

# Dyadic Netherlands

## Efficient Saccharification of Lignocellulosic Feedstocks Using the C1-Technology Platform

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Science Director



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# Dyadic Netherlands



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- Dyadic Nederland BV, Wageningen, The Netherlands
- [www.dyadic.nl](http://www.dyadic.nl)

## Focus

- Discovery and development of enzymes for the bioenergy, food/feed, and paper and pulp industries.

## Tools

- Fungal Genomics
- Molecular biology
- Enzymology
- Fermentation technology



C1: developed into an efficient protein production system

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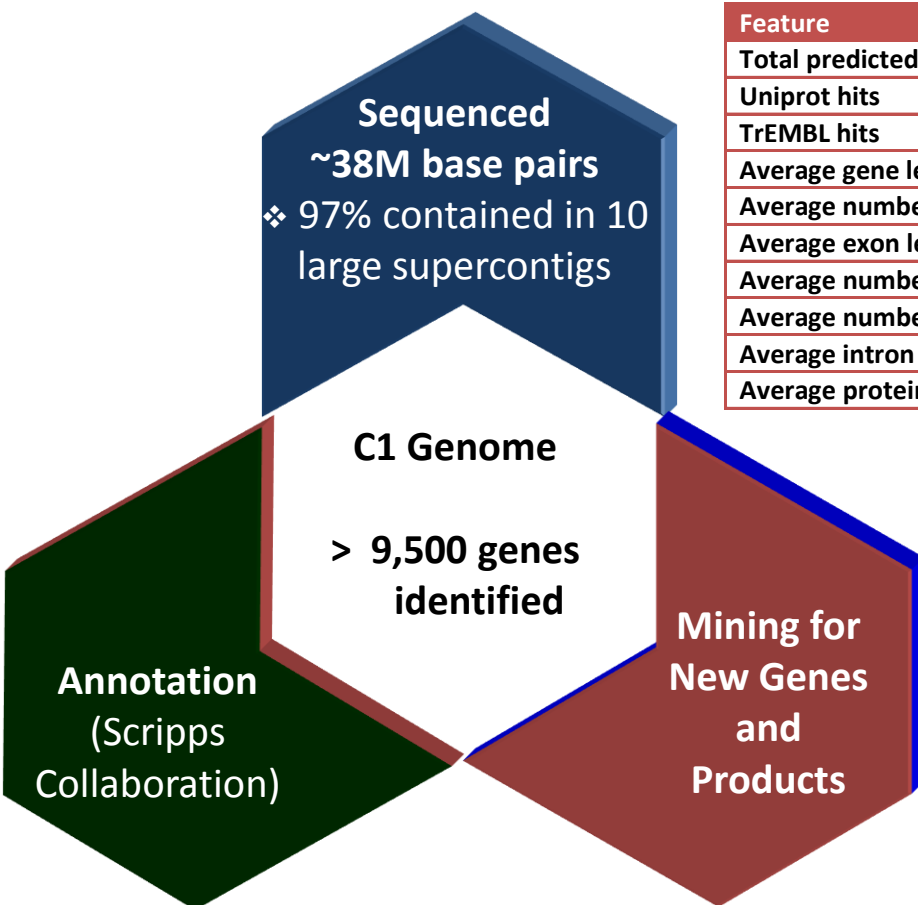


***Myceliophthora thermophila C1*** (formerly *Chrysosporium lucknowense C1*)

- Isolated from soil in eastern Russia as producer of neutral cellulases.
- Developed into a proprietary mature enzyme production system.
- Main features:
  - Low viscosity, fermentation to very high densities
  - High production levels (up to 100 g/L protein), easy scaling
  - Versatile genetic tools and hosts developed
  - Genome sequenced and annotated
- Self affirmed GRAS status of a C1-cellulase product was acknowledged by the FDA (2009)



# Exploration of the C1-enzymatic potential by genomics



Feature	Genemark	Geneid	Glimmer
Total predicted genes	9807	16012	8545
Uniprot hits	3151	4234	3831
TrEMBL hits	5007	3813	2856
Average gene length <sup>1</sup> (bp)	2223	2271	2313
Average number of exons per gene <sup>1</sup>	3.47	3.66	3.70
Average exon length <sup>1</sup> (bp)	549	499	503
Average number of introns per gene <sup>1</sup>	2.79	3.10	2.91
Average number of introns per gene (incl. 0) <sup>1</sup>	2.47	2.66	2.70
Average intron length <sup>1</sup> (bp)	130	169	169
Average protein length <sup>1</sup> (aa)	636	609	621

A large number of genes putatively encoding **industrially important** enzymes discovered:

- ~**250** Carbohydrate-active Enzymes (CAZy)
- ~**150** proteases
- ~**700** oxido-reductases
- ~**75** lipases / esterases.

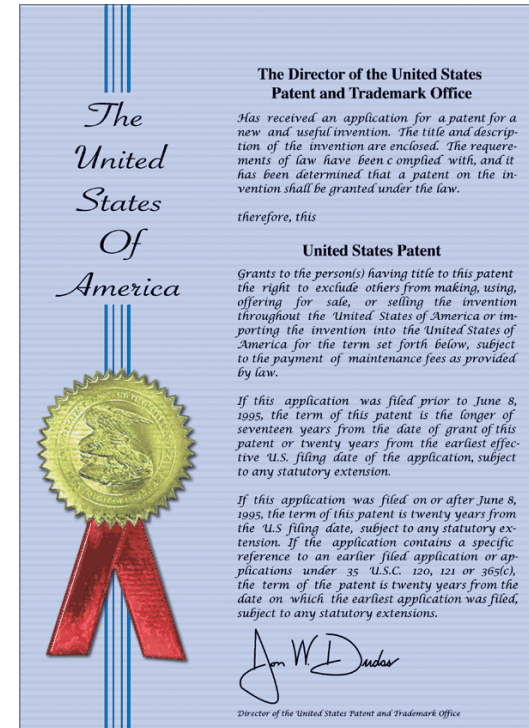


Dyadic fully owns the C1 fungus and technology:

- 10 US Patents granted
- 10 US Patent applications pending
- 74 Foreign patents issued
- 23 Foreign patents pending

