Novel Highly Productive Production System for Biotherapeutics: Filamentous Fungus *Myceliophpthora thermophila* C1

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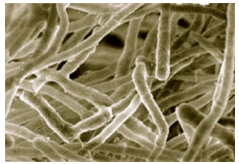
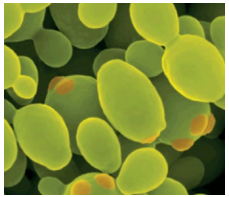
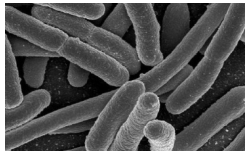
VTT Cell Factories for Recombinant Protein Production



Trichoderma reesei



Pichia pastoris

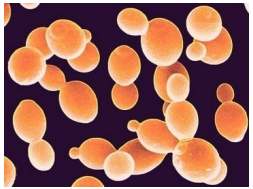


Escherichia coli

Host systems

- Prokaryotic
  - *Escherichia coli*
- Yeasts
  - *Saccharomyces cerevisiae*
  - *Pichia pastoris*
- Filamentous fungus
  - ***Trichoderma reesei***
  - ***Myceliophthora thermophila***
- Higher eukaryotes
  - Plants and plant cell cultures

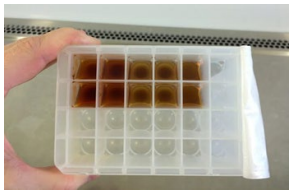
Nicotiana benthamiana

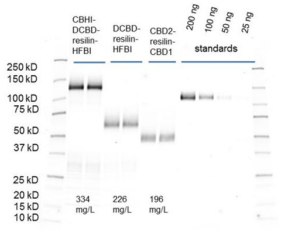


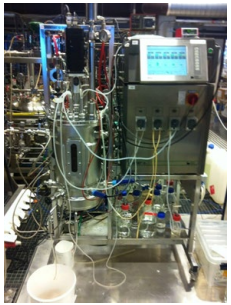
Saccharomyces cerevisiae

From gene to protein

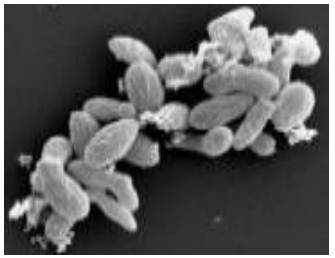
We can provide the customer with complete tailor-made service for producing a protein of interest.





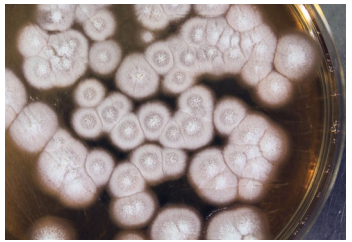


Myceliophthora thermophila *(Thermotelomyces heterothallica)*



- Thermophilic fungus originally isolated from alkaline soil in Russia
- Designated earlier as *Chrysosporium lucknowense* and currently as *Myceliophthora thermophila*
- Isolated for its ability to produce neutral/alkaline cellulases for use in textile applications
- Dyadic Announced Successful Completion of Sequencing of C1 Fungal Genome (May/2005)
- Genome sequenced 2011 by JGI (Nat. Biotechnol. 2011 Oct 2;29(10):922-7)

- **Dyadic International Inc. has developed an industrially proven expression system based on the fungus *Myceliophthora thermophila*, C1 Expression System**
  - Improved production strains with unique morphology
  - C1 received a Generally Recognized As Safe (GRAS) designation from the FDA, with no viruses detected, no mycoplasmas, and no detectable mycotoxins (FDA, 2009)
  - Highest production level of enzymes >120 g/L
  - Highest production level of an individual recombinant enzyme 80 g/L



Our Goal

To further develop C1 into efficient gene expression system of **biologic vaccines and drugs**, to help speed up the development, lower production costs and improve the performance at flexible commercial scales.

Efficient Expression

- Library of promoters, carrier proteins, signal sequences and terminators
- Synthetic Expression System (SES)
- Dual vectors
- Split-marker technology
- Marker recycling
- Site-specific or random integration

Reducing Proteolytic Activity

- Identification of key proteases
- Deletion of protease genes
- Characterization and utilization of protease deletion strains

