Ingeniería Química y Biotecnología
FACULTAD DE CIENCIAS FÍSICAS Y MATEMÁTICAS
UNIVERSIDAD DE CHILE

Domeyko
PROGRAMA DE INVESTIGACIÓN

CONICYT
COMISIÓN NACIONAL DE INVESTIGACIÓN CIENTÍFICA Y TECNOLÓGICA

Identification, heterologous expression and characterization of novel cellulases from white rot fungi

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SEMINARIO DE CLAUSURA

"OPTIMAL TREATMENT PROCESSES OF LIGNOCELLULOSES FOR BIOETHANOL – CONSORTIUM: OPTBIO"

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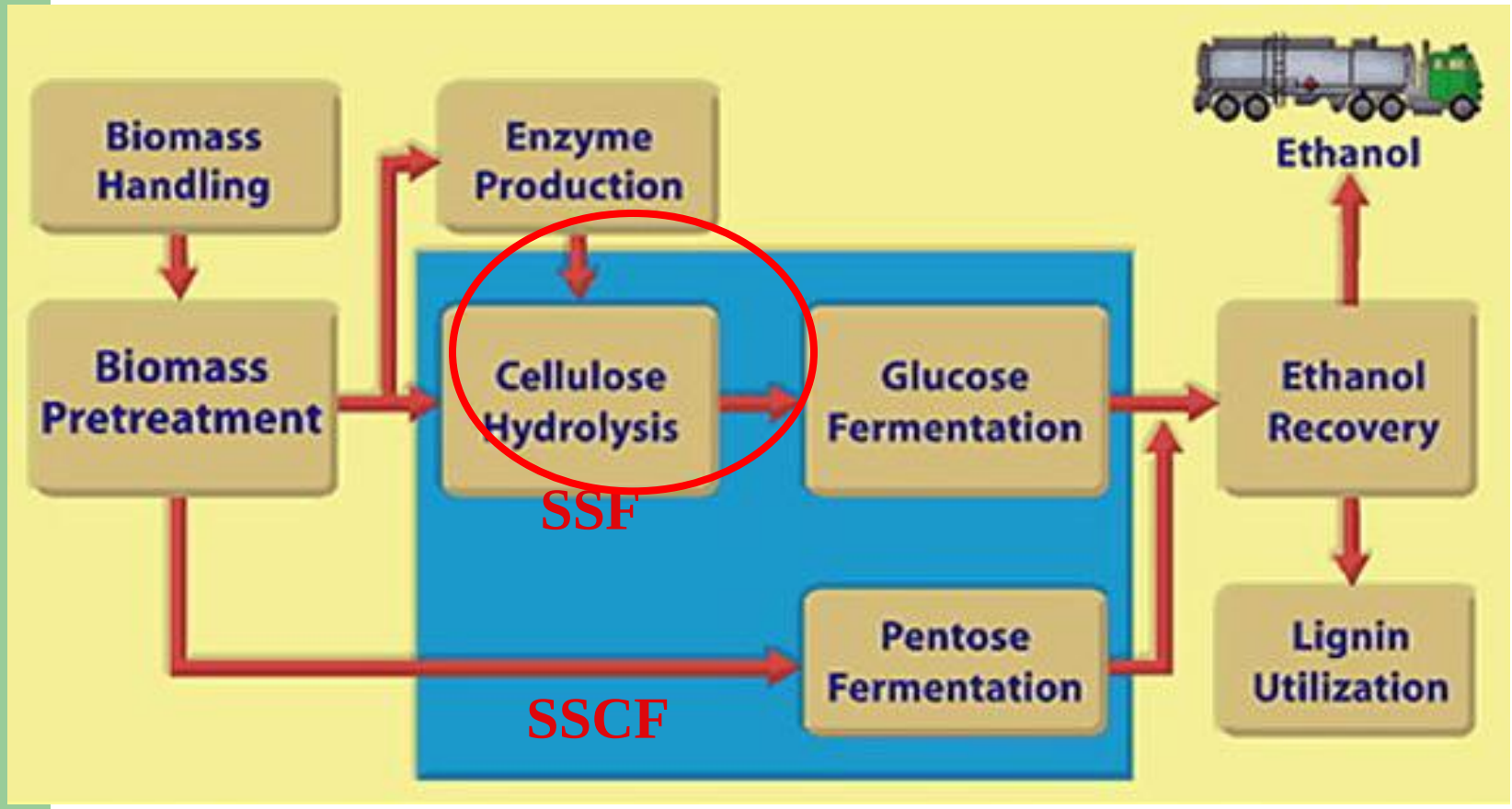
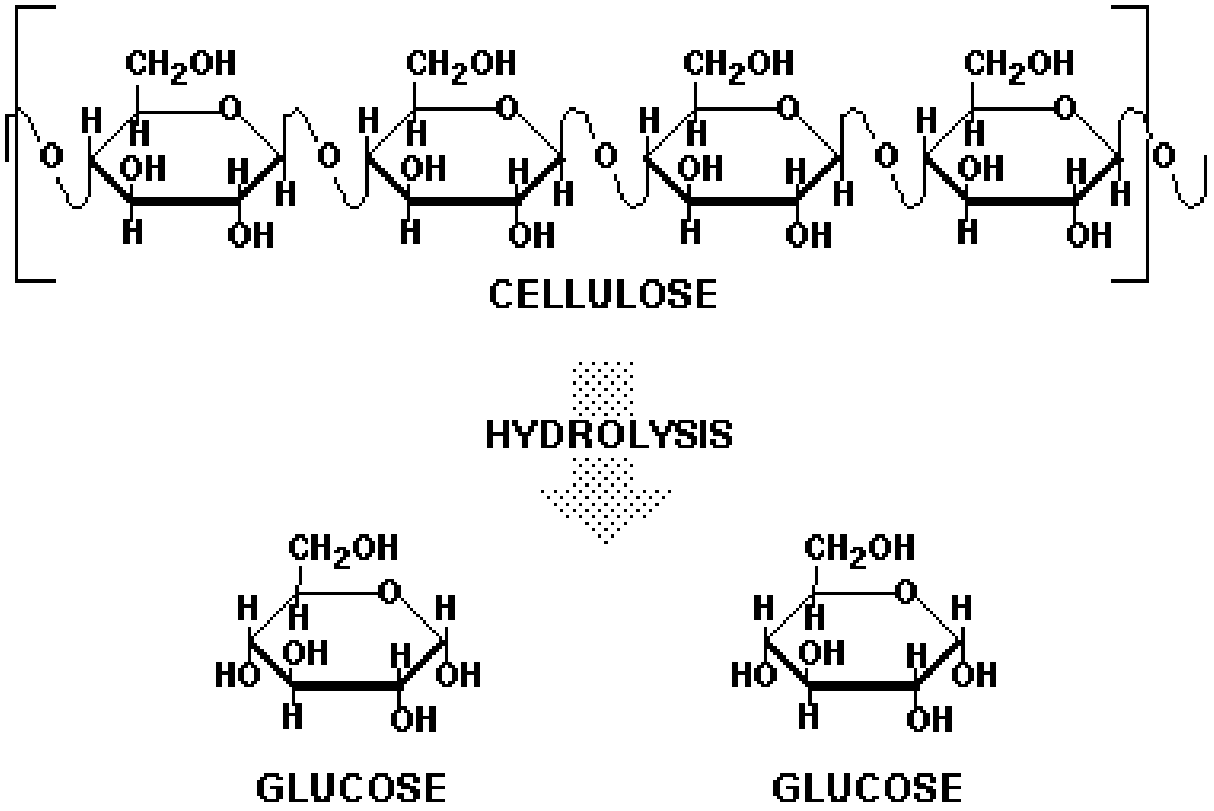
Outlook

- **Introduction**
  - Cellulose hydrolysis in the bioethanol process
  - Enzymes for cellulose hydrolysis
  - White rot fungi as source of cellulases
- **Main Goals**
- **General Methodology**
- **Results**
  - Identification of cellulolytic fungi
  - Identification of novel cellulases and characterization
  - Cellulase gene cloning and recombinant expression
- **Conclusions**