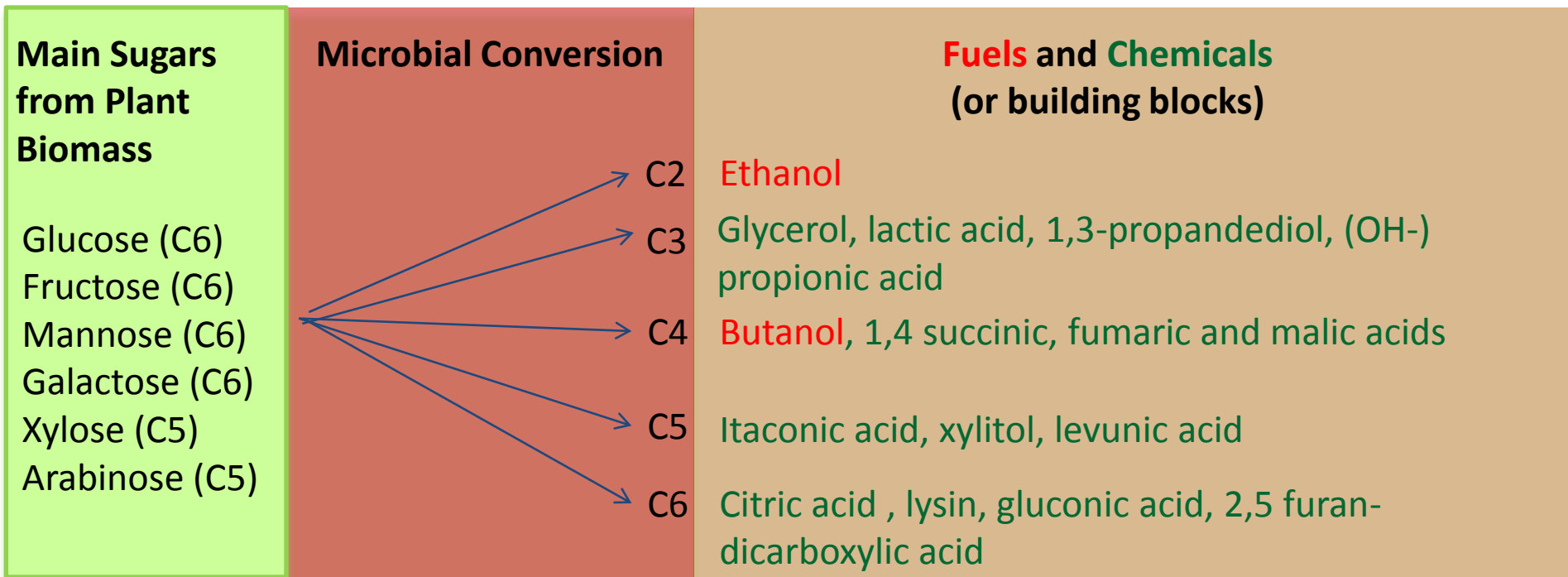


Large freedom to operate



# Biobased fuels and chemicals

Replacing chemicals now derived from fossil oil with **sugar-based** fuels and chemicals



**First generation:** sugars from **edible** parts of plants (starch, sucrose)  
**Second generation:** sugars from **non-edible** parts of plant (ligno-cellulose)



# Chain involved in 2nd generation processes

Integration of different processes is imperative

## 1. Thermo/Chemical Pretreatment

**Biomass**  
 --Corn stover  
 --Wheat straw  
 --Bagasse  
 --Energy crops



## 2. Enzyme treatment

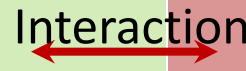
**Dyadic**

Liberate C6 and C5 sugars from (hemi-) cellulose fibers

## 3. Microbial conversion

**Fuels and Chemicals**

--Ethanol  
 --Butanol  
 --Chemicals (Building blocks)



# Matching Enzyme Activity to Microbial Conversion (SSF conditions)

Examples of organisms producing relevant fuels and chemicals

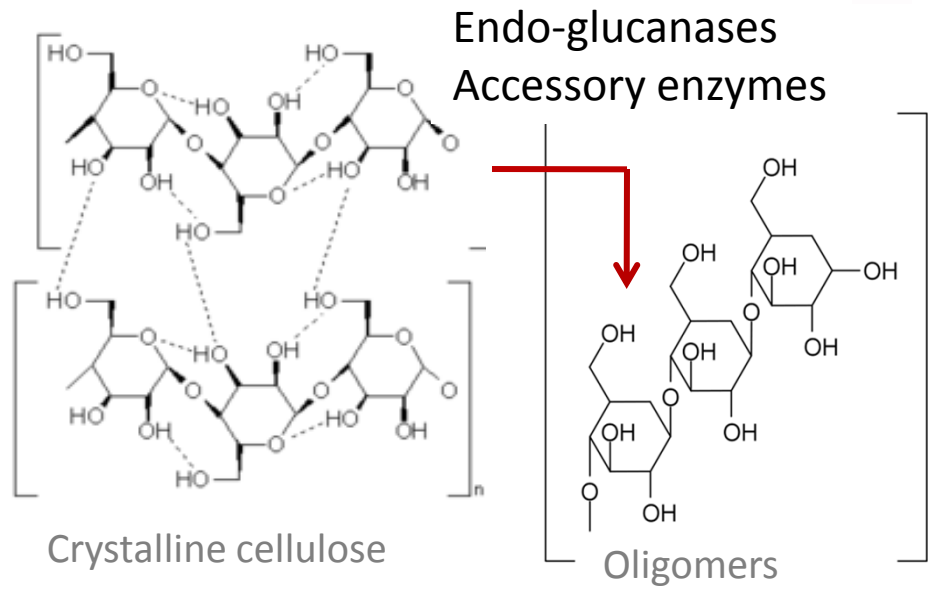
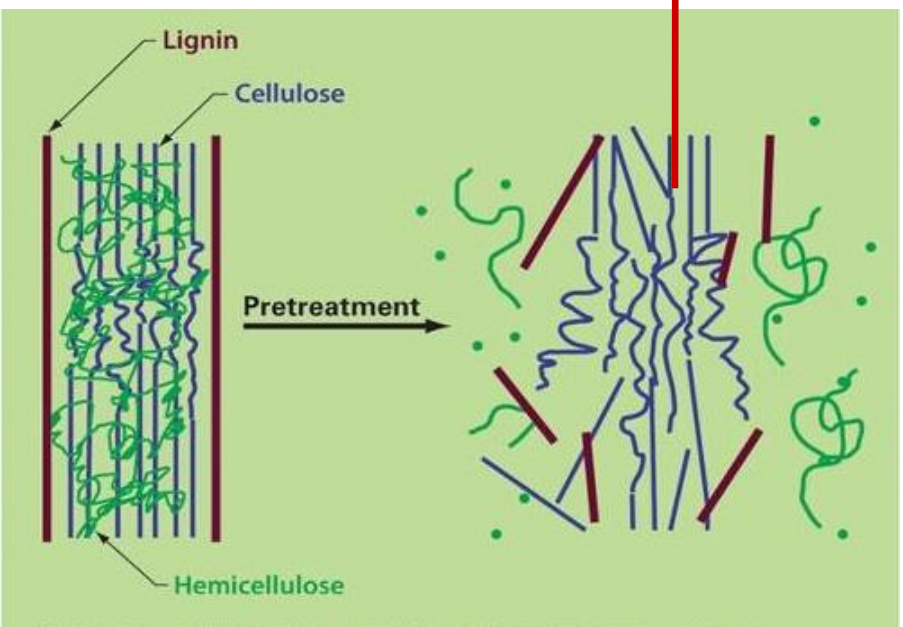
Fuel/chemical	Micro-organism	T (°C)	pH-range	Selection of Companies
Ethanol	Yeast	32-37	4-5	Nedalco, DSM, Mascoma (CBP)
Ethanol	<i>Z. mobilis</i>	30	7	Dupont/Genencor
Ethanol	<i>E. coli</i>	37	6-7	
Ethanol	<i>T. saccharolyticum</i>	50-60	5-6	Mascoma
Ethanol	<i>T. mathranii</i>	50-80	6.5-7.5	Biogasol
Ethanol	<i>C. phytofermentans</i>	35	6 - 9	Qteros
Butanol	<i>C. acetobutylicum</i> , <i>E.coli</i> , yeast	30-37	4-7	BP/Dupont, Butalco, Gevo, Tetravitae
1,3-Propanediol	<i>E. coli</i>	37	6-7	Dupont/Genencor
Succinic acid	<i>E. coli</i> and yeast	30-37	3-7	DSM/Roquette, Myriant
Fatty acids (diesel)	<i>E. coli</i>	37	6-7	LS9/JBEI (DOE)
Farnesene (building blocks, biodiesel)	Yeast	32-37	4-5	Amyris
Isoprene (building block chemical)	<i>E. coli</i>	37	6-7	Genencor/Goodyear
Lactic acid	Bacteria and Fungi	30-60	4.5-6.5	ADM, Purac, Myriant, Galactic
DHA, Fuel precursors, Lubricants	Algae	25-30	7-9	Martek biosciences, Solazyme



