Hydrolytic properties of two novel cellulases of *M. albomyces* expressed in *T. reesei*

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Renewable Resources and Biorefineries

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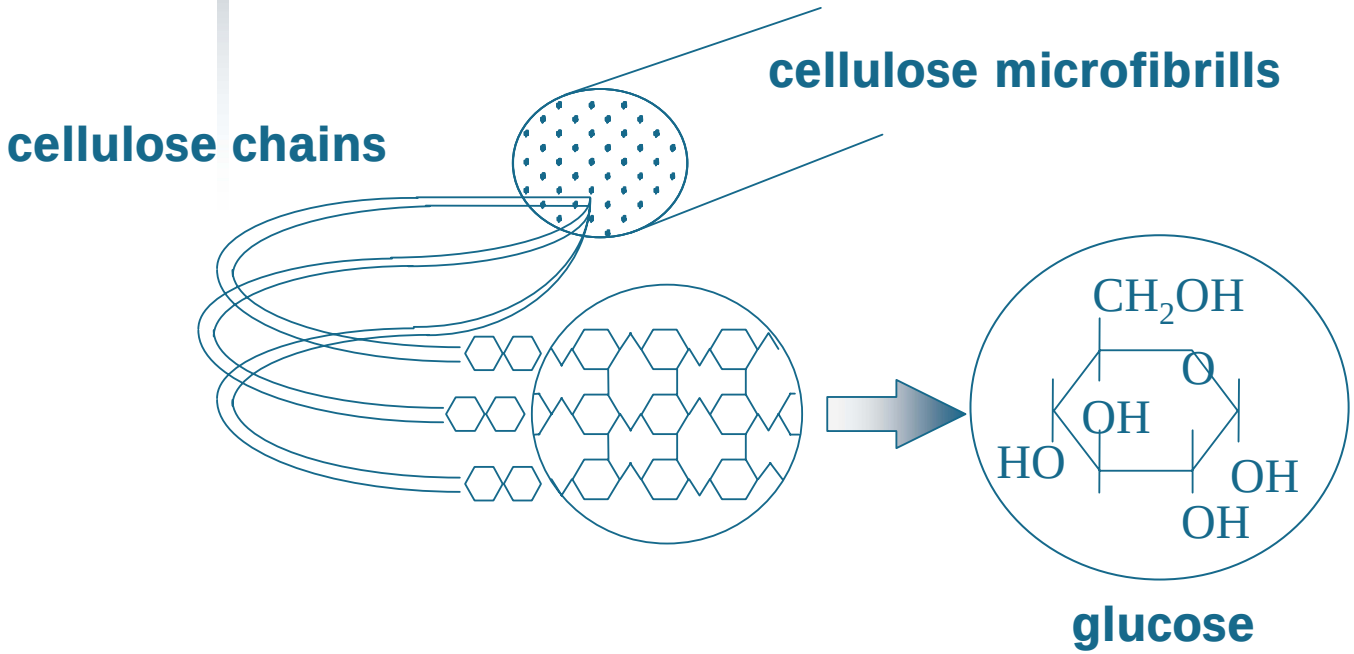
Cellulose



- insoluble linear chains of β–1,4–linked D-glucose units
- major structural polysaccharide of plants
- annual production of dry plant material: 1.5 x 10^{11} tons

> 50% → **cellulose is a unique renewable resource**

Cellulose degrading enzymes

” Cellulases ”

- a set of enzymes (different specificity & mode of action):

endoglucanases (EG)
random cleavage of β-glucosidic bonds

cellobiohydrolases (CBH)
release of cellobiose from the end of chains

β-glucosidases (BGlu)
hydrolysis of cellobiose to glucose units

synergistic action on substrate

complete degradation of cellulose

- modular structure: catalytic domain & cellulose binding domain (CBD)
- classified upon sequence similarities to glycosyl hydrolase families
- microbial origin (aerobic & anaerobic bacteria, filamentous fungi)
- major role in global carbon cycle (biodegradation)
- extracellular enzymes

Cellulases in the industry

substantial advantages over chemical catalysts

- derived from renewable resources (biodegradability)
- relatively mild operating conditions (T, pH)
- exquisite selectivity in reactant & product stereochemistry

commercial sales: 110 million euro/year (2000)

- cellulase enzyme production stands for more than 10% of total industrial enzyme production worldwide

industrial application: 23,000 tonnes/year (2000)

- food & feed industry
- beer & wine biotechnology
- textile & laundry
- pulp & paper industry
- research & development

Cellulases in food industry

extraction, clarification and stabilization of fruit and vegetable juices

- decreased viscosity of pulp mash
- increased filtration rate & reduced processing time
- increased juice yield & improved extraction of valuable components

production of fruit nectars and purees

- lower viscosity
- easier concentration
- improved mash stability & texture

extraction of olive oil

- better performance under cold processing conditions
- improved oil extraction
- better centrifugal fractionation
- slower induction of rancidity

Cellulases in animal feed industry

important sector of agro business

annual production rate: > 600 million tonnes
annual commercial sales: > 50 billion USD

supplementation of feed by cellulases

- degradation of certain cereal components (improved nutritional value)
- supplementation of animals' own digestive enzymes
- improved feed conversion rate (better digestion, adsorption, weight gain)
- utilization of less expensive feed components

limitations to successful enzyme use

- enzyme stability in feed (during & after processing)
- ability of added enzyme to hydrolyze plant cell wall polysaccharides
- ability of animals to utilize reaction products efficiently

Cellulases in beer & wine biotechnology

beer brewing

- poor quality barley (seasonal variations, different cultivars, poor harvest)
  - presence of 6-10 % non-starch polysaccharide
  - low levels of endogenous β- glucanase activity
  - gel formation during the brewing process
  - poor filtration (slow run-off times, low extract yields)

