Fruit and vegetable processing | Seeds, peels, waste water, husks, shells, stones, rejected whole fruit and juice | Animal and fish feed, some seeds for oil extraction |
| Sugar cane other sugar products | Bagasse | Burnt as fuel |
| Oils and oilseed plants<br>Nuts, cotton seeds, olives, soybean etc. | Shells, husks, lint, fibre, sludge, presscake, wastewater | Animal feed, fertiliser, burnt fuel |
| Animal waste | Manure, other waste | Soil conditioners |
| *Forestry-paper and pulp*<br>Harvesting of logs | Wood residuals, barks, leaves etc. | Soil conditioners, burnt |
| Saw-and plywood waste | Woodchips, wood shavings, saw dust | Pulp and paper industries, chip and fibre board |
| Pulp & paper mills | Fibre waste, sulphite liquor | Reused in pulp and board industry as fuel |
| Lignocellulose waste from communities | Old newspapers, paper, cardboard, old boards, disused furniture | Small percentage recycled, others burnt |
| Grass | Unutilised grass | Burnt |

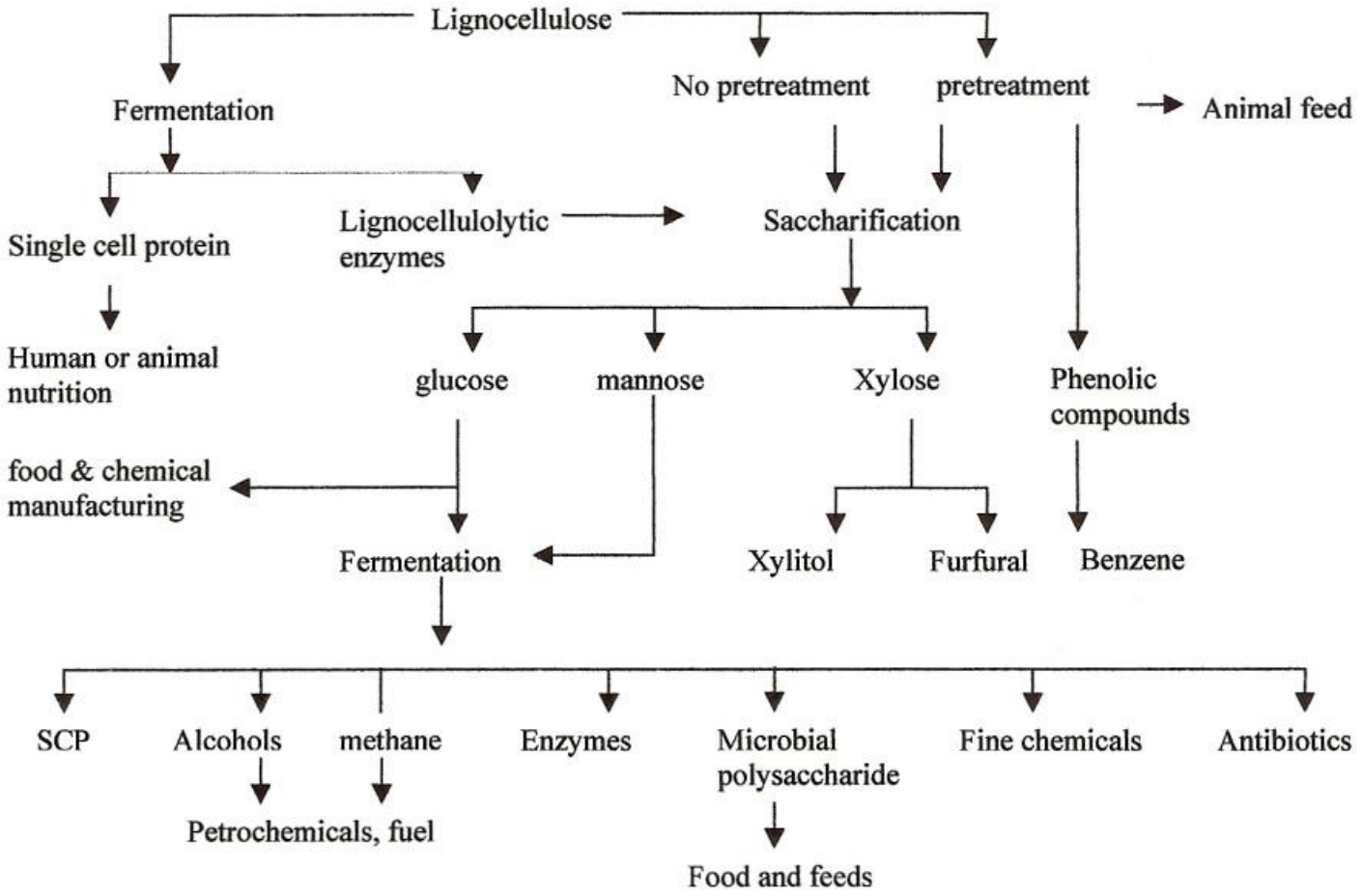
**Howard *et al*., 2003. African J. Biotechnol. 2: 602-619**

# Lignocellulose as a Source of Holocellulose




Neves *et al*., 2007.
DBPBMB, 1: 1-14

# Products of Lignocellulose Conversion




**Howard *et al*., 2003. African J. Biotechnol. 2: 602-619**.

# Some Conclusions!!

- An effective hydrolysis of holocellulose requires a hetero- and homosynergistic action of different hydrolases
- It is crucial an optimization of hydrolases action, specially in the insoluble phase of holocellulose
- Genomic Enzymology: “a strategy for understanding the interplay of structure and function, requiring correlated functional and structural characterization ”

“There are more things in heaven and earth, Horatio,
Than are dreamt of in your philosophy.”
**Hamlet,** scene v , William Shakespeare

# My research group!

# THANK YOU

# A LOT !!

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