# General picture: enzymes for plant biomass saccharification



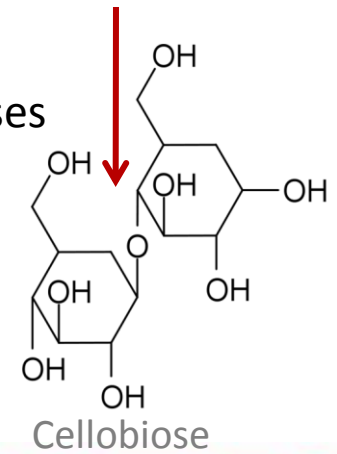
## Cellulases



Crystalline cellulose

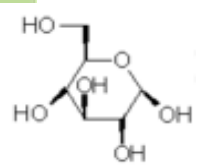
Oligomers

Exo-gluconases/  
cellobiohydrolases



Cellobiose

$\beta$ -glucosidase



Glucose

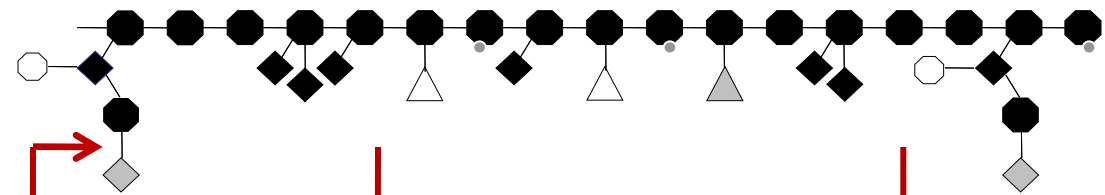
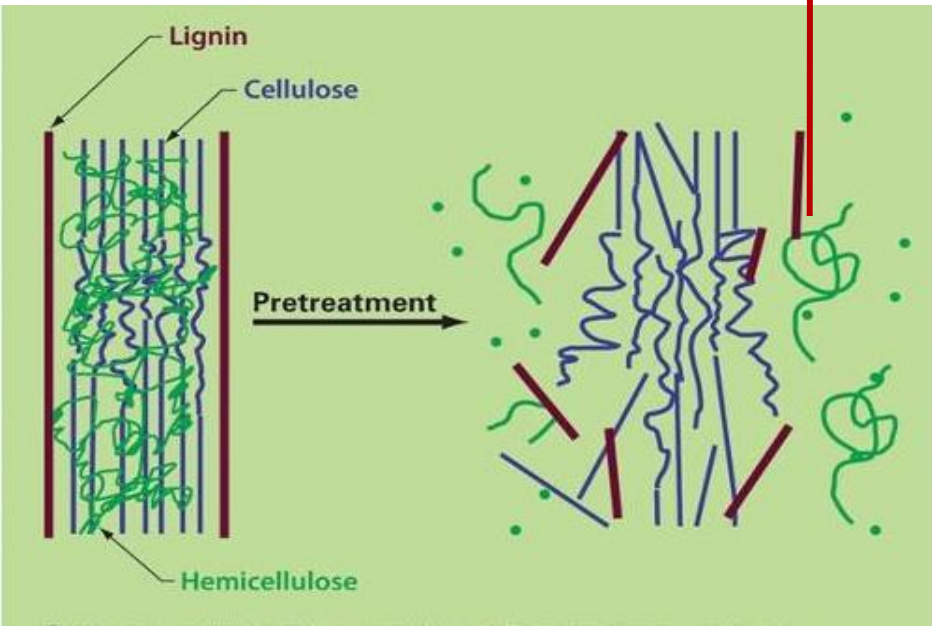
Fuels  
Chemicals