- microbial β-glucanases added during mashing or fermentation
  - hydrolysis of β-glucan
  - reduction of viscosity

wine production

- better skin degradation
- improved colour extraction
- easier must clarification & extraction
- improved wine quality & stability

Cellulases in textile & laundry

bio-stoning of denim

- alternative for stone-washing to gain faded, worn, aged appearance
- removal of excess dye from denim fabrics
- softening fabrics without fibre damage (less low-quality garment)
- short treatment times and increased productivity of washing machines
- reduced wear & tear of machines

bio-polishing of cellulosic fabrics

- removal of excess microfibrils from fabric surface
- prevention of pilling
- smooth & glossy appearance
- improved colour brightness & uniformity

production of environmentally friendly washing powders

- restoration of softness & colour brightness of cotton fabrics
- enhanced detergent performance
- removal of small, fuzzy fibrils from fabric surface

Cellulases in pulp & paper industry

bio-mechanical pulping

- enzymatic modification of mechanical pulp
- improved drainage of recycled fibres
- increased runnability of paper mills
- decreased energy consumption

bio-deinking

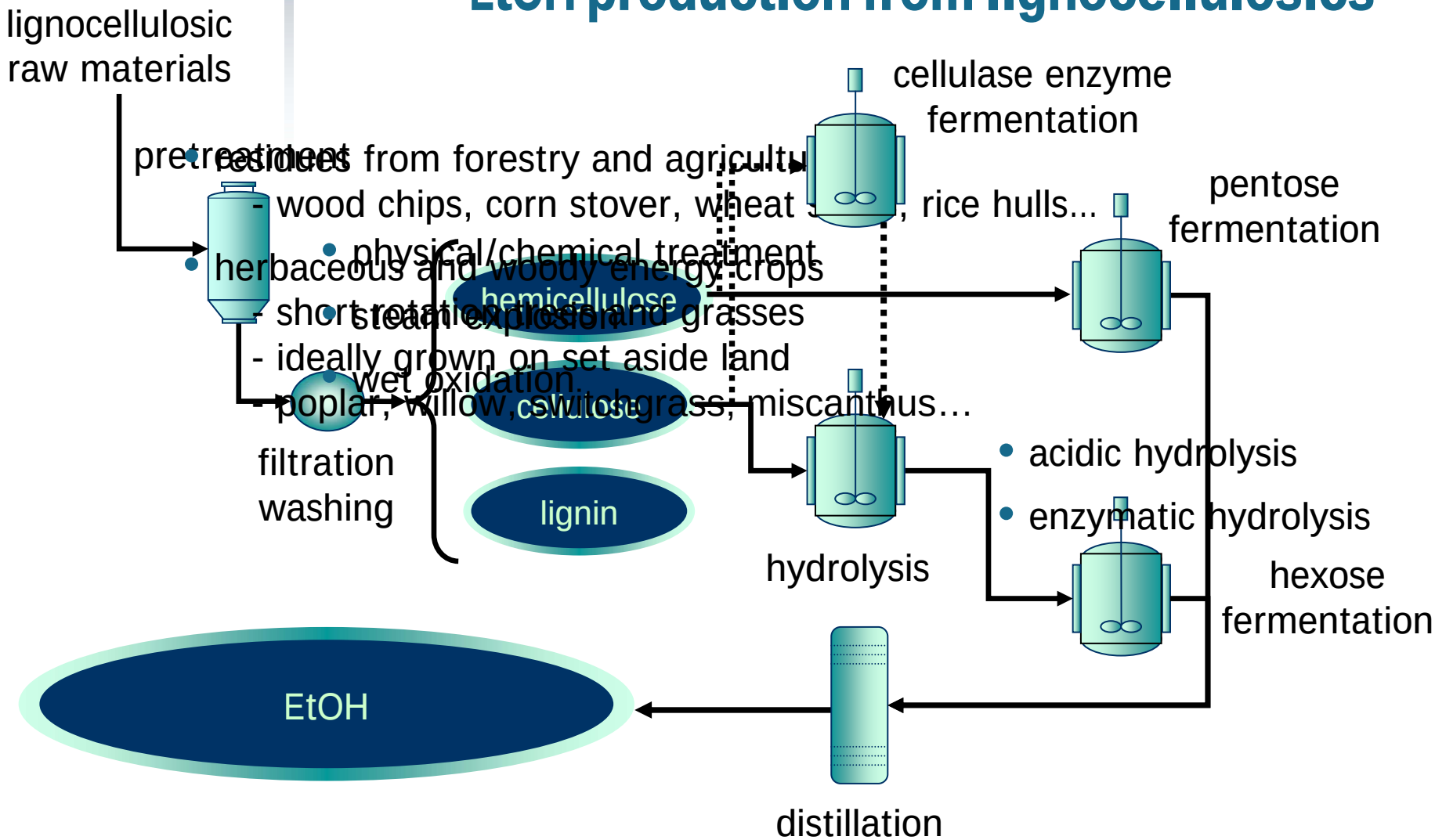
- release of ink from fibre surfaces by partial hydrolysis of carbohydrate
- prevention of alkaline yellowing by using enzymes at acidic pH
- improved brightness of pulp
- reduced environmental pollution

bio-characterization of pulp fibres

- selective solubilisation & characterization of pulp carbohydrates

Cellulases in R&D activities

EtOH production from lignocellulosics



Cellulase production

Aerobic fermentation by filamentous fungi

strains

- *Trichoderma, Aspergillus, Humicola* species...

substrate

- carbon source & inducer of cellulase production

fermentation method

- solid state fermentation → surface technology

  agricultural cellulosic residues as substrates (stalks, hulls, fibers...)