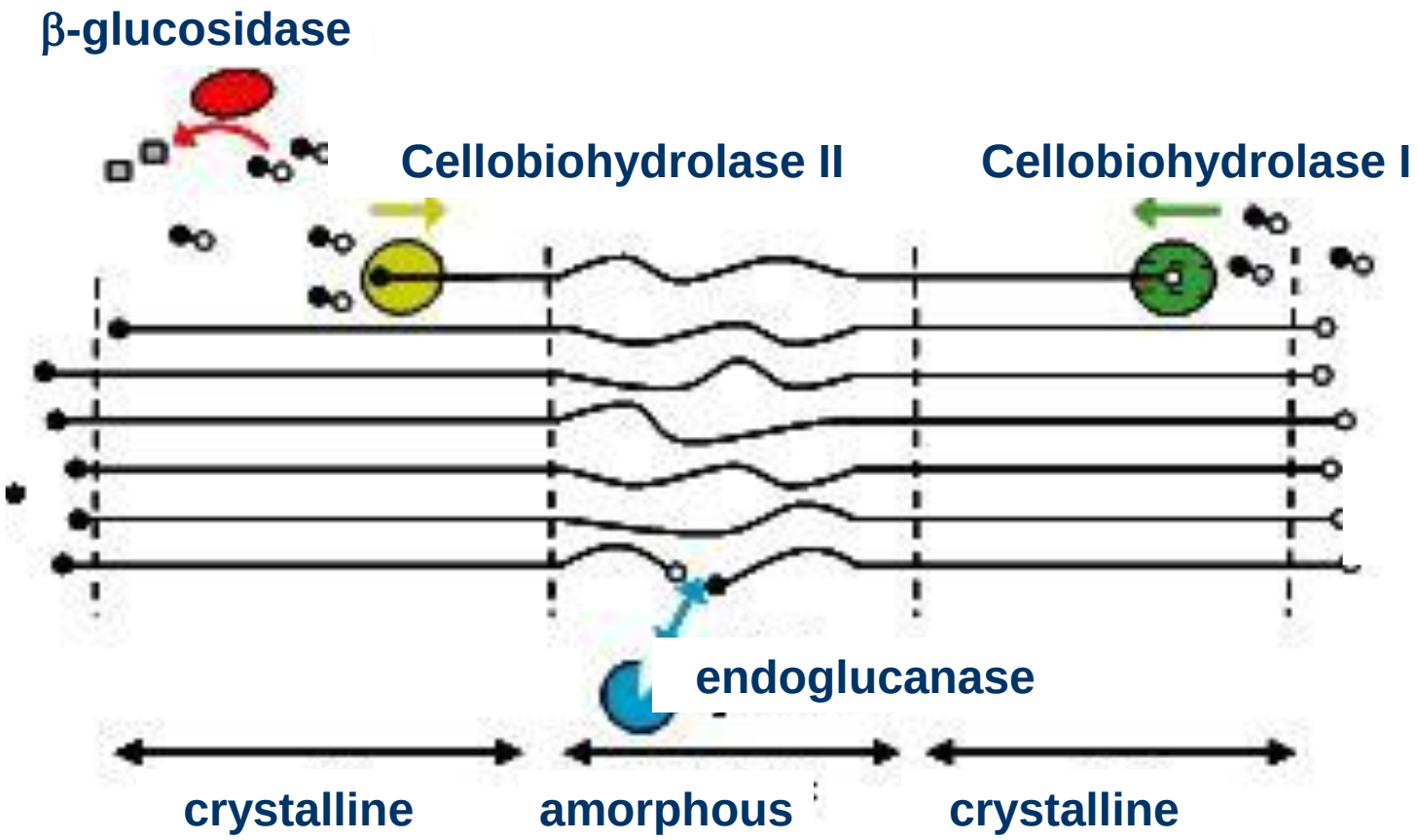
The process of bioethanol production from lignocellulosic biomass



Cellulose hydrolysis



Cellulose hydrolysis is carried out by an enzymatic complex, cellulase



What are the requirements for cellulases to be used for lignocellulose biomass degradation?

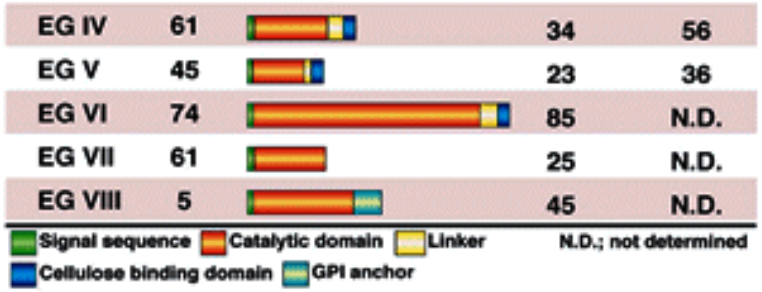
- High activity for extensive hydrolysis and low loading in reactors
- Thermostability for long-time (days) operation at 50°C.
- Tolerance to inhibitors (chemicals, glucose, ethanol, etc)
- High capacity to adsorb to cellulose and low adsorption to lignin

Microorganisms producing cellulases

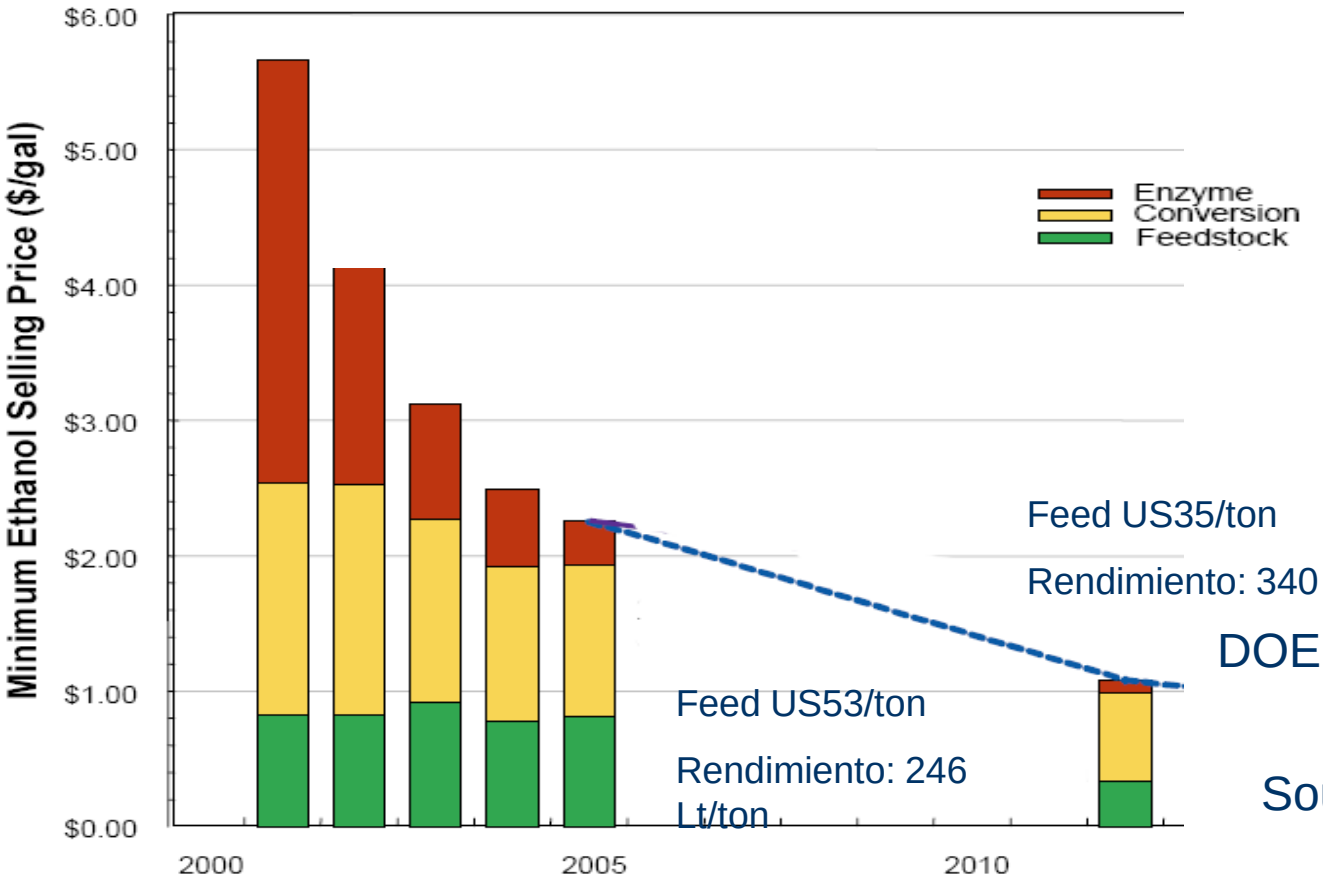
- *Clostridium*, *Cellulomonas, Tricho-derma*, *Penicillium*, *Neurospora*, *Fusarium*, *Aspergillus* etc, have cellulolytic and hemicellulytic activity.
- Main producer is *Trichoderma reesei,* containing
  - 8 endoglucanases
  - 2 exoglucanases
  - 1 cellobiase
- **Enzyme properties and productivity have been enhanced but further improvement is still needed**