Fermentation

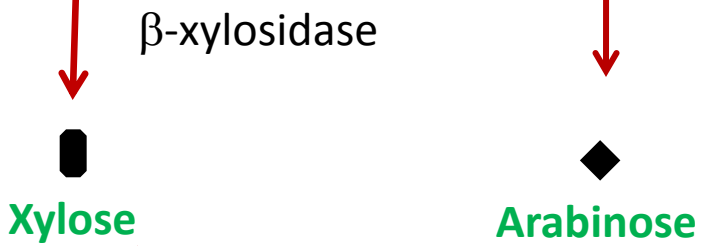
our enzymes; nature at work

# General picture: enzymes for plant biomass saccharification

## Hemi-cellulases



- Endo-/exo xylanases
- Arabinofuranosidases
- Esterases



**Xylose**      **Arabinose**

Fermentation

**Fuels**  
**Chemicals**

- = xylose
- ◆ = arabinose
- ▲ = glucuronic acid
- = ferulic acid
- = acetyl group



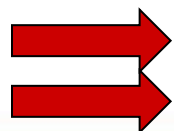
# Putative (Hemi-) cellulase genes in the C1 genome

Comparison of the (hemi-)cellulolytic potential of C1 and *Trichoderma reesei*  
 (currently the main industrial source for second generation biofuel enzymes)

Genes encoding	Number in C1	Number in <i>T. reesei</i> *	Biomass Fiber
Endo-glucanases, Cellobiohydrolases, $\beta$ -glucosidases	~ 20	~ 11	Cellulose
Cellulose binding domains (CBM1-type)	~ 46	~11#	
Xylanases/Xylosidases	~ 18	~ 6	Hemi-cellulose
Arabinofuranosidases/arabinases	~ 8	~ 5	
Esterases (Axe, Fae)	~ 18	~ 4#	
Cellulase boosters (GH61)	~ 26	~ 3	Cellulose/Hemi-cellulose

\*From Martinez et al. (2008) *Nature Biotechnol.* 26:553-560 and Foreman et al. (2003) *J. Biol. Chem.* 278: 31988–31997

# Based on literature and JGI database searches



C1 is a rich source of genes encoding lignocellulolytic enzymes!

But, these are not necessarily expressed under applied growth conditions

our enzymes; nature at work



# Many putative “cellulase-boosters” in the C1 genome

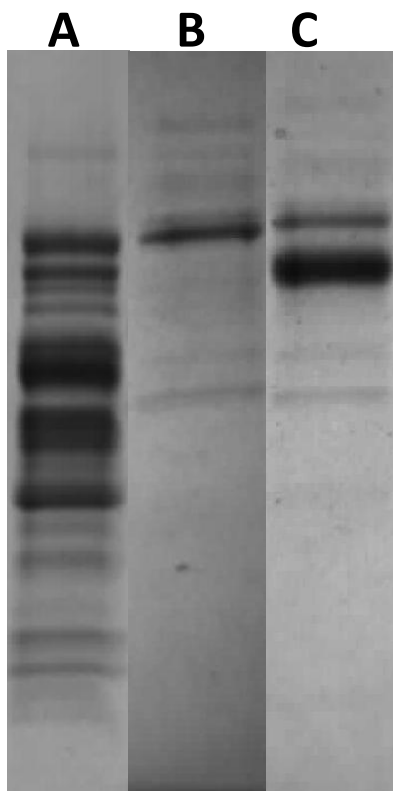
- General observations regarding GH61-proteins
  - Enhancers of activity of cellulases mixtures<sup>1</sup>
  - Very weak cellulase activity by themselves<sup>1</sup>
  - Contain binding site for divalent metal ions and dependent on them<sup>1</sup>
  - Oxidative degradation of crystalline polymers shown for structurally related CBM33 proteins<sup>2</sup>
  - GH61 enzymes may create knicks in most inaccessible parts of crystalline polymers and disrupt crystalline packing<sup>2</sup>
- Abundance of different GH61 proteins in C1
  - Powerfull auxiliary mechanism of degradation of different polymers?
  - Genomic co-location with cellulases, pectinases, hemicellulases observed.
  - Assessment of boosting effect in lignocellulose saccharification ongoing.

<sup>1</sup> Harris et al. (2010) Biochemistry 49:3305-3316

<sup>2</sup> Vaaje-Kolstad (2010) Science 330:219-222



# Characterization of all individual (hemi-)cellulosic enzymes



← Individual target enzyme

- (A) Baseline C1-strain:  
 High cellulolytic activities  
 Diverse enzyme mixture  
 Up to 100 g/L total protein
- (B) Low background C1 Strain:  
 Almost no cellulolytic activities  
 Very few endogenous secreted  
 Suited for enzyme characterization
- (C) Up to 80% target protein  
 Up to 30 g/L of target protein achieved

Establish a characterized enzyme library



# Conclusions individual (hemi-) cellulases

- Collection of about 70 functional single (hemi-) cellulase expressing strains has been obtained.
- Detailed biochemical and mechanistic studies performed<sup>1,2</sup>.
  - Endo/exo-glucanases, Cellobiohydrolases,  $\beta$ -glucosidases, endo/exo-xylanases, acetyl-esterases, ferulic acid esterases, arabinofuranosidases,  $\beta$ -xylosidases.
- Great diversity of activities discovered, important for synergy and versatility.
- Ongoing activity in collaborative projects.

 Use this information to develop efficient (hemi-) cellulosic enzyme mixtures

<sup>1</sup> Hinz, S.W.A., Pouvreau, L., Joosten, R., Bartels, J., Jonathan, M.C., Wery, J., Schols, H.A. (2009). J. Cereal Sci., 50: 318-323.

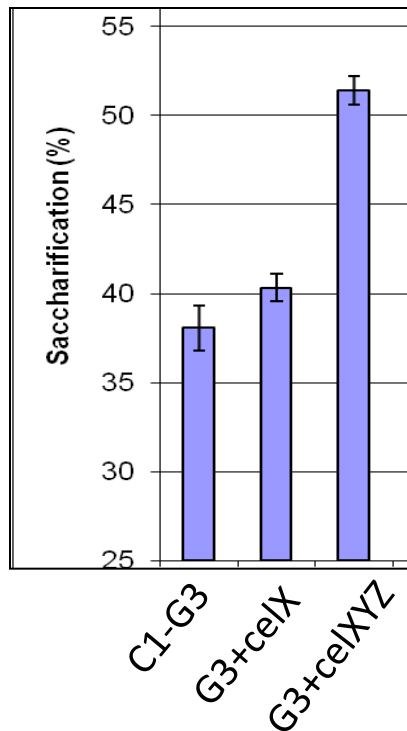
<sup>2</sup> Kühnel, S., Hinz, S.W.A., Pouvreau, L., Wery, J., Schols, H.A. Gruppen, H. (2010) J. Biores. Technol., 101:8300-8307