- submerse fermentation → bulk process

  soluble substrates (sophorose, lactose)

  insoluble substrates (purified cellulose or lignocellulosic residues)

  complex laboratory medium

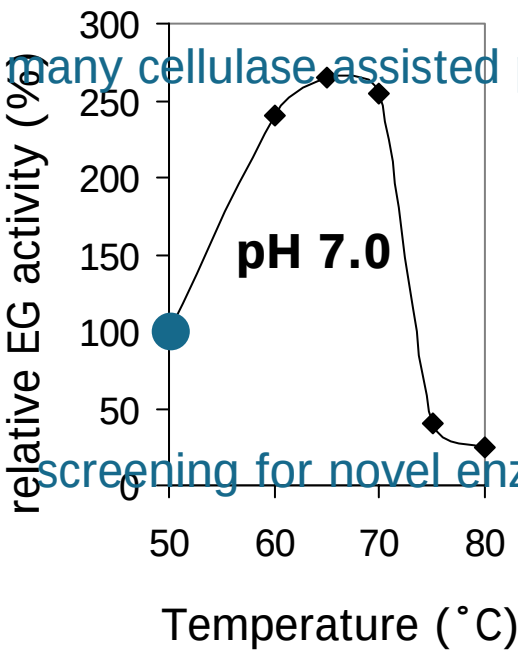
product recovery

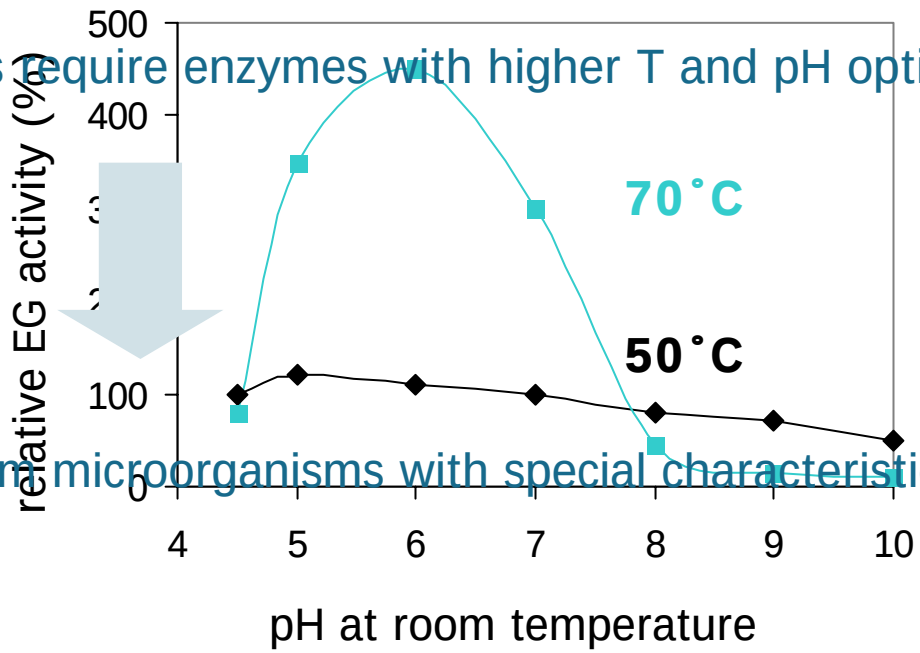
- extracellular enzymes → no cell-disintegration is needed
- extraction and/or phase separation; concentration

Operational parameters of cellulases

optima of conventional cellulases of mesophilic fungi (e.g. *Trichoderma*) ~ 50˚C pH 5.0

Temperature and pH dependence of EG activity of a novel neutral cellulase from the thermotolerant fungus *Melanocarpus albomyces*





many cellulase assisted processes require enzymes with higher T and pH optima

screening for novel enzymes from microorganisms with special characteristics

Miettinen-Oinonen *et al.*, 2004

Cellulases of *Melanocarpus albomyces*

Cel7A (50kDa) → EG **Cel7B (50kDa) → CBH**

- low production level by native host
- no cellulose binding domain (CBD)

heterologous expression in a hyper producing recombinant *T. reesei* strain (deficient in its main cellulases) under the strong *cbh1* promoter of the host

native structure (no CBD)

Cel7A / EG

Cel7B / CBH

modified form (with CBD of *T. reesei* CBH1)

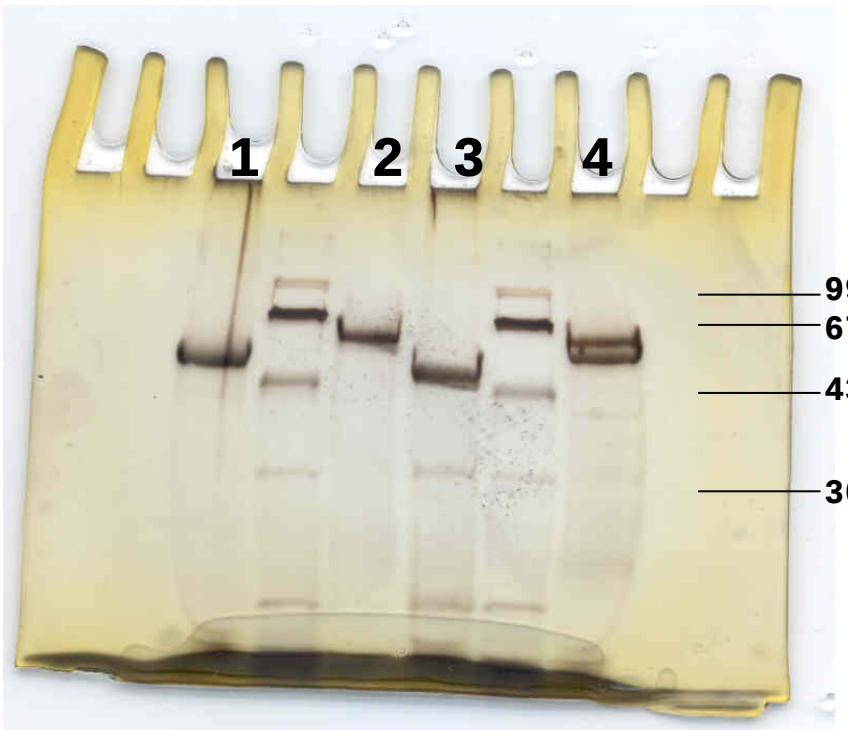
Cel7A (+CBD) / EG (+CBD)