Cellulases from *Trichoderma reesei*

Enzyme	GHF	Domain structure	Molecular weight (kDa) Calculated	*T. reesei*
Cellobiohydrolase				
CBH I	7		58	68
CBH II	6		47	58
Endoglucanase				
EG I	7		46	54
EG II	5		42	50
EG III	12		25	25
EG IV	61		34	56
EG V	45		23	36
EG VI	74		85	N.D.
EG VII	61		25	N.D.
EG VIII	5		45	N.D.



Cost of cellulase in the bioethanol process



DOE target cost

Source: NREL

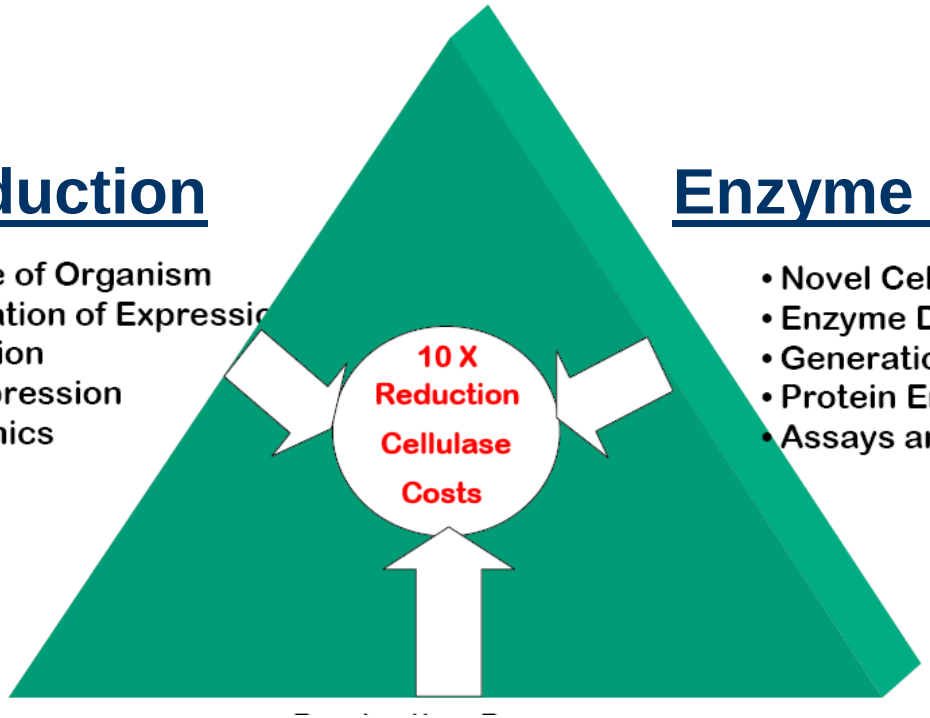
Strategies used to reduce cellulase cost

Enzyme production

- Choice of Organism
- Regulation of Expressi
- Induction
- De-repression
- Genomics

Enzyme performance

- Novel Cellulolytic Activities *
- Enzyme Discovery
- Generation of Diversity
- Protein Engineering *
- Assays and Screens



Reduced $/gm enzyme

Production process

Reduced gm enzyme/vol EtOH

White Rot Fungi (WRF)

- Grow in the forests
- Degrade wood of trees
- Posses a enzyme system of ligninases, oxidases, peroxidases, hemicellulases and cellulases
- Very low number of cellulases from WRF have been cloned and tested for industrial degradation of cellulose

Main Goals

- To evaluate the efficiency of diverse enzymatic complexes previously isolated from WRF fungi.
- To isolate and clone cellulase genes from WRF
- To evaluate the recombinant enzymes performance on the degradation of cellulosic substances to fermentable sugars.
- To study the characteristics of the interaction between cellulose and the wild type and recombinant cellulolytic enzymes.

General strategy

Phase 1

Screening of the fungal library for the best cellulase producers

Phase 2

Study of the potential of the cellulase for wood biomass saccharification

Phase 3

Cellulase production and characterization

Phase 4

Cellulase genes isolation, cloning and recombinant expression

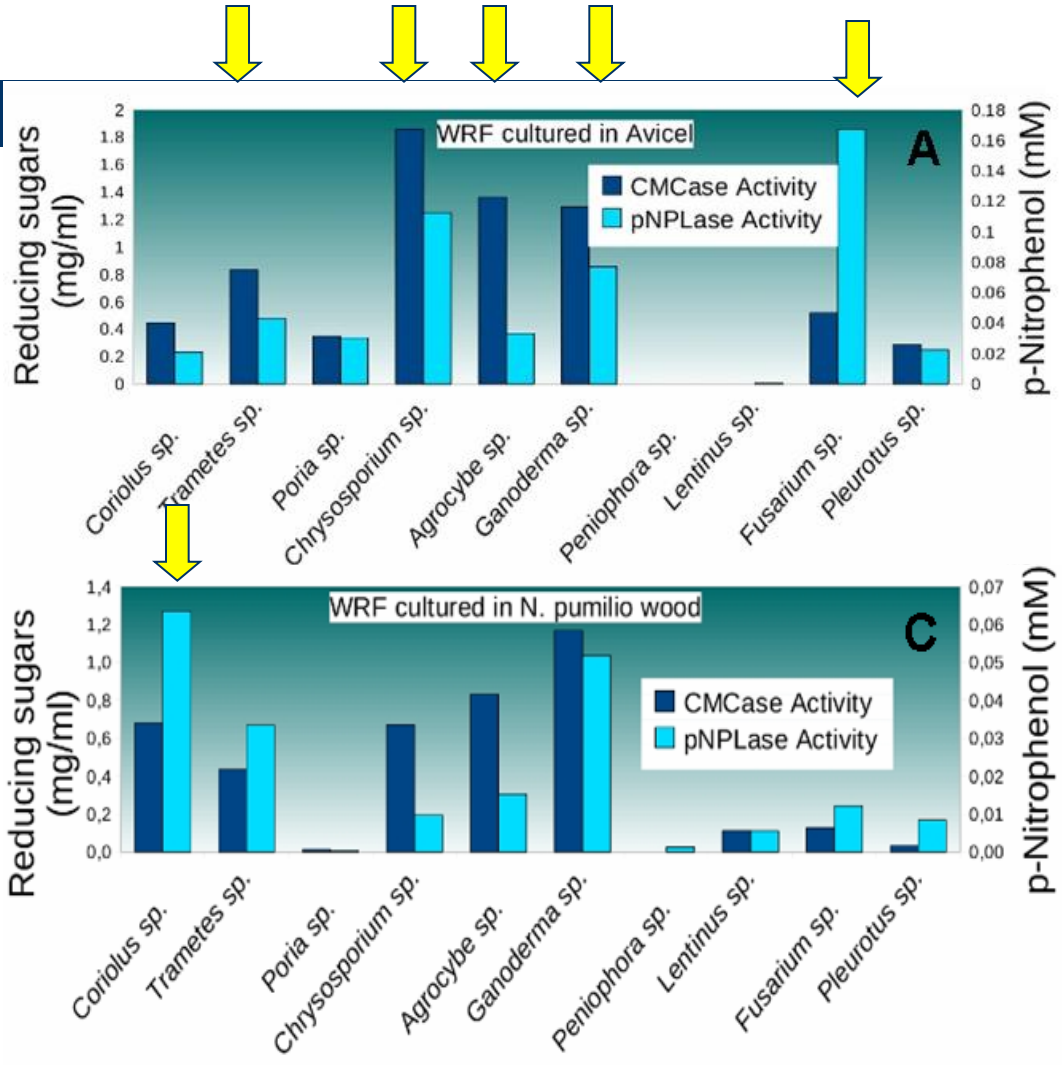
In the future:

Protein engineering to improve:

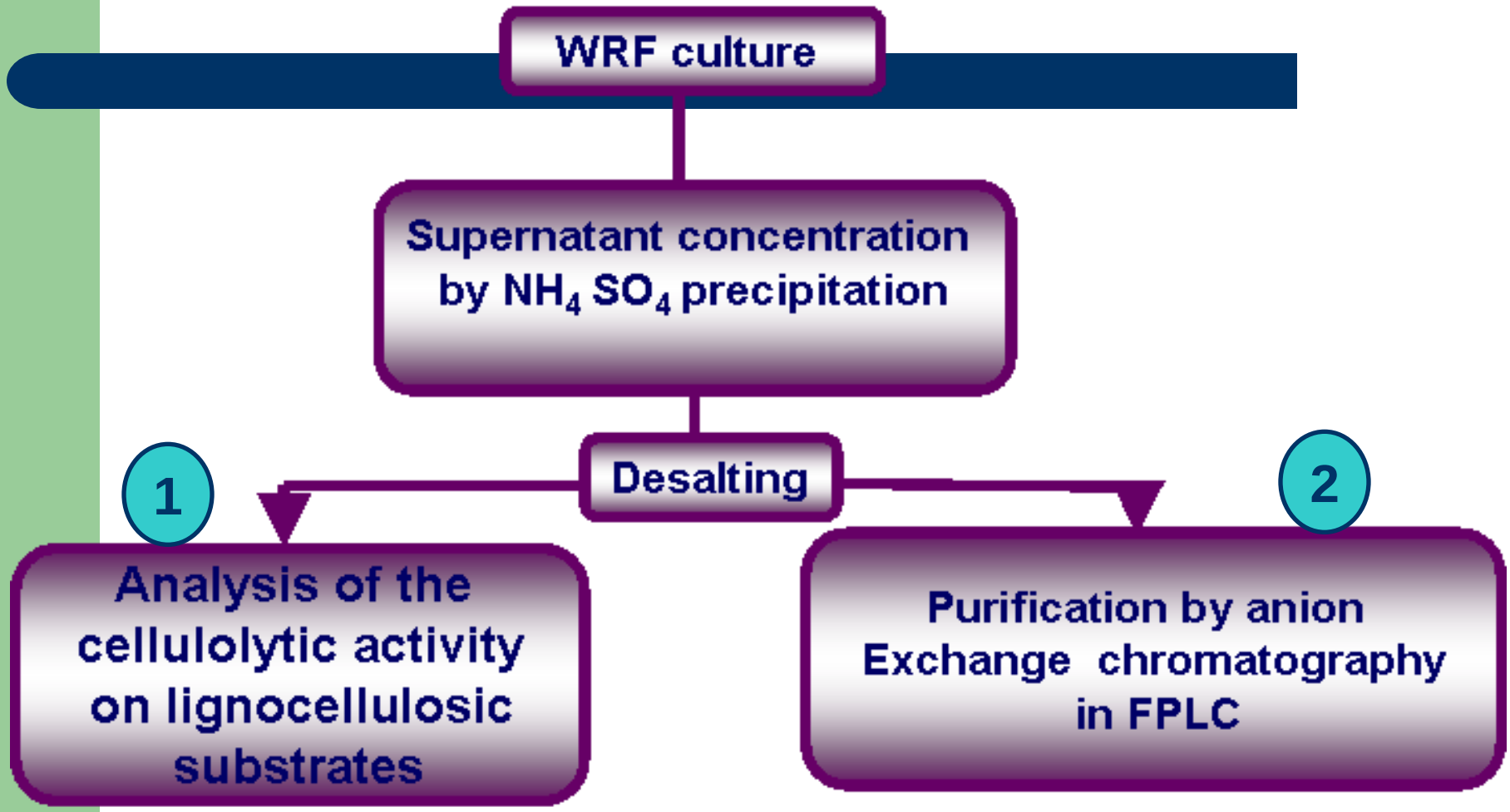
- activity
- thermostability
- tolerance to inhibitors derived from the pretreatment
- Cellulose adsorption

Phase I: Identification of the best WRF producers of cellulase activity

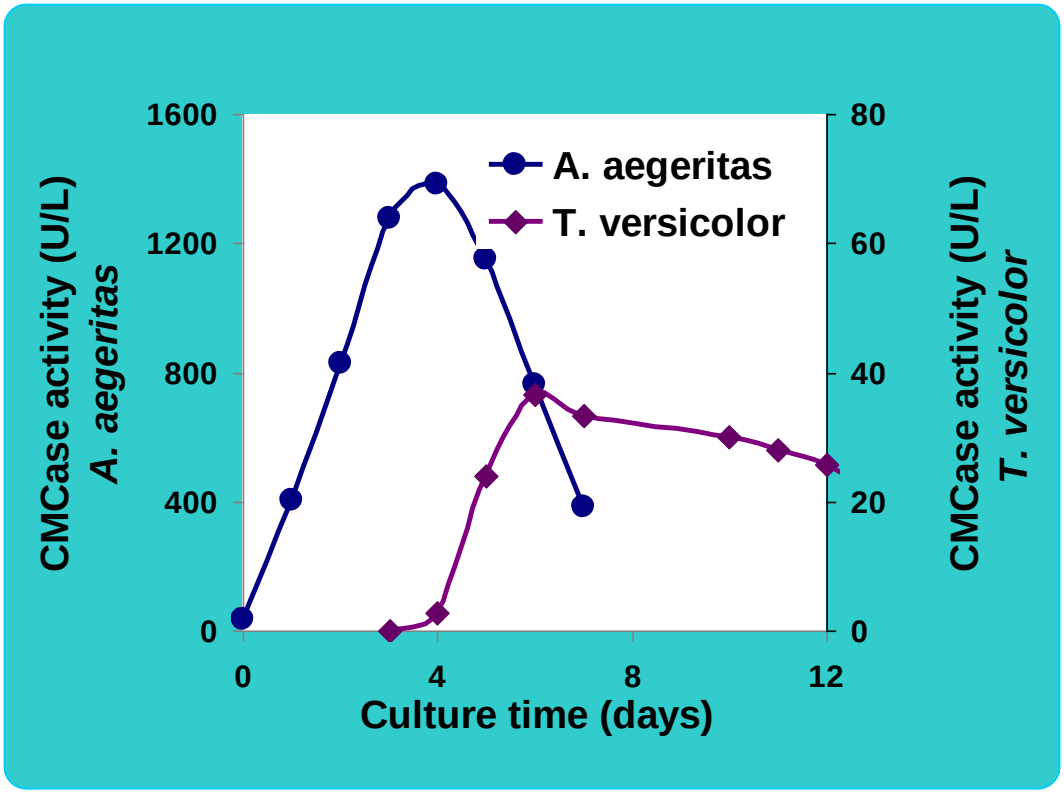
- Fungi were cultured in saline medium with cellulose as the only carbon source
- Production of endoglucanase and exoglucanase activity was evaluated using specific substrates for endoglucananses (CMC) and exoglucanases (pNPL)
- Six fungal isolates were selected for the next step



Phase II: Cellulolytic activity of WRF cellulases for wood biomass saccharification