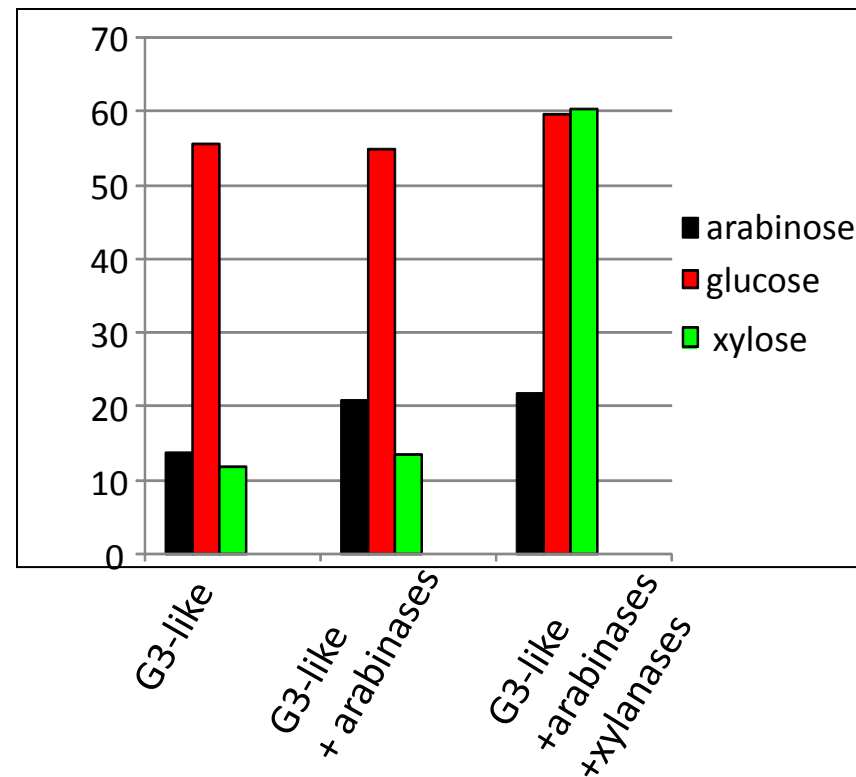
# Aim: Construct one strain producing the optimal (hemi-) cellulase mixture

Approach: pinpoint limitations in C1-mixtures by mixing with library enzymes.

Pretreated corn stover:  
Mainly cellulose



Mildly pretreated wheat bran:  
Cellulose + hemicellulose



➔ Strains developed producing efficient cellulosic and hemicellulosic enzyme mixtures



# Enzyme mixtures for ligno-cellulosic substrates

Pretreated  
wheat straw



Pretreated  
wheat straw (20% DM w/w)



C1-enzyme  
mixture

Fast liquefaction

Hydrolysed  
wheat straw



Cellulose: 50-55%  
Hemicellulose: 5%

Insoluble cellulose ( $\approx 100\text{g/kg}$ )

Glucose ( $\approx 100\text{g/kg}$ )



# Experimental C1-biofuel enzymes under industrial conditions

## In specially designed reactors:

- High biomass loadings possible (>20% DM)
- Controlled pH, Temperatures
- Combined saccharification and yeast fermentation (SHF/SSF) possible
- Online ethanol measurements