Glycoengineering

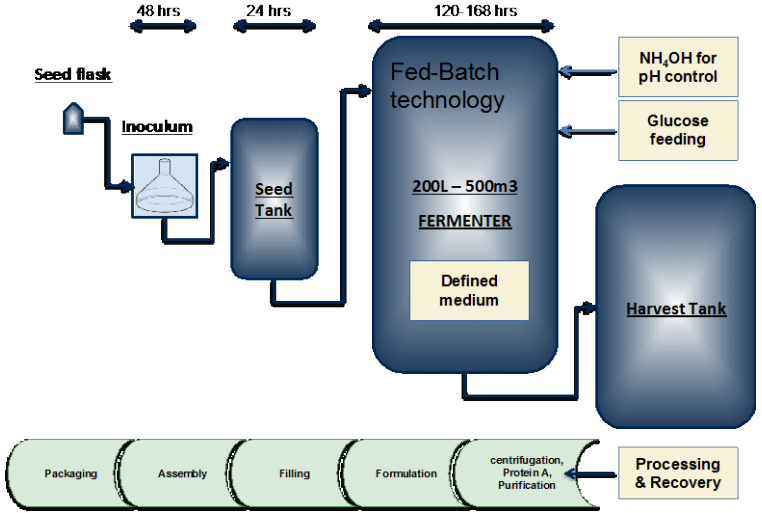
- Generation of humanized protein-glycan structures
- Engineering a G0-glycan producing C1

C1 Fermentation Technology

Fed-batch Process

- Easily available defined media components – glucose, salts, micro and macro elements, AA and vitamins
- Fed-batch technology with glucose feeding
- Low viscosity culture due to morphology changes (propagule)

- No need for induction
- Protein is secreted to the media
- 20-30 % biomass
- pH: 5-8, Temp: 20 - 45°C
- 1L to 500,000 L fermentation scale

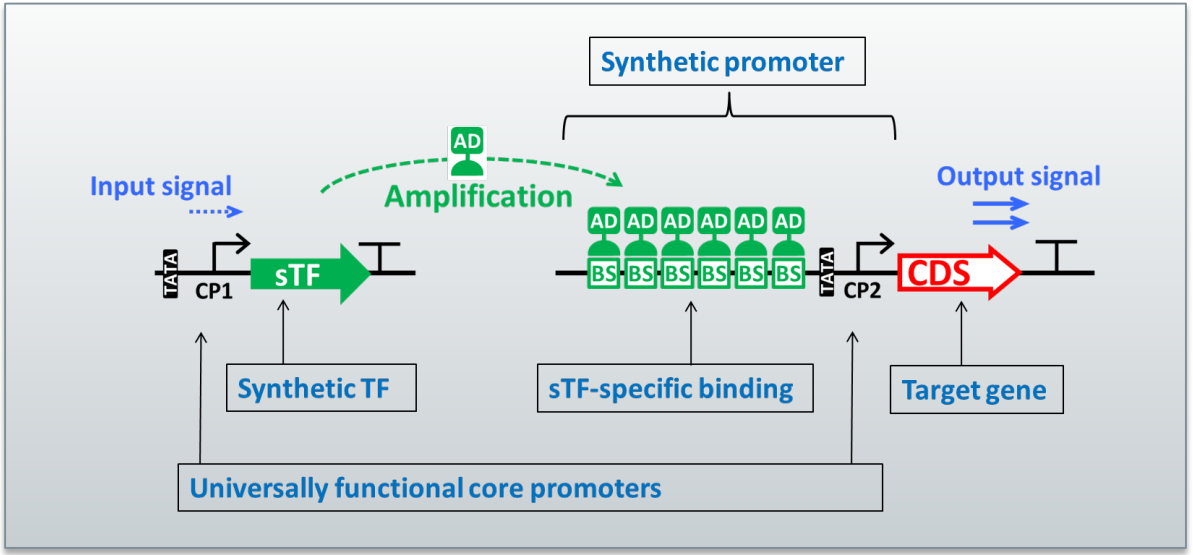


From MTP to Large scale; productivity of mAbs (example)

24-wells MTP – 1 mg/4ml
1L fermentor – 1.7 g/l/d
30L fermentor – 2.4 g/l/d

VTT Synthetic Expression System (SES)

- Constitutive and extremely high-level expression permits use of any production conditions
- Expression level is tunable by changing the number of sTF binding sites
- Has been shown to function in multiple organisms: yeasts, filamentous fungi, plants and mammalian cells