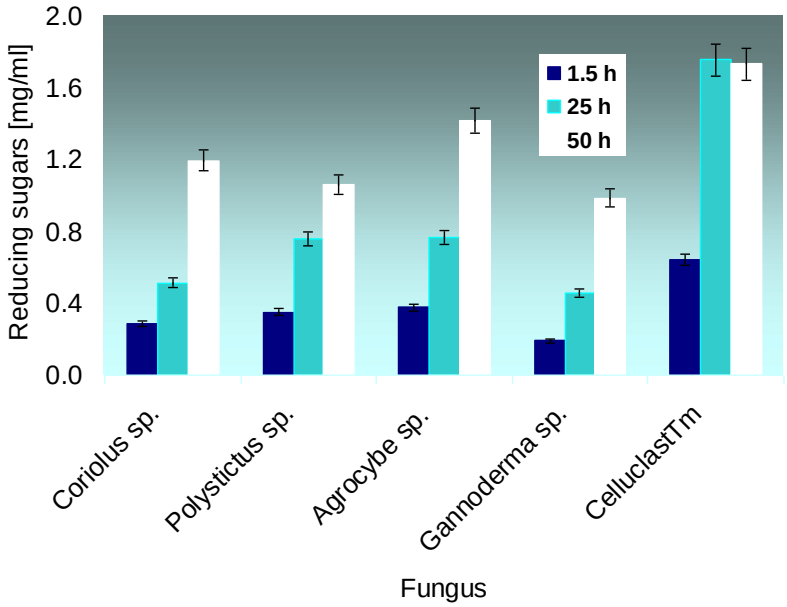


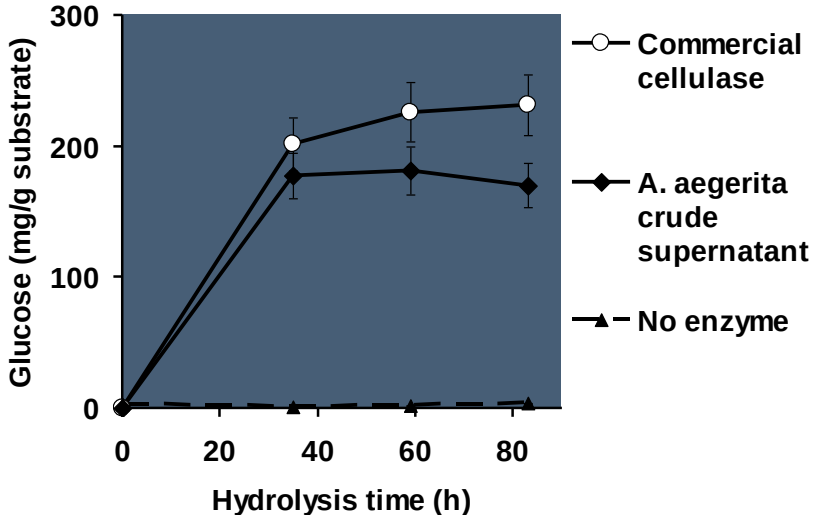
Cellulase production by WRF



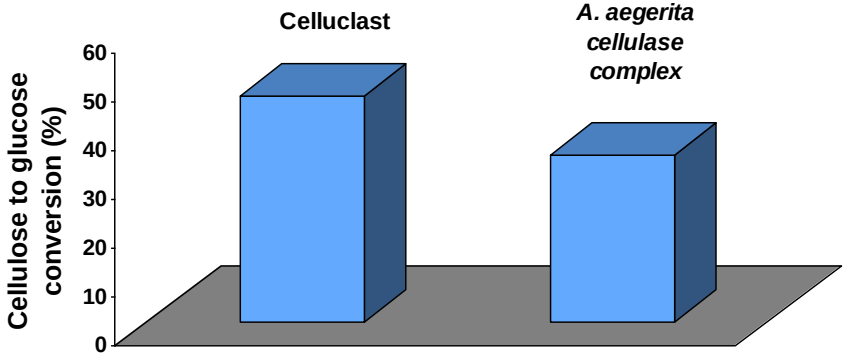
Phase II. Cellulolytic activity in WRF crude extracts on lignocellulosic substrate

Degradation of *N. pumilio* (Lenga) wood chips by enzymes from WRF

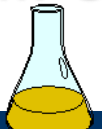




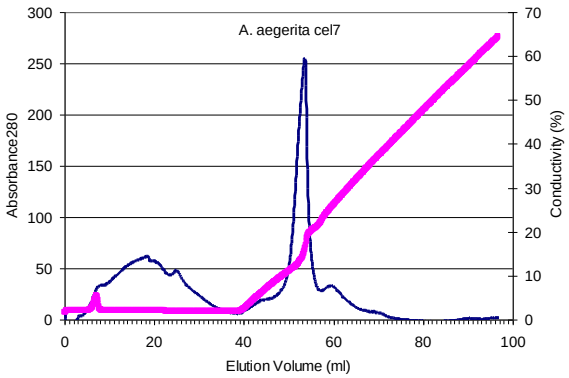
Fungal cultures supernatants were incubated with wood chips (1%) at 50 ºC at pH 5 for 1.5, 25 or 50 hours.



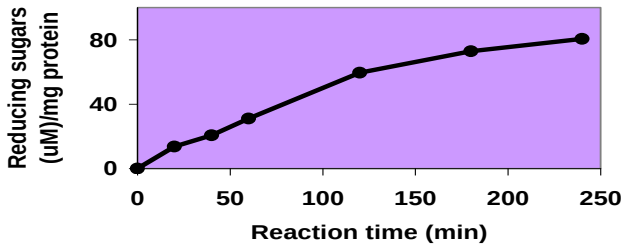
Phase III. Cellulases production and characterization



Anionic Exchange chromatography

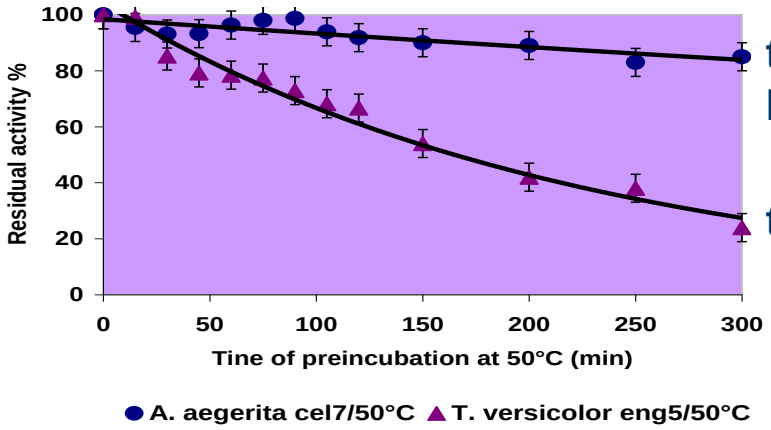


Activity of cel7 on microcrystalline cellulose



Specific activity =0,5 U/mg prot.