0.5h



1h



2h



5h



48h



20% solids  
100 g/L glucan



Fast viscosity reduction and formation of glucose

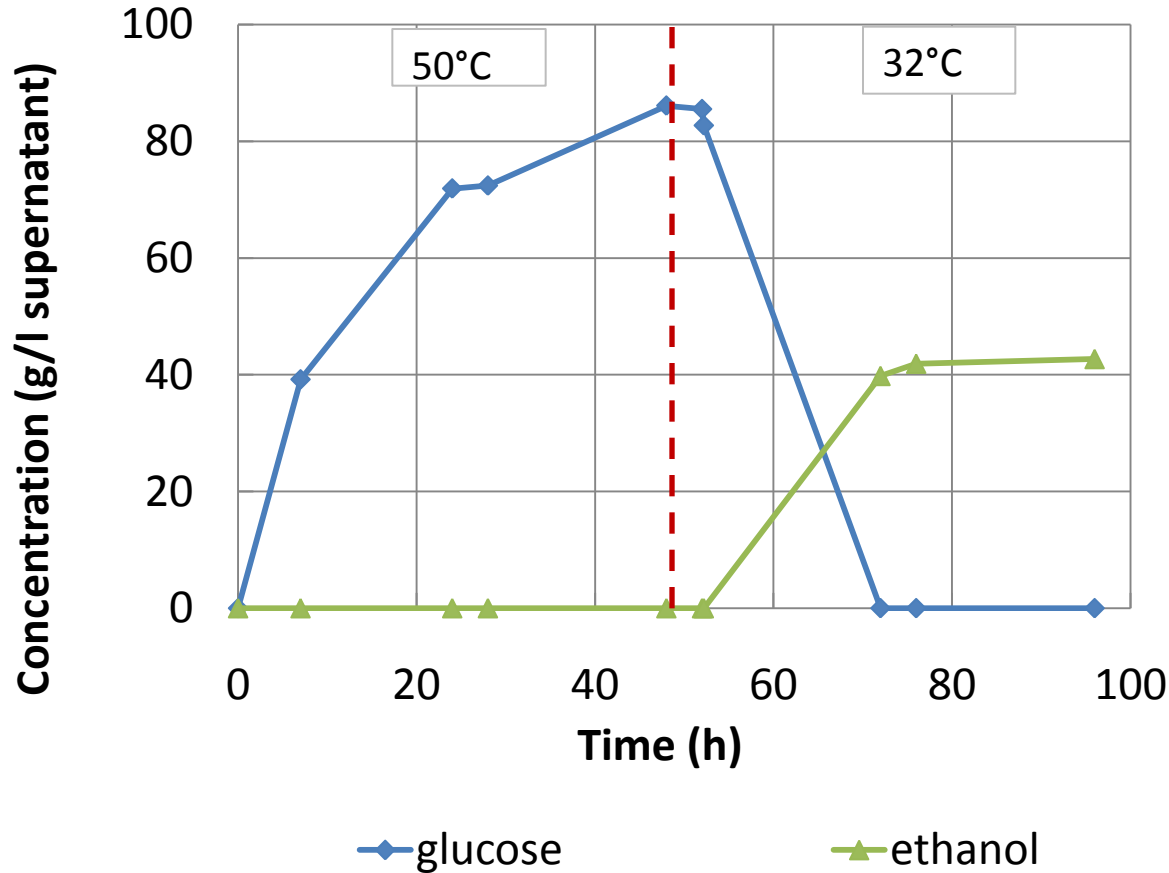
~ 90 g/L glucose

our enzymes; nature at work



# Hybrid saccharification and fermentation by G3

Example: dilute acid pre-treated wheat straw, 20% DM

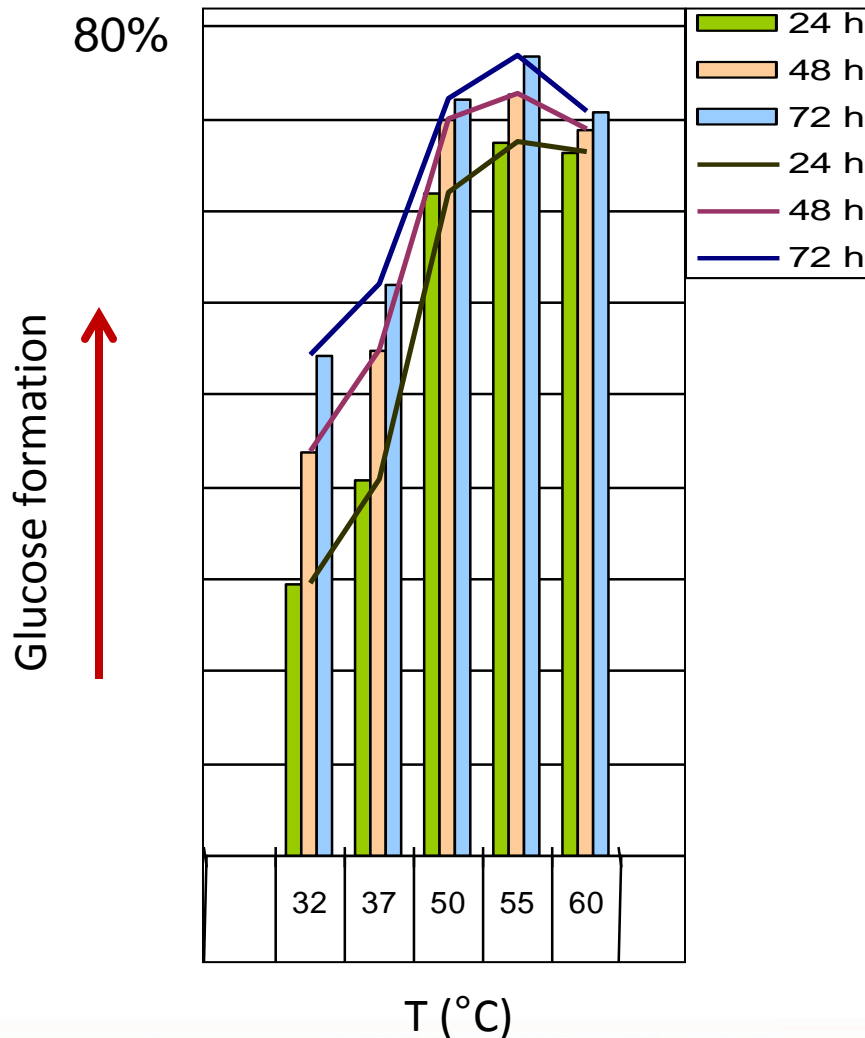


## Conclusions:

- Efficient conversion of glucan to ethanol enabled by G3 (80%) in 3 days.
- Crude fungal fermentation broth leads to efficient ethanol production

# C1-biofuel enzymes show a broad active temperature range

Dilute acid pre-treated corn stover, 10% DM



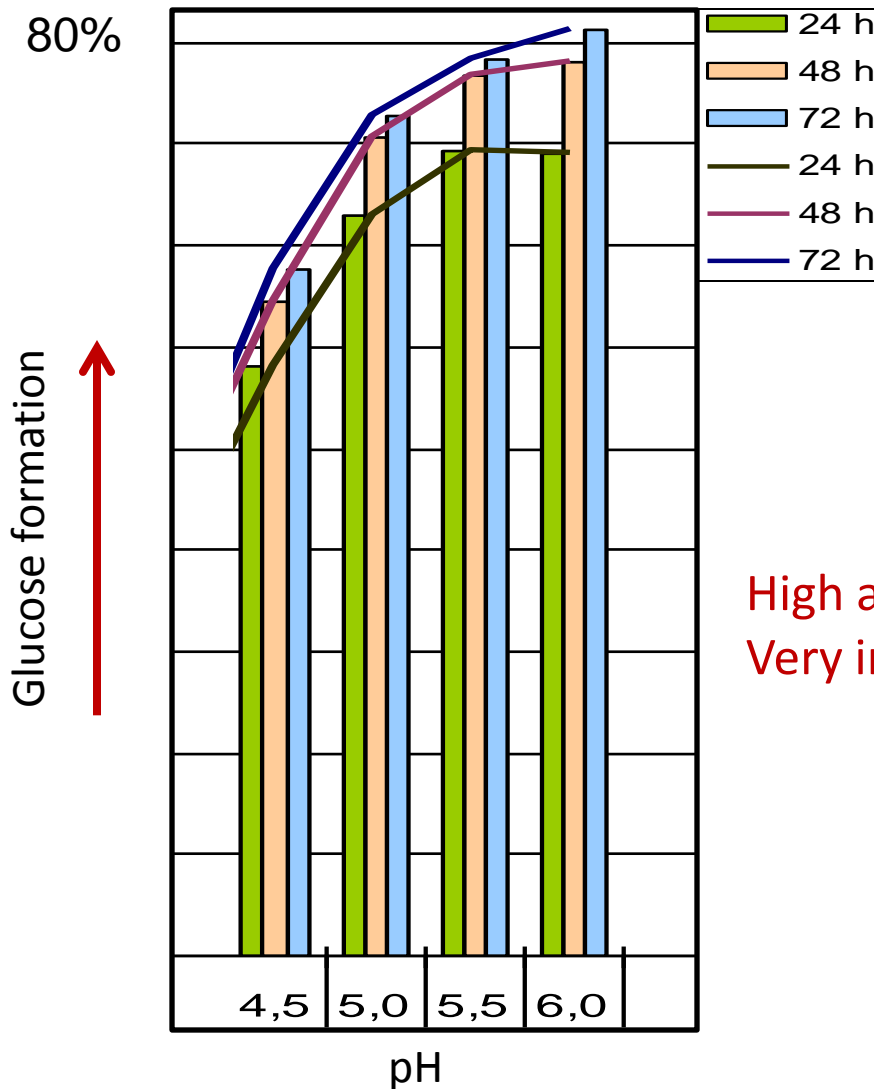
Ability to add enzyme in early stage after thermo-pretreatment.