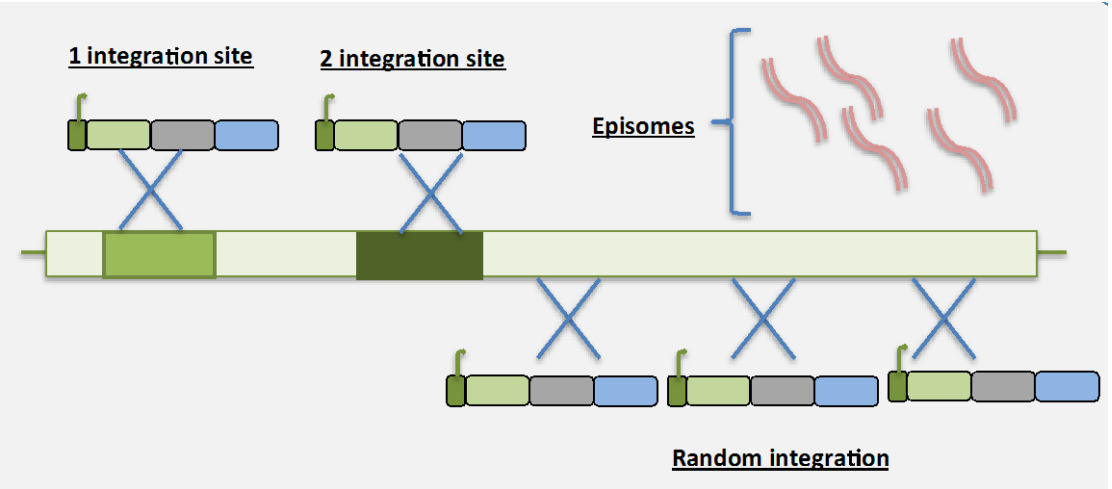
Rantasalo A *et al.*, A universal gene expression system for fungi. Nucleic Acids Res. 2018 Oct 12;46(18)

C1 Expression Technology

Transformation efficiency

Different genetic modification methods can be applied:

- Single site directed integration
- 2 sites directed integration
- Random integration
- Episomal vectors – Transient expression system



- Transformation procedure based on chemical (PEG) method with protoplasts or electroporation
- Frequencies for 1µg DNA:
  - >20 transformants for site specific integration
  - Up to 100 transformants for random integration
  - ~13,000 transformants for telomeric vector transformation