Thermal stability of purified cellulases

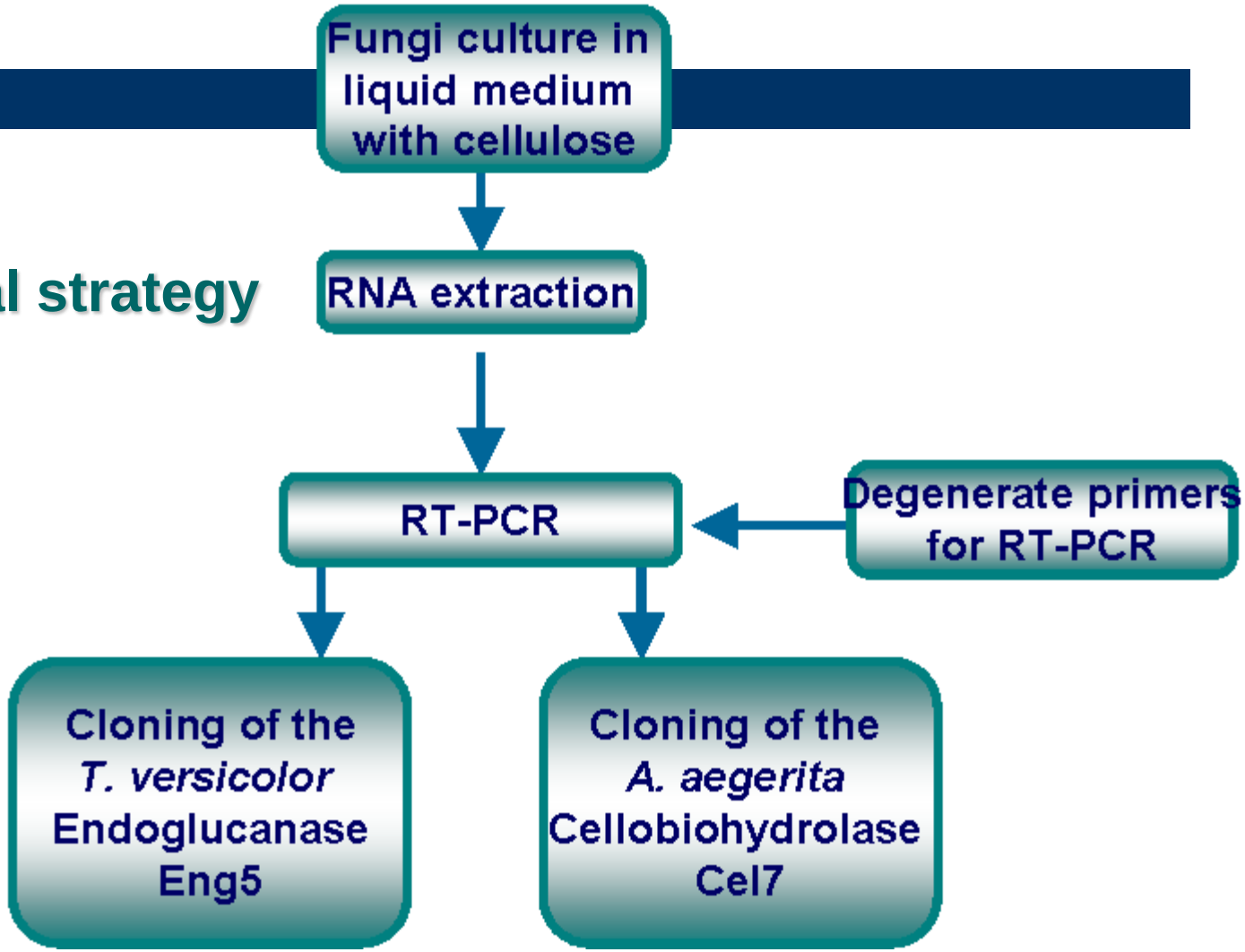


$t1/2^{50}$~10-12 h

$t1/2^{50}$~2,5 h

Phase IV. Identification of the cellulase genes, and recombinant expression

General strategy



Partial sequences for several cellulase genes were identified using molecular techniques

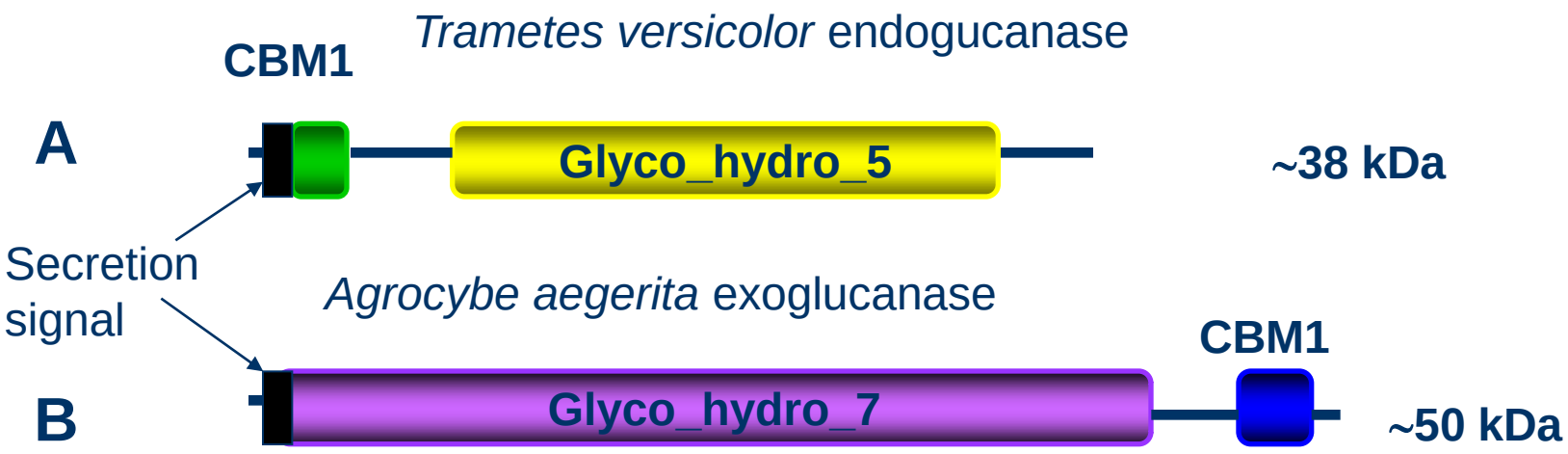
Comparison of WRF cellulase sequence to described cellulases

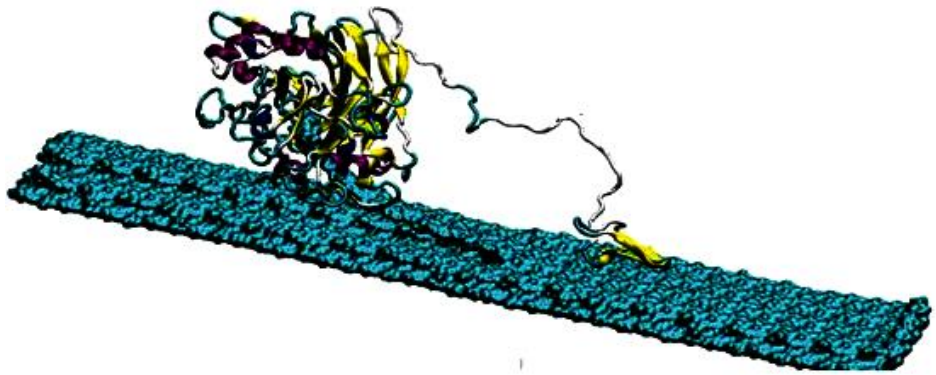
Fungus	Enzyme specificity	Sequenced (%)	Identity[a] (%)
Trametes versicolor	GH5 endoglucanase	46	33
	GH6 exoglucanase	58	89
	GH7 exoglucanase	49	79
Fusarium oxysporum	GH7 endoglucanase	25	47
	GH61 endoglucanase	29	54
Ganoderma applanatum	GH61 endoglucanase	29	37
Phanaerochaete chrysosporium	GH7 exoglucanase	60	100
Coriolus versicolor	GH7 exoglucanase	20	100
Agrocybe aegerita	GH7 exoglucanase	42	89

Symbols

Gene fragments selected to complete the sequence

Domain architecture of the cellulases isolated from *Trametes versicolor* and *Agrocybe aegerita*





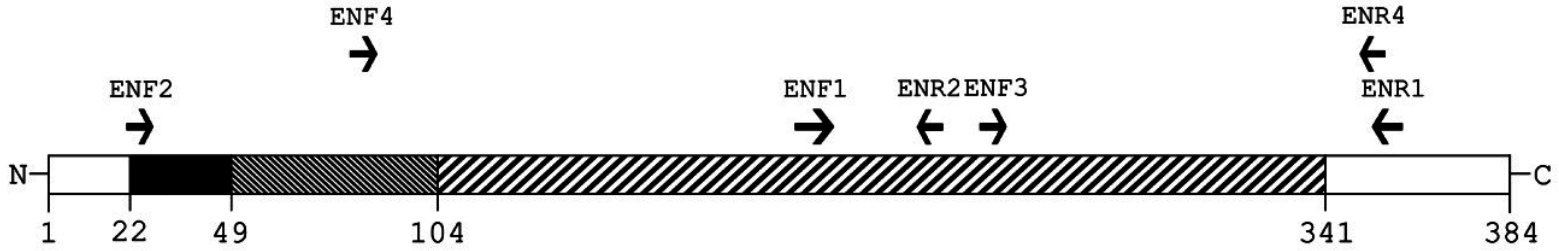
Are these novel cellulases?