Advantages:

- Less processing time
- Lower viscosity of biomass at high temperature



# C1-biofuel enzymes show a broad active pH range

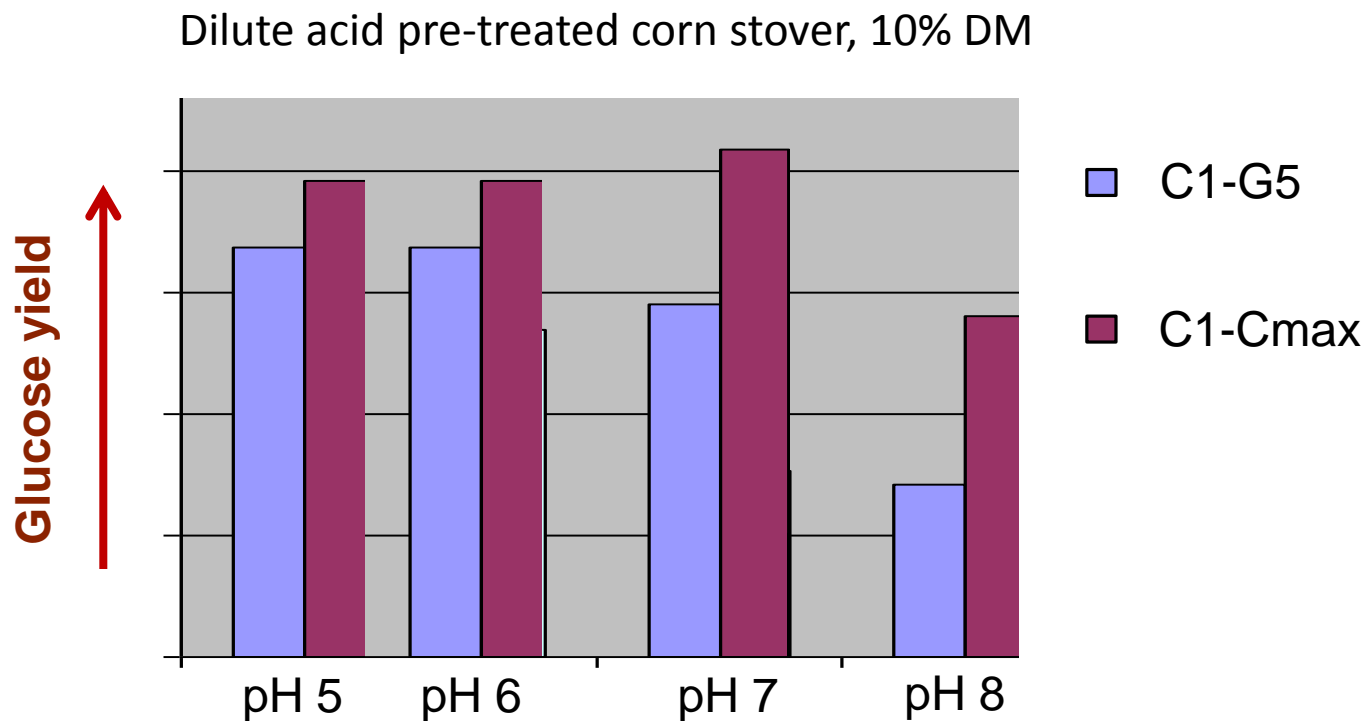


High activity at higher pH's:  
 Very important for neutral (bacterial) processes



# C1-biofuel enzymes show a broad active pH range

Testing the upper limits of relevant SSF pH's: pH 5-8



➔ Activity under conditions where other fungal enzyme mixtures no longer work



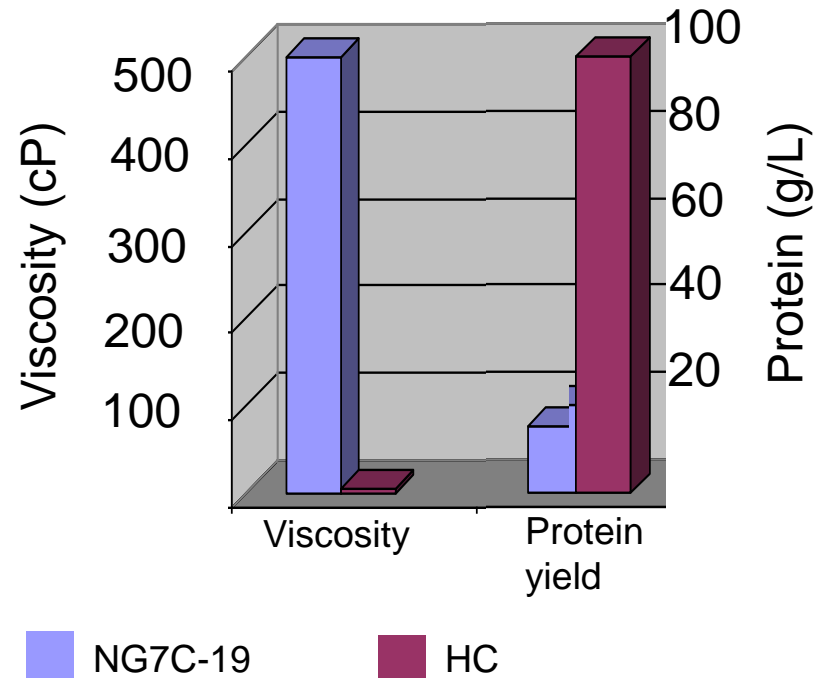
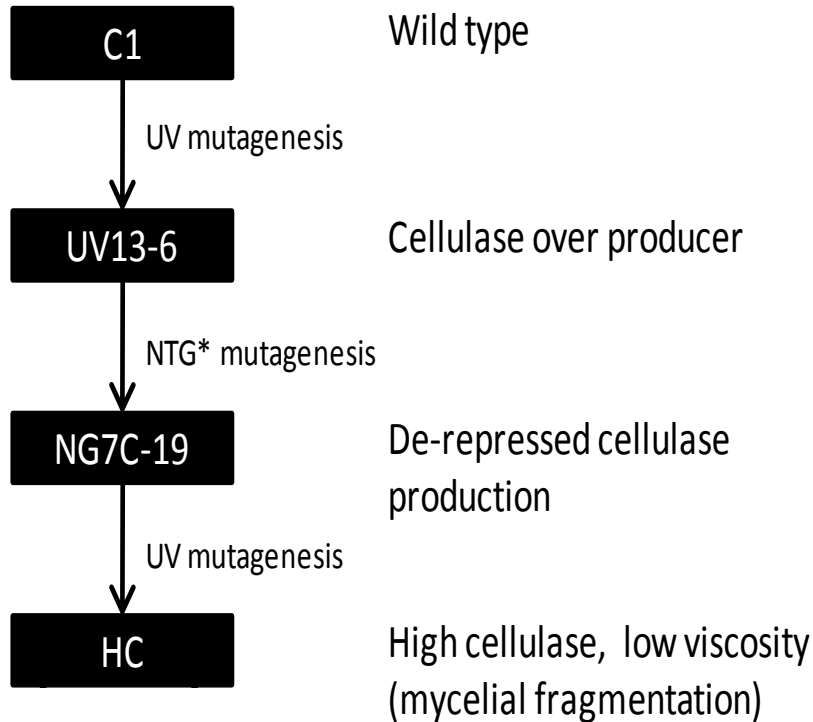
## General observations