Isolation and identification of extracellular proteases

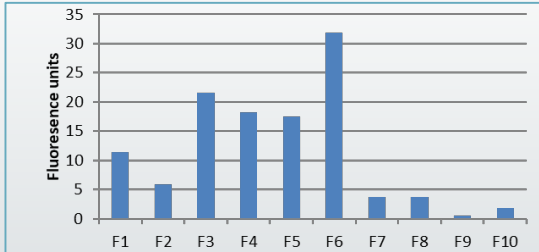
Fermentation

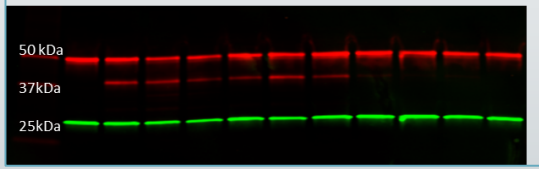
Culture supernatant

Affinity Chromatography

Analysis of fractions

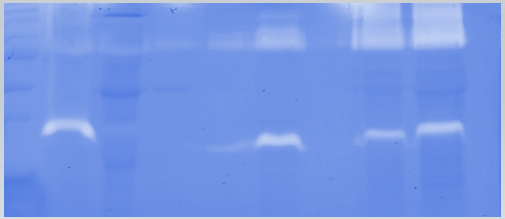
Protease activity assays





Spiking with target proteins

Zymogram gels with target protein



Fractions with proteolytic activity

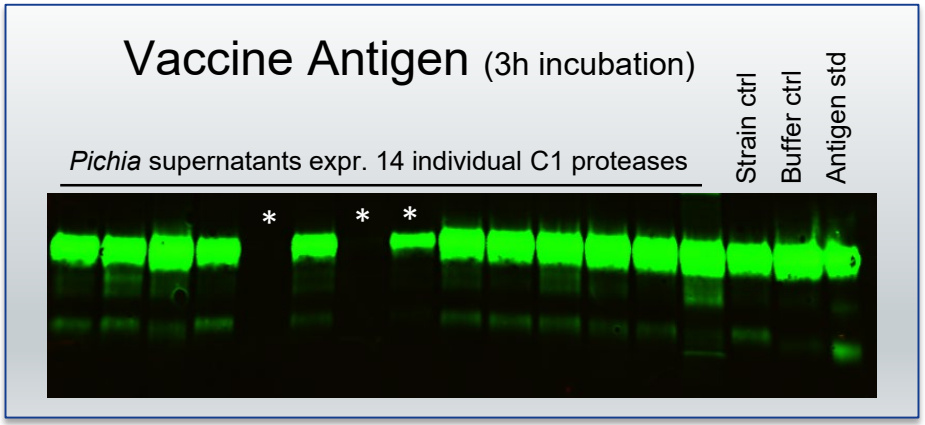
LC-ESI MS/MS

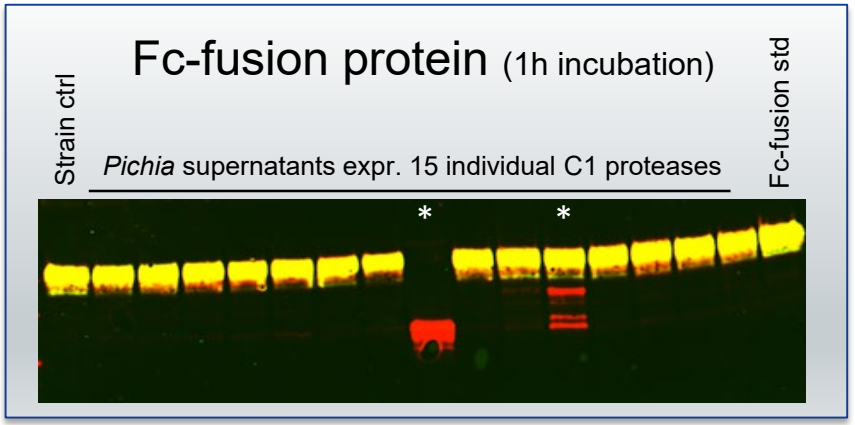
Protease A
Protease B
Protease C
Protease D
Protease E

C1 Protease library in *Pichia pastoris*

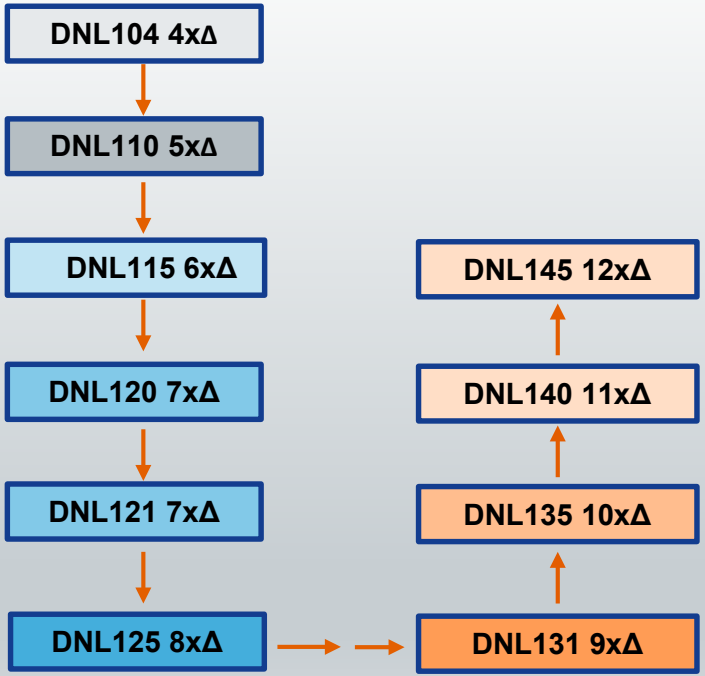
- More than 50 proteases were expressed individually in *Pichia pastoris*
- Culture supernatants of each strain expressing a C1 protease were tested in spiking experiments

→ Identification of critical proteases which are problematic for many sensitive target proteins



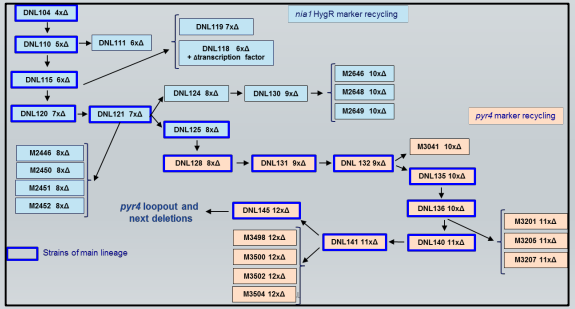


C1 Lineage of protease deficient strains



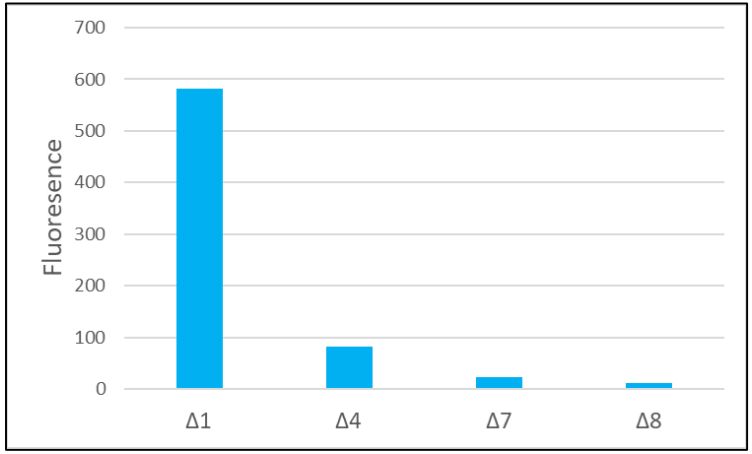
Systematic deletion of protease genes based on:

- Isolation and identification of extracellular proteases
- C1 protease library in *Pichia pastoris*
- Effect of different protease inhibitors on protease activity
- mRNA sequencing data
- Protease gene annotation

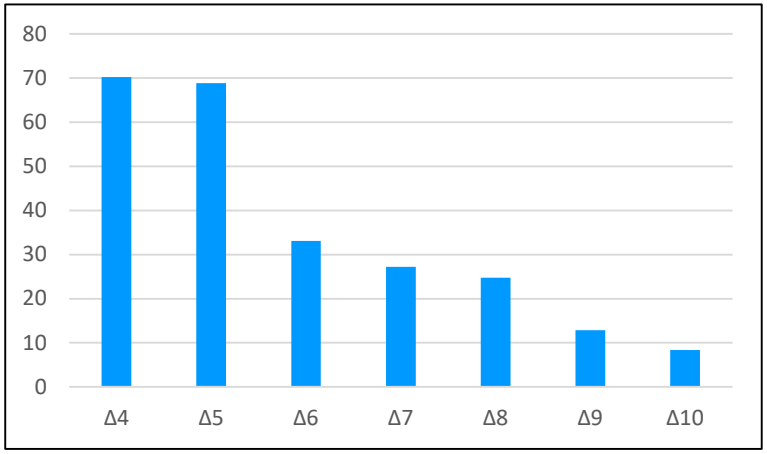


Total extracellular protease activity is greatly reduced in C1 protease deficient strains

- Direct fluoresence-based assay with casein substrate
- The activity in the 8xΔ strain is reduced over 50-fold compared to the 1xΔ strain (fermentation cultures)
- The activity in the 10xΔ strain is reduced 10-fold compared to the 4xΔ strain (24-well plate cultures)

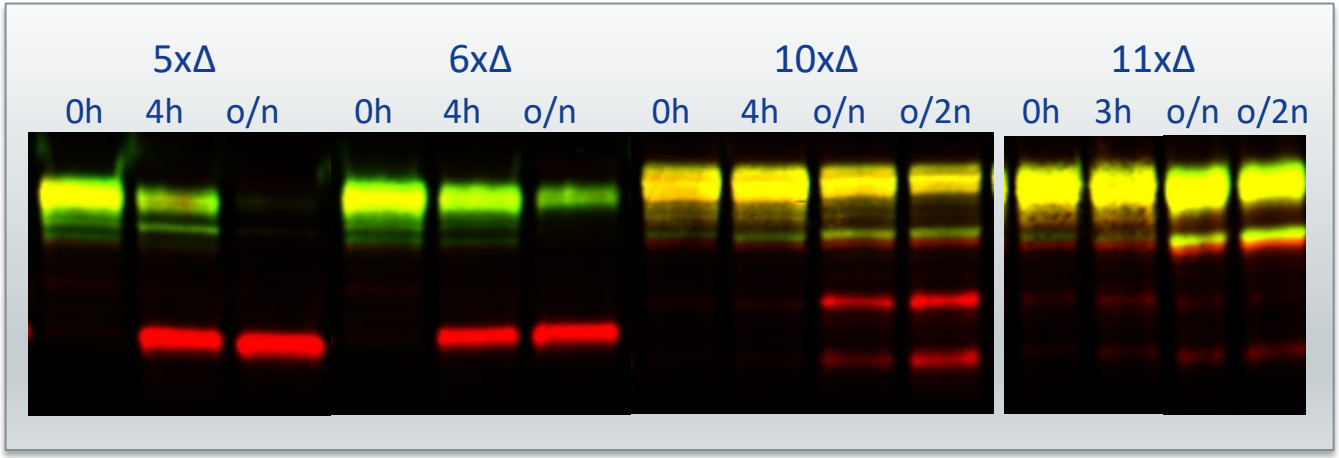


Fermentation culture



MTP culture

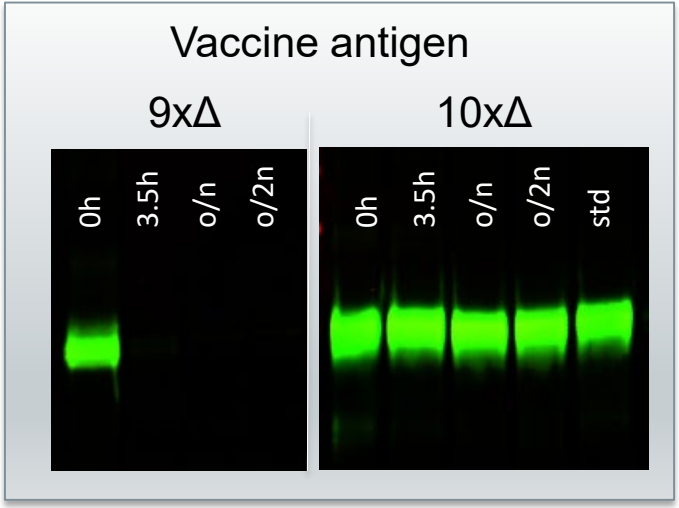
Protease Deletions are Improving Stability and Production of a Fc-fusion protein