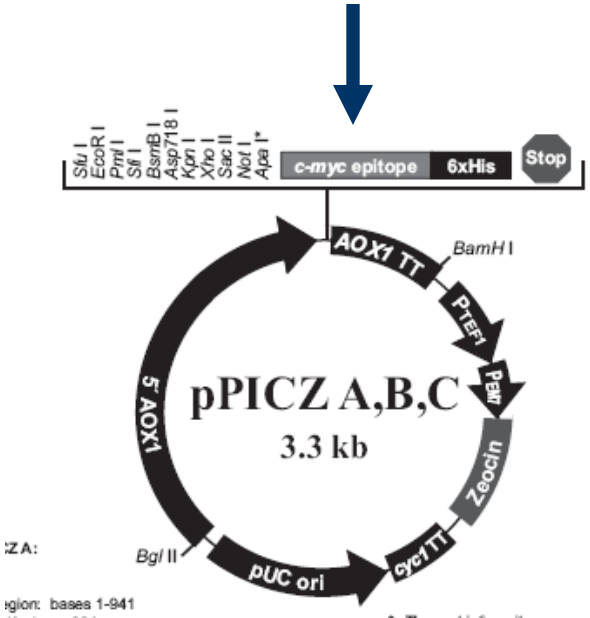
Comparison of WRF cellulases amino acid sequence to known fungal cellulases available in databases.

	Identity (%)			
	T. hirsuta	*T. reesei*	*H. insolens*	*A. niger*
Eng5	83	26	42	44
	T. reesei	*P. chrysosporium*	*T. emersonii*	*P. chrysosporium*
Cel7	57	78	59	78

According the sequence criterium, genes isolated from *T. versicolor* and *A. aegerita* encode for novel cellulases, not described so far.

Gene cloning and recombinant expression of the two novel cellulases





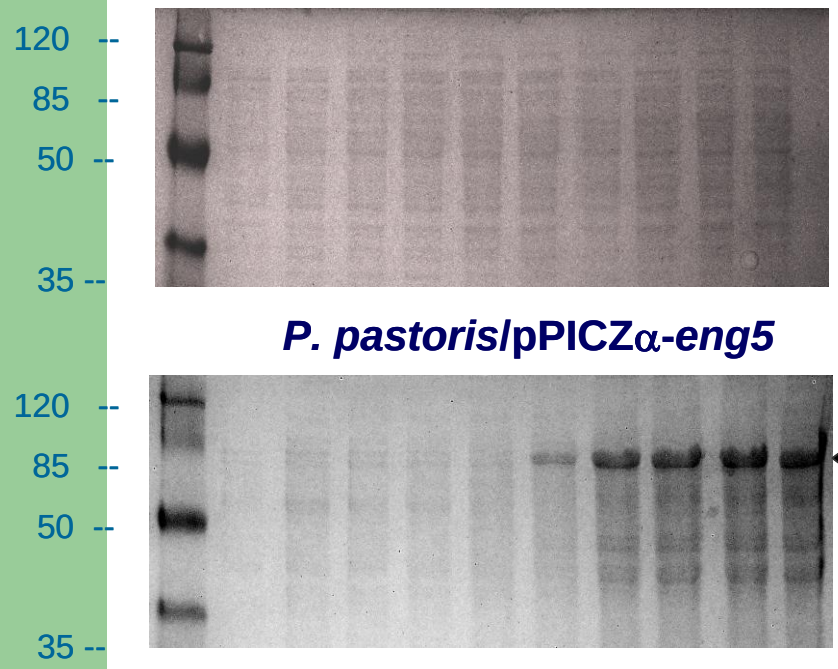
Yeast *Pichia pastoris*

? Cellulases

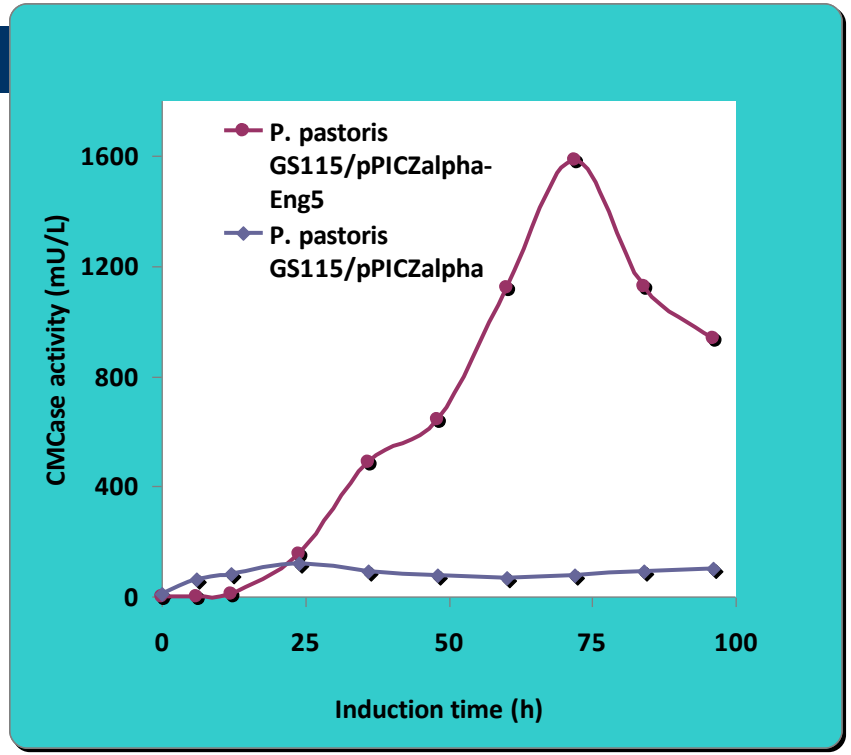
Recombinant expression of Eng5 in the yeast *P. pastoris*

***P. pastoris*/pPICZα (control)**

0 → 96 h



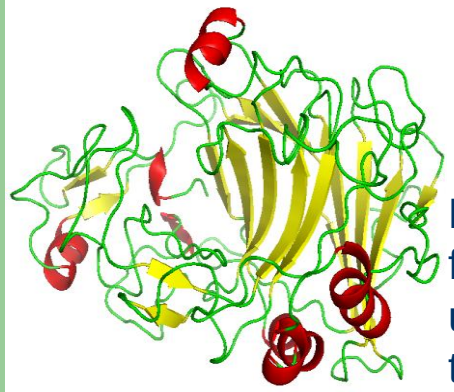
P. pastoris*/pPICZα-*eng5



T. versicolor* Eng5 is expressed as a <u>functional cellulase</u> in *Pichia pastoris

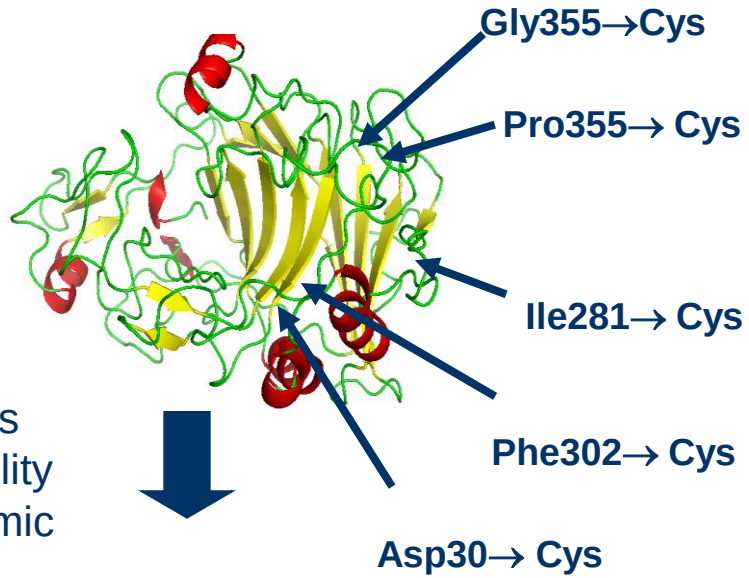
Protein engineering for cel7 thermostabilization

Homology modelling



Model of the three-dimensional structure

Prediction of mutations for higher thermostability using molecular dynamic tools



Experimental validation

Concluding Remarks

- White rot fungi *Trametes versicolor* and *Agrocyte aegerite* produce cellulase activities able of degrading *Nothofagus pumilio* wood chips and soluble cellulose (CMC).
- Under our experimental conditions, values of cellulose to glucose conversion were similar to those exhibited by Celluclast 1.5L, a commercial preparation.
- Sequences encoding for one endoglucanase and one cellobiohydrolase were cloned from *T. versicolor* and *A. aegerita*, respectively. The new cellulase genes share variable sequence identity (55 – 90 %) to cellulases described in literature. To our knowledge these genes have not been previously described in the mentioned fungi or any other fungus
- Sequence alignment showed that the these sequences encode for glycosyl hydrolases belonging to families 5 and 7. One Family 1 carbohydrate binding module was identified in the amino end of Eng5 and the carboxy end of Cel7.
- Eng5 was cloned into the vector pPICZα and transformed into *P. pastoris* GS115, resulting in functional expression of the endoglucanase to the extracellular space.
- **White rot fungi are a promising source of cellulases, con high potential to be used in the bioethanol production process.**

Main Outcomes

Students formation

- 2 undergrade and 5 postgrade students

International meetings:

- II Latin America Congress Biorefineries, May 2009. Concepción, Chile
- The 13 European Congress in Biotechnology, September 2009, Barcelona, Spain.
- 14th International Biotechnology Symposium and Exhibition. Biotechnology for the Sustainability of Human Society. September 2010 Rimini Italy.