- **Fast saccharification:** Ethanol process completed in 72h.
- **Broad temperature range:** Active between 32°C and 65°C.
- **Broad pH-range:** High activity between pH 4.5 and 8.
- **Active on a variety of biomass substrates:** Corn Stover, Wheat Straw, Wheat bran, Sugar Cane Bagasse, Switch Grass, Sorghum, Wood and Paper Waste



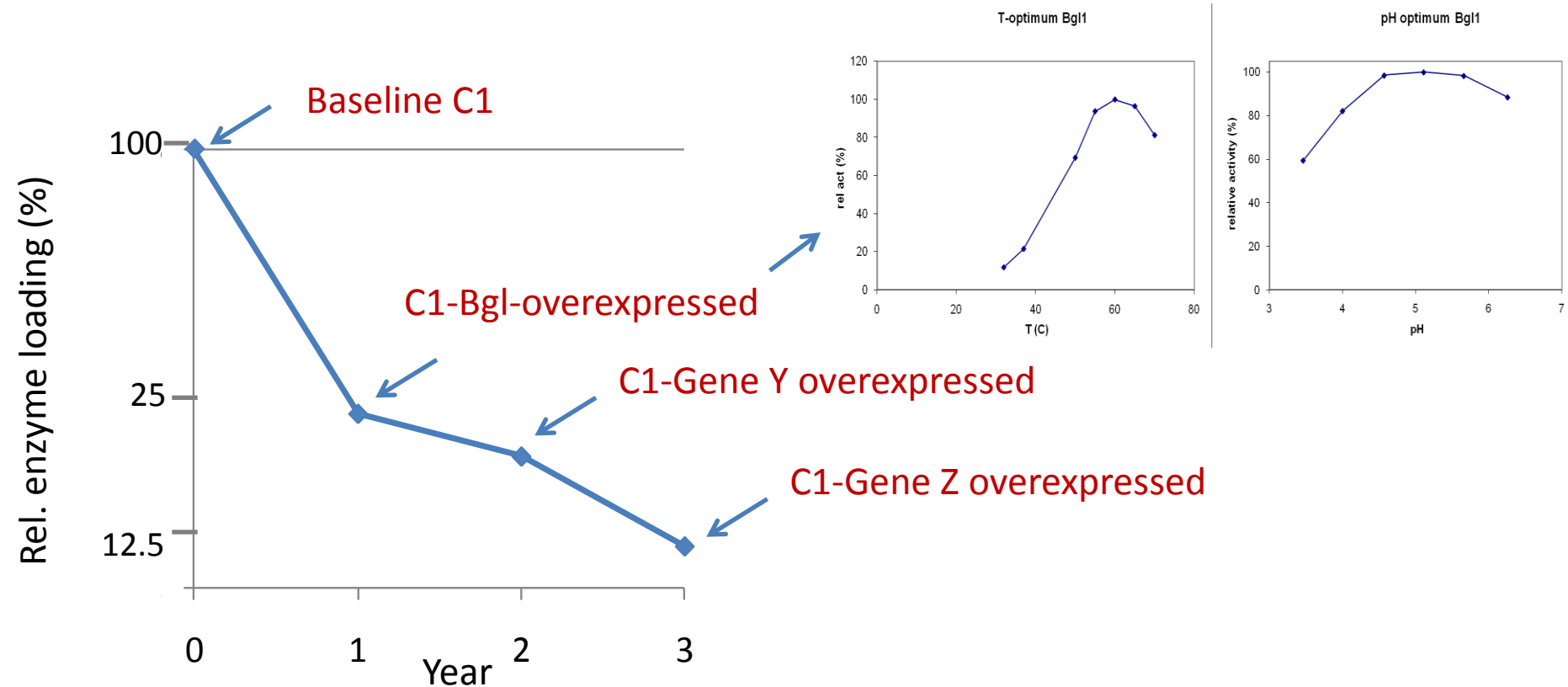
# Enzyme productivity and enzyme loading determine cost in use

## Enhancement of enzyme productivity in C1



# Enzyme productivity and enzyme loading determine cost in use

Reductions in enzyme loading obtained within 3 years in one strain lineage\*



Large reductions obtained, BUT ligno-cellulolytic potential C1 by far not exploited to the fullest.

\* General trend combining results acid pretreated corn stover and wheat straw from different experiments. Confirmation in a single comparative experiment in progress.



# Take home message

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- The variety of single (hemi-)cellulase enzymes in C1 is much higher than in the traditional *Trichoderma* host.
  - Better position to develop tailor-made enzyme mixtures for a variety of second generation feedstocks.
- Broad operating conditions allow applications in different process set-ups.
  - Active at conditions where competitors' enzymes no longer work
- C1-strains have been developed that already produce very efficient and versatile (hemi-) cellulosic enzyme mixtures.
- Several leads have been obtained towards immediate further optimization.
- Excellent outlook for additional significant enzyme cost reductions at the short term.





# Acknowledgements

## Academic partners:



## Team Dyadic Inc. Biofuels lab:



## Partnering Industries:



## European Union funded projects



our enzymes; nature at work