Cel7B (+CBD) / CBH (+CBD)

Production of pure enzyme preparations

- submerse fermentation in lactose medium
- filtration and concentration of broth
- heat treatment (60˚C, 2h)
- protein purification (DEAE-IEX)

SDS PAGE (silver staining)



1 Cel7A

2 Cel7A (+CBD)

3 Cel7B

4 Cel7B (+CBD)

Activities of *M. albomyces* enzymes

heterologously produced & purified (DEAE-IEX) enzymes

	protein content	against CMC (pH 6.0, 50˚C)		against HEC (pH 6.0, 50˚C)	
	mg/ml	nkat/ml	nkat/mg	nkat/ml	nkat/mg
Cel7A	3,25	4723,0	1453,2	647,7	199,3
Cel7A (+CBD)	3,64	5175,0	1421,7	748,8	205,7
Cel7B	3,02	12,9	4,3	3,4	1,1
Cel7B (+CBD)	3,53	52,9	15,0	7,7	2,2

Hydrolysis experiments

Goal:

- comparison of novel enzymes (no complete hydrolysis is targeted!)

Conditions:

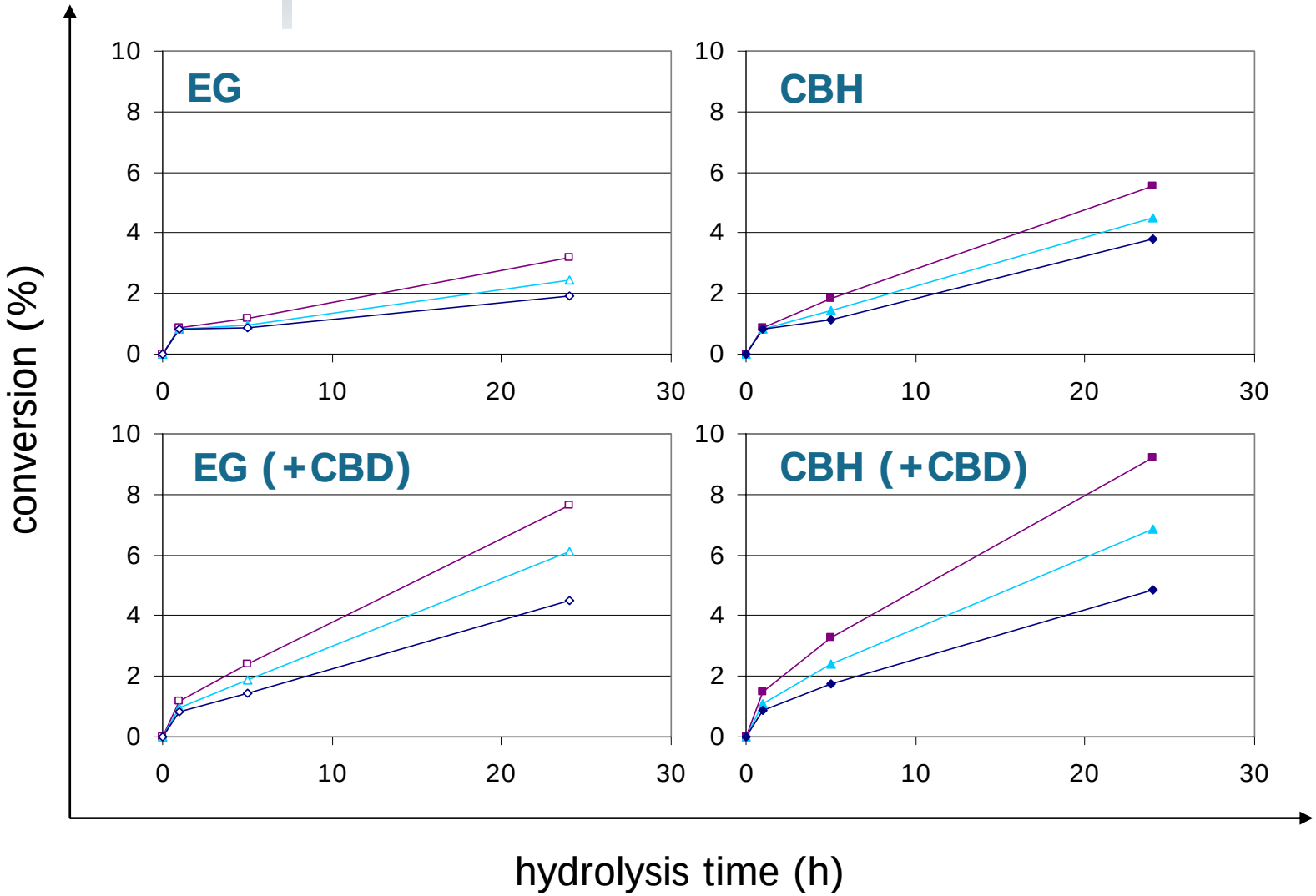
- model substrates (10 g/l): **Avicel** (microcrystalline cellulose) → insoluble
  Walseth (amorphous cellulose) → soluble
- enzyme dosage (0.5 - 50 mg/g substrate) set as protein loading
- 60˚C / pH 6.0 (50 mM sodium phosphate buffer) / 24h

Monitoring of hydrolysis performance:

- sampling: 1h, 5h, 24h
  (boiling for 5 min; phase separation by centrifugal force)
- reducing sugars liberated during hydrolysis assayed by DNS method
  (colourimetric detection of the complex formed with dinitrosalicylic acid)
- conversion: released sugar as glucose per net carbohydrate content (%)