Publications:

- Two manuscripts in redaction, containing results about cloning, recombinant expression and characterization of the two novel cellulases
- One manuscript including the thermostabilization of the cellulase cel7.

Acknowledgements

- Undergrade students:
  - Alejandra Guerrero
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  - Iván Gajardo
  - Marcela Vega
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  - Erika Jorquera
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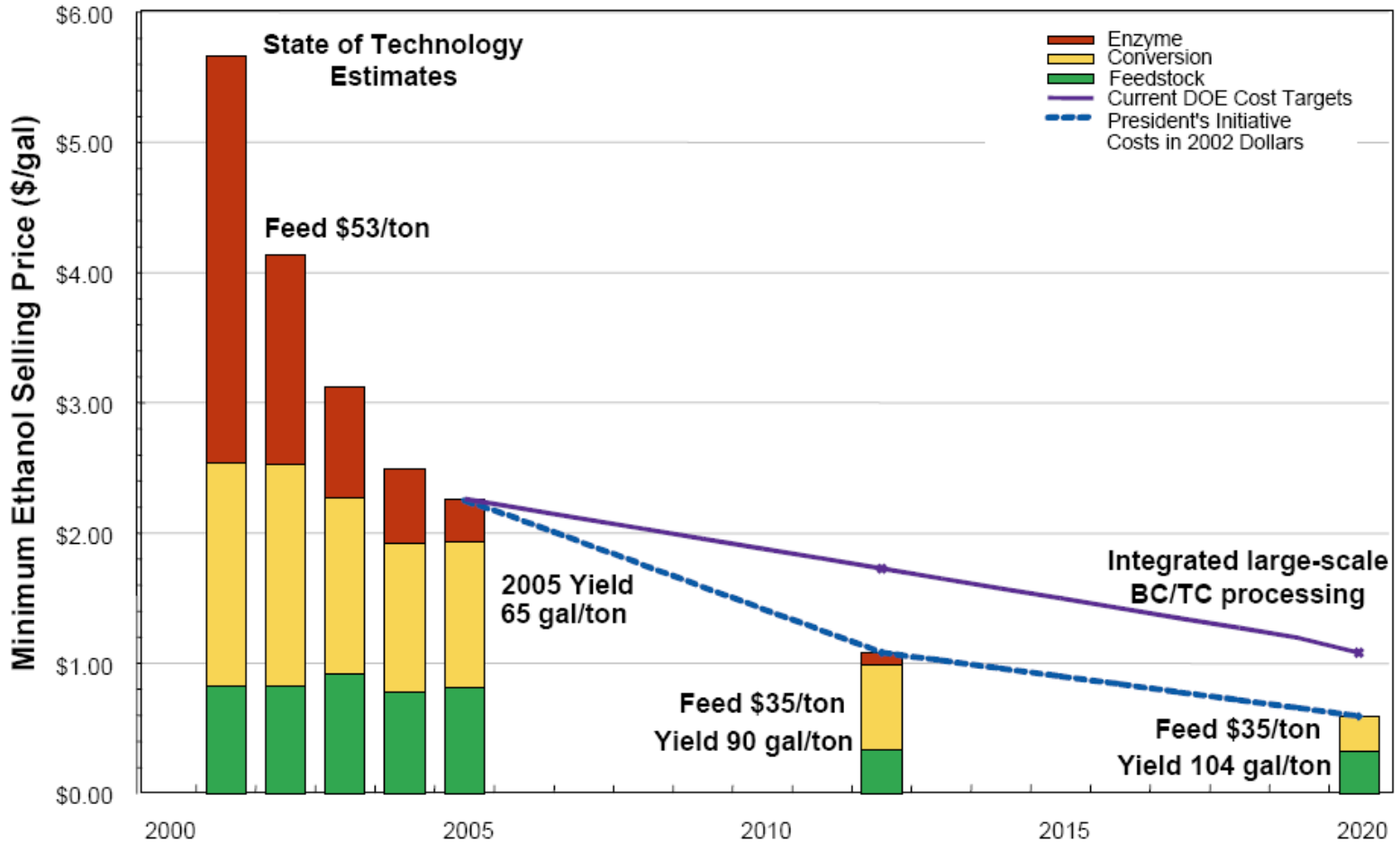
Thanks !


Minimum Ethanol Selling Price ($/gal)
$6.00
$5.00
$4.00
$3.00
$2.00
$1.00
$0.00
2000
2005
2010
2015
2020
State of Technology Estimates
Feed $53/ton
2005 Yield
65 gal/ton
Feed $35/ton
Yield 90 gal/ton
Feed $35/ton
Yield 104 gal/ton
Integrated large-scale BC/TC processing
Enzyme
Conversion
Feedstock
Current DOE Cost Targets
President's Initiative
Costs in 2002 Dollars

Cellulases are grouped in families, depending on their sequences

- Fungal Endoglucanases
  - Family GH5
  - Family GH7
  - Family GH61
- Fungal Exoglucanases
  - Family GH6
  - Family GH7
  - Family GH74

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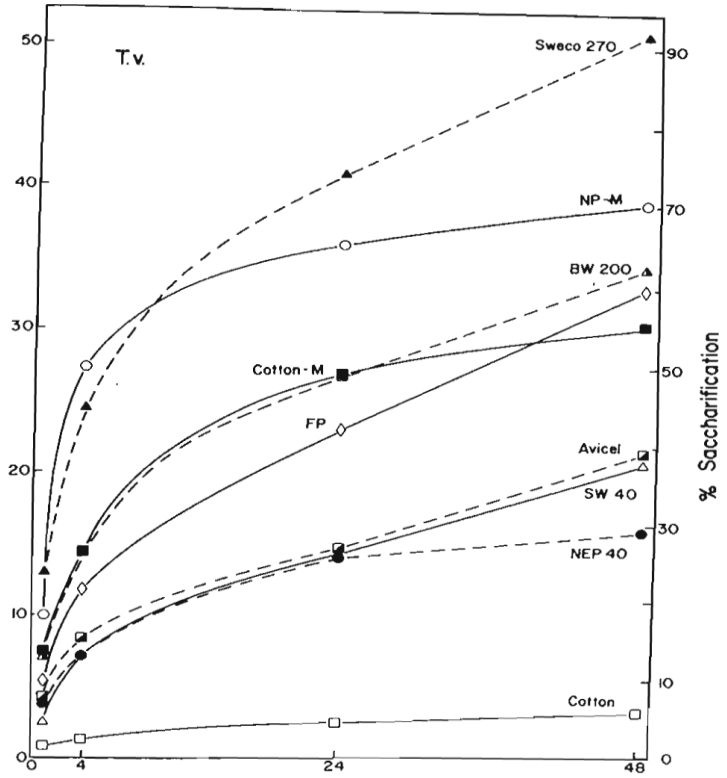
T.v.
Sweco 270
NP-M
BW 200
Cotton-M
FP
Avicel
SW 40
NEP 40
Cotton
% Saccharification
0
10
20
30
40
50
0
10
30
50
70
90
0
4
24
48

Enzymes

- Functional proteins; catalyse biochemical reactions
- Primarily made up of chains of amino acid linked togheter by peptide bonds.
- Found in all living organisms
- Work under mild conditions
- Replace harsh chemicals such as strong acids
- Biological degradable
- A “clean” technology