Yellow full-length
Red degradation product

Target protein was spiked into the culture supernatant of the different protease deletion strains

Protease deletion strains are improving stability of target proteins - Difficult-to-express protein



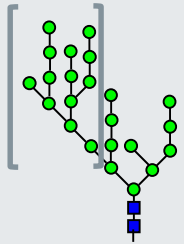
Target protein spiked into the culture supernatant of the protease deletion strains

Advantage of C1 over Yeast and CHO

- C1 glycan structure is more mammalian like than typically in yeasts
  - The native C1 glycans are mostly high mannose type (Man3-Man9) including some hybrid glycans
  - Less engineering steps needed for C1
  - Stable genome - defined glycan structure is stable from culture to culture and batch to batch

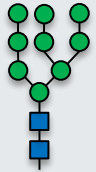
- We aim at defined mammalian glycan forms G0, G0F, G2 and G2F in our glycoengineering efforts

Typical Yeast Glycan Structure



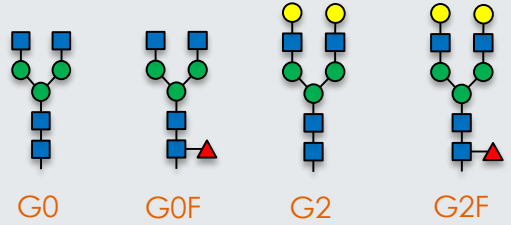
Man_{30-50}

Dyadic C1 Glycan Structure



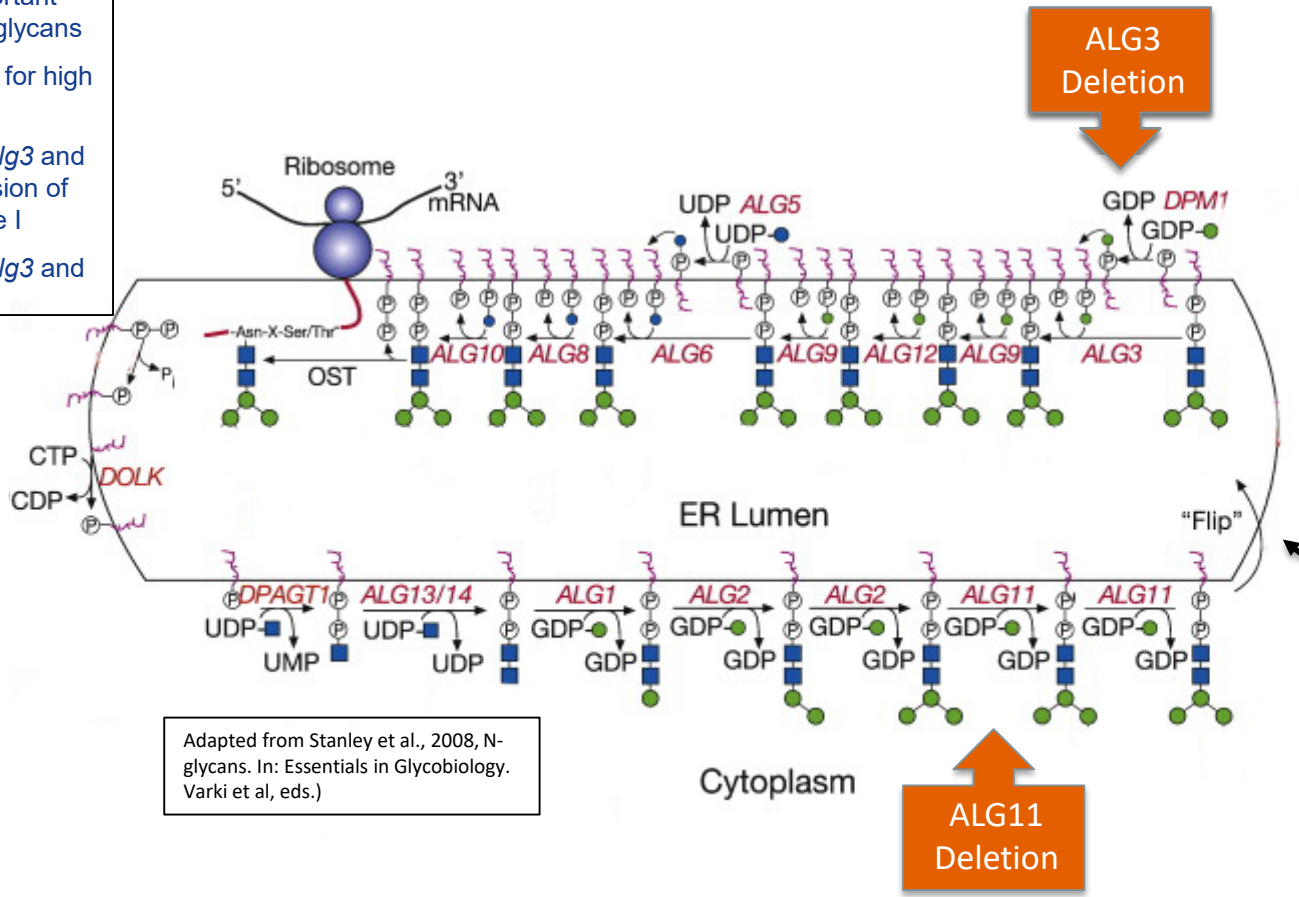
Man_{3-9}

Targeted Mammalian Glycoform Structures

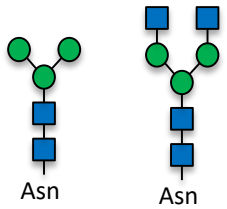


Two main approaches to gain high Man3

- Man3 is the important precursor of G0 glycans
- Two approaches for high Man3:
  1. Deletion of *alg3* and over-expression of Mannosidase I
  2. Deletion of *alg3* and *alg11*



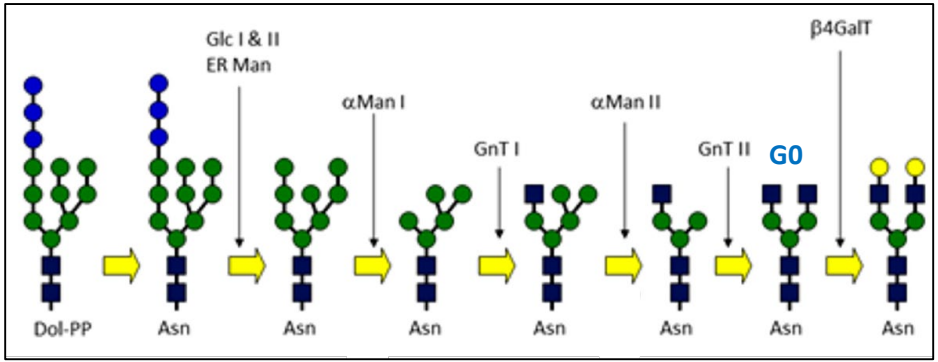
Adapted from Stanley et al., 2008, N-glycans. In: Essentials in Glycobiology. Varki et al, eds.)



Man3 G0

Flippase is the membrane protein enabling turning of the lipid-linked oligosaccharide to ER lumen side

Alternative approach for G0: classical animal pathway



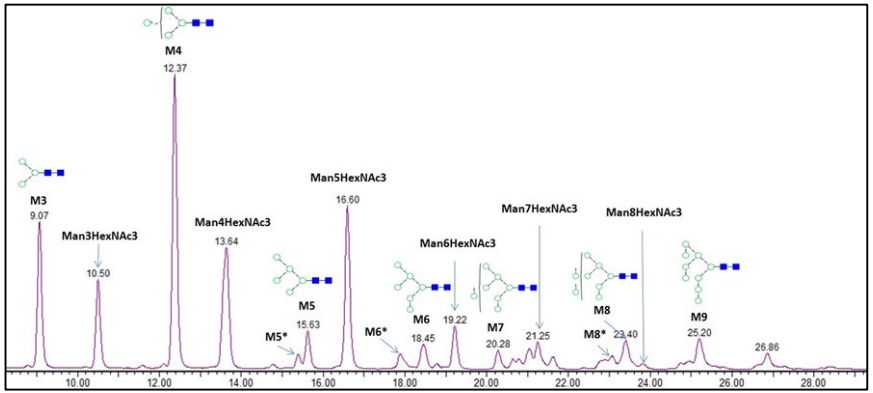
Modified from Nagae and Yamaguchi, *Int. J. Mol. Sci.* 2012, *13*(7), 8398-8429.