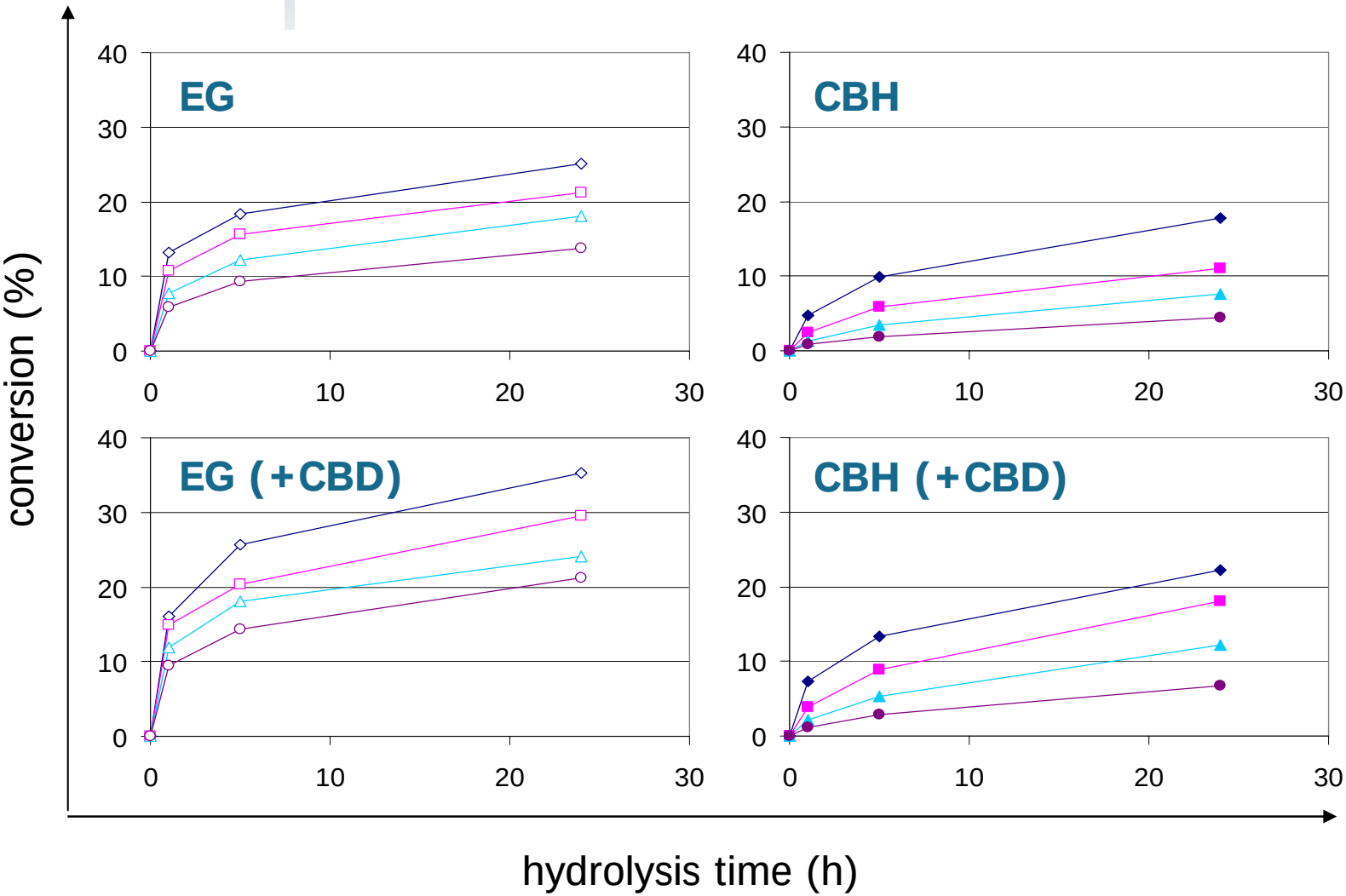
Hydrlolysis of Avicel

enzyme dosage: 5, 10, 20 mg / g Avicel



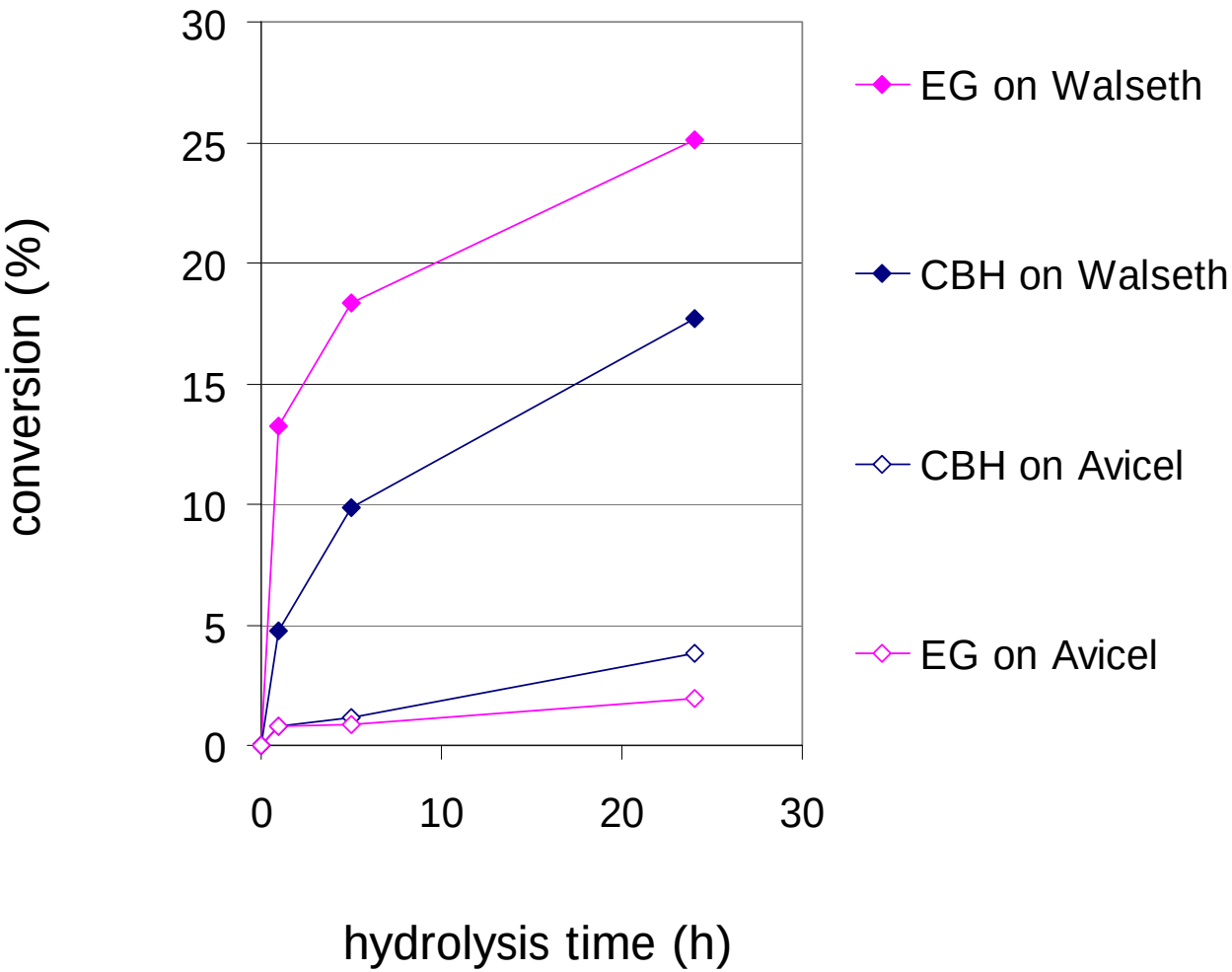
Hydrlolysis of Walseth

enzyme dosage: 0.5, 1, 2, 5 mg / g Walseth



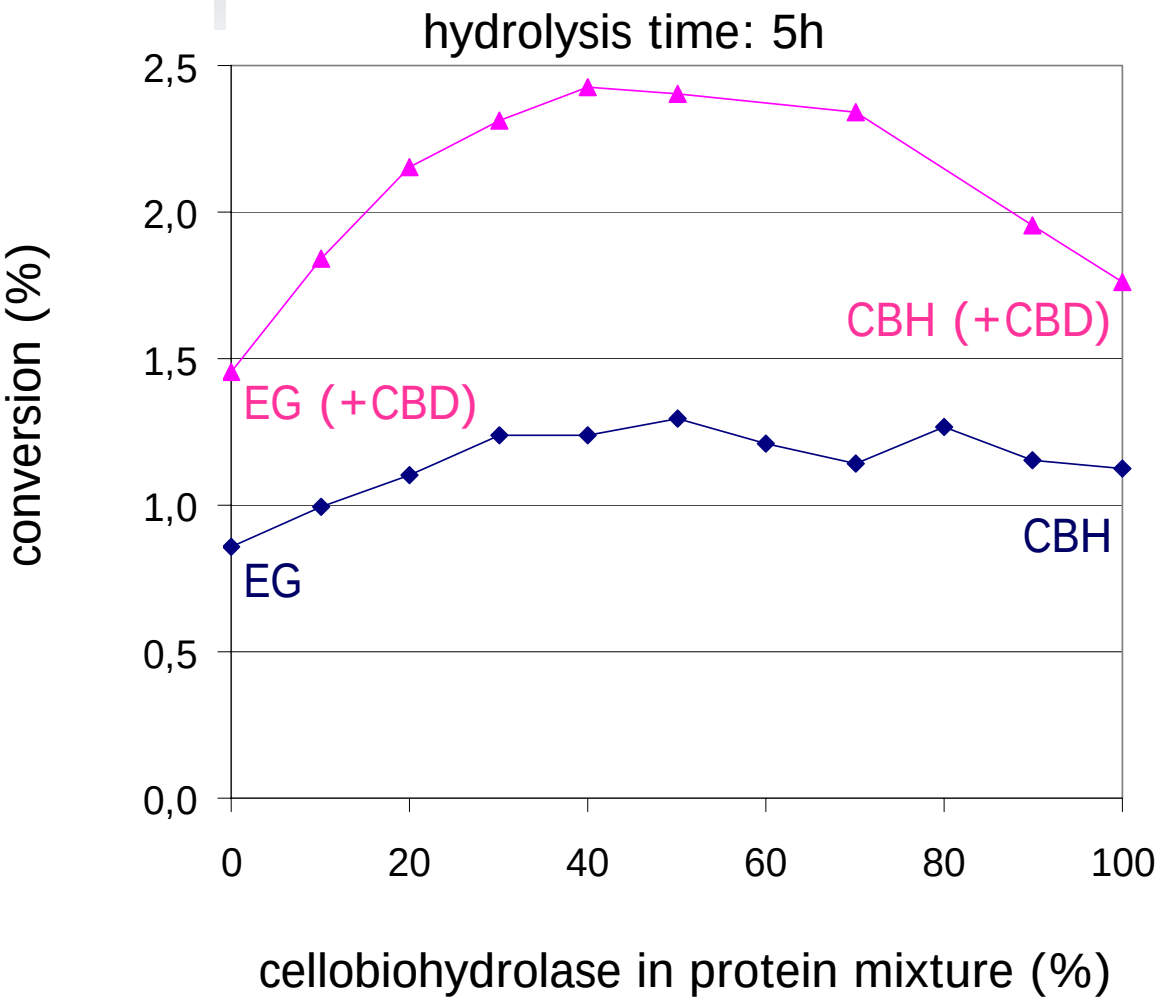
Accessibility of substrates

enzyme dosage: 5 mg / g substrate



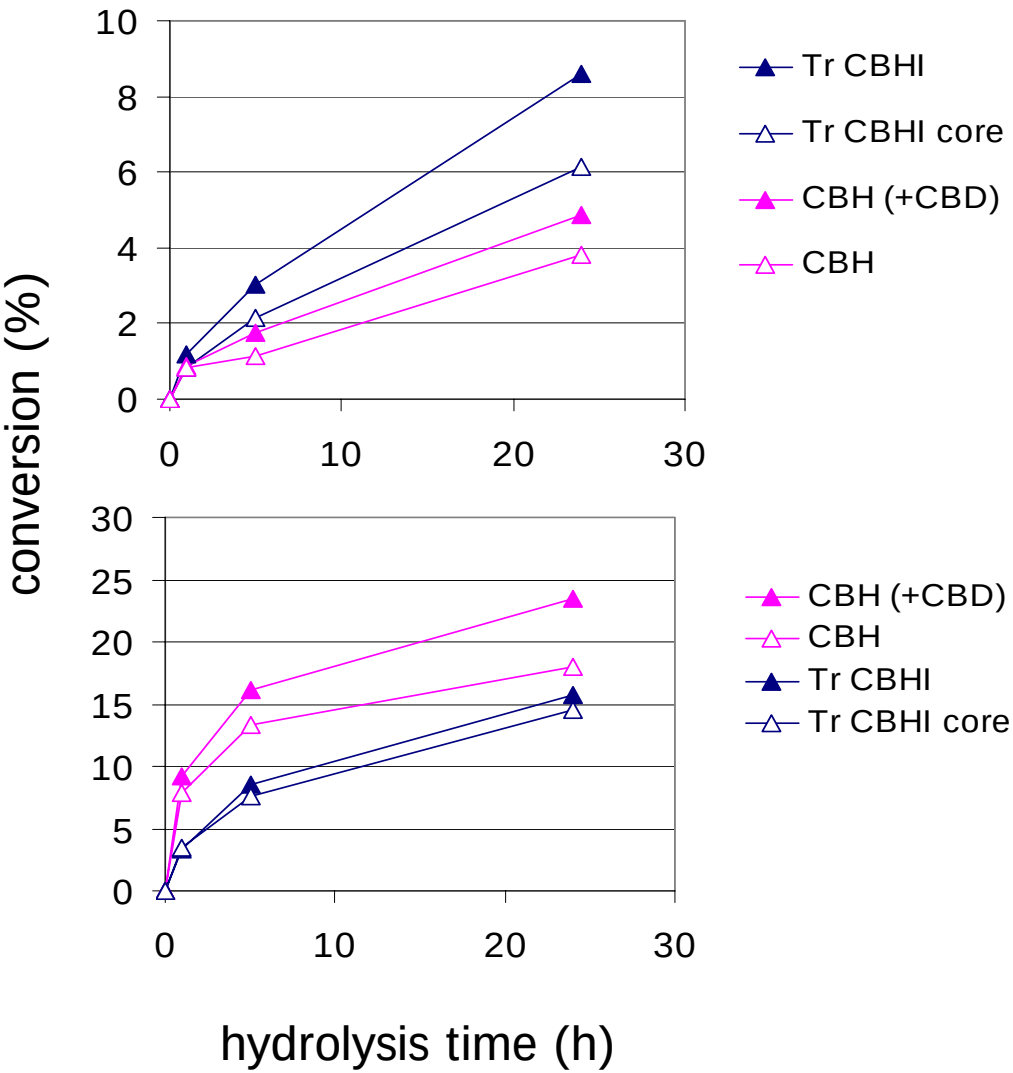
Synergism on Avicel

net protein dosage: 5 mg enzyme / g Avicel



Comparison to *T.reesei* CBHI & CBHI core

enzyme dosage: 5 mg / g substrate



Avicel substrate

Walseth substrate

Concluding Remarks

- cellulase enzymes have a great potential in a large variety of processes
- their role in fuel ethanol production from lignocellulosics is of special interest
- enzymes with special characteristics are of particular importance
- cellulases of *M. albomyces* are promising in applications at elevated pH and T
- they are specially efficient with CBD and in synergism
- Cel7B of *M. albomyces* can compete with the traditional CBHI of *T. reesei*

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“Leonardo da Vinci Program”

European Community Vocational Training Action Program

Thank you for your attention !

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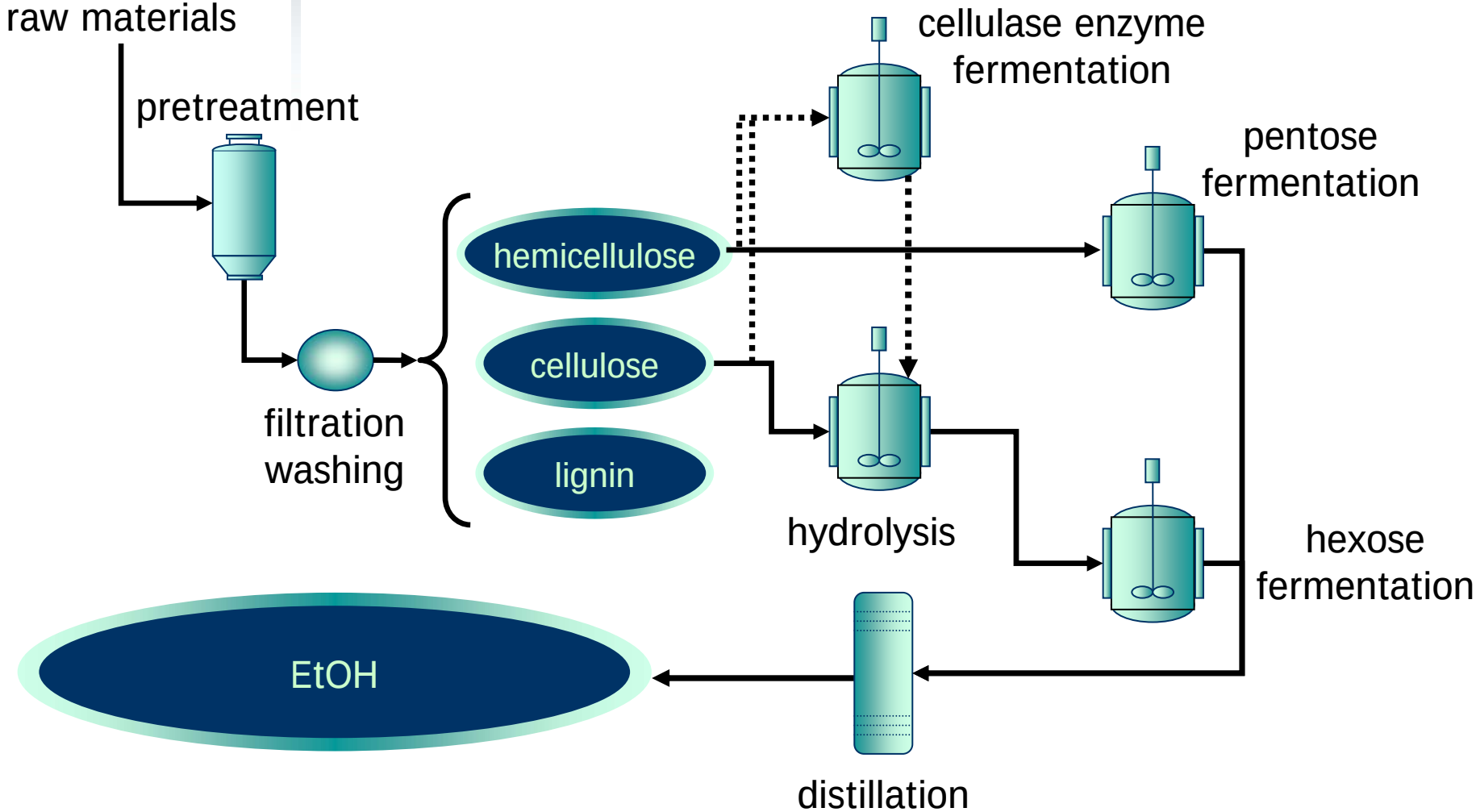

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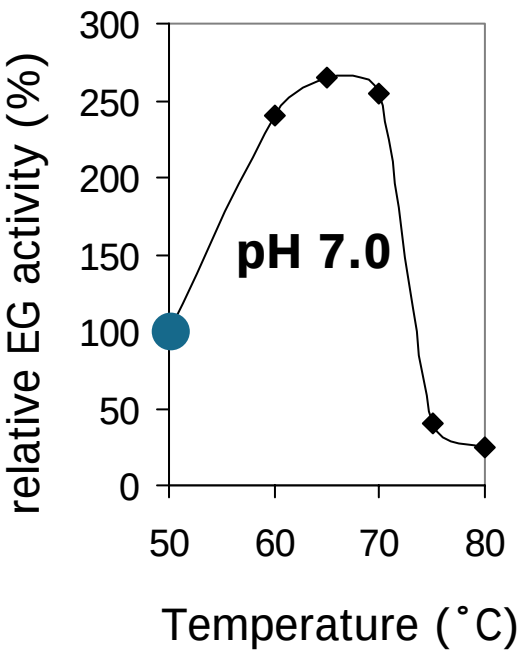

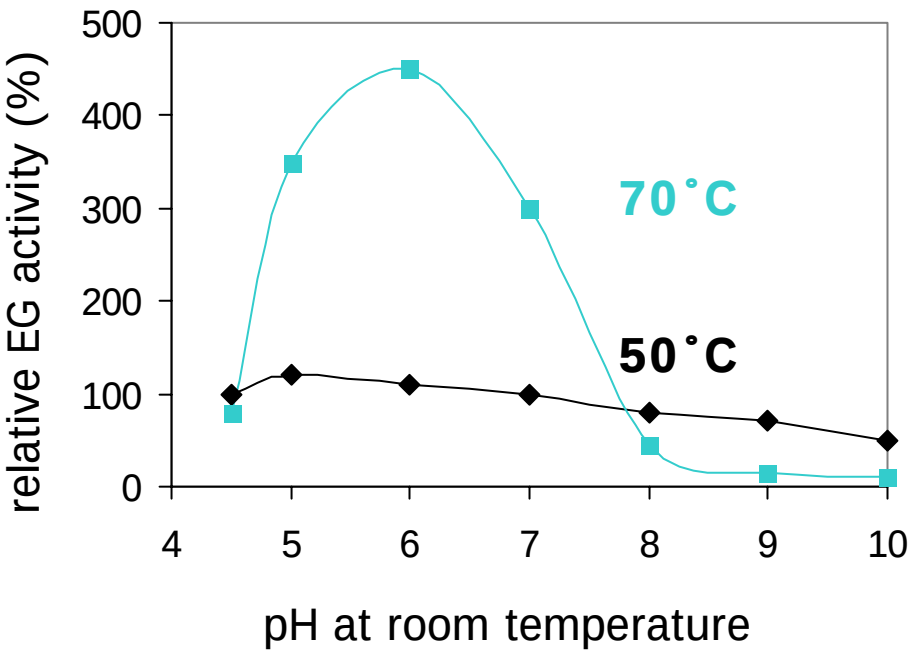

Miettinen-Oinonen *et al.*, 2004