The animal glycan modification pathway coud be built into C1

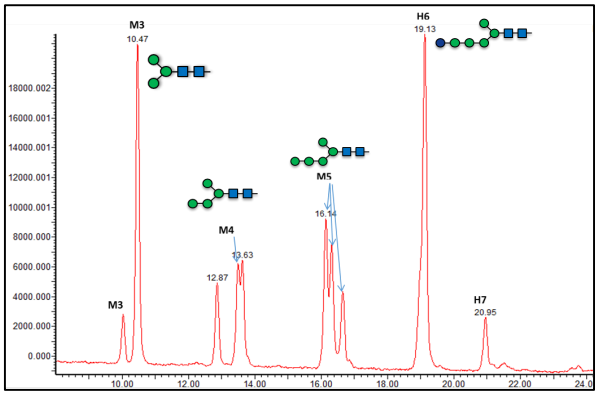
- Requires expression of Mannosidase I, GNT I, Mannosidase II and GNT II to gain G0 glycans
- Is more complicated than *alg3* or *alg3/alg11* approaches
- Not pursued in C1 presently

The effect of *alg3* deletion in C1

Wild type strain



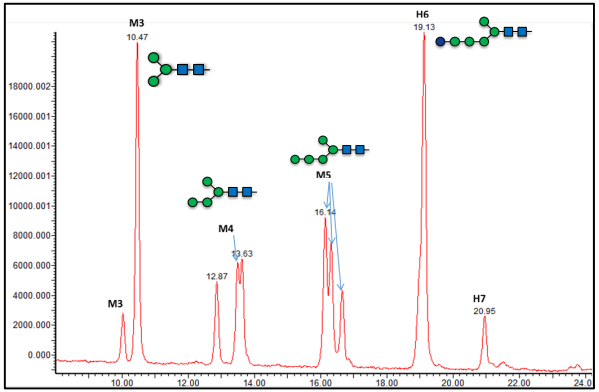
***Alg3* deletion strain**



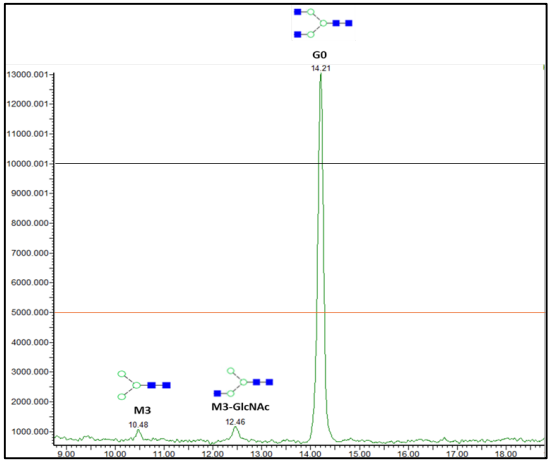
- The *alg3* gene was deleted by replacing it with a marker gene. Glycans on native proteins were analyzed
- The glycan pattern became much more simple
  - All higher MW glycans and hybrid glycans were omitted
  - Substantial amounts of Hex6, Man5 and Man4 glycans remained – further engineering along this strategy is in progress

Applying *alg3-alg11* deletion strategy to produce G0 glycans

***Alg3* deletion strain**



***Alg3-alg11* deletion + GNT1+2**



RT	Glycoform	Area (Abundance)	Relative Abun (%)
10,48	M3	30,93	1,63
12,46	M3GlcNac	85,79	4,52
14,21	**G0**	1781,79	**93,85**
	sum	1898,51	100,00

- The *alg11* gene was deleted from an *alg3* deletion strain. Simultaneously heterologus GNT1 and GNT2 were expressed from the *alg11* locus. Glycans on native proteins are shown.
- G0 glycan levels of up to 95% have been reached with this strategy. In addition to G0, only Man3 and GlcNAcMan3 remain in the glycan pattern.
- Different Golgi localization signals for GNT1 were tested.

The effect of flippase expression

No flippase o/e

RT	Glycoform	Area (Abundance)	Relative Abun (%)
10,48	M3	69,34	7,05
14,21	**G0**	914,85	**92,95**
	Sum	984,19	100,00

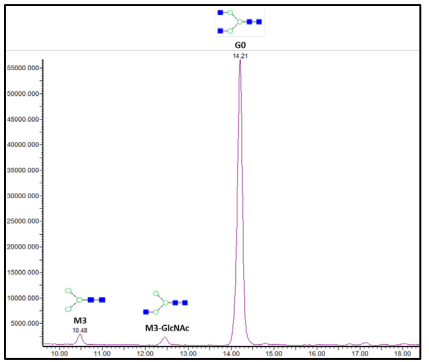
Expression of flippase 1

RT	Glycoform	Area (Abundance)	Relative Abun (%)
10,48	M3	116,77	2,84
12,46	M3GlcNac	95,17	2,32
14,20	**G0**	3894,9	**94,84**
	Sum	4106,84	100,00

Expression of flippase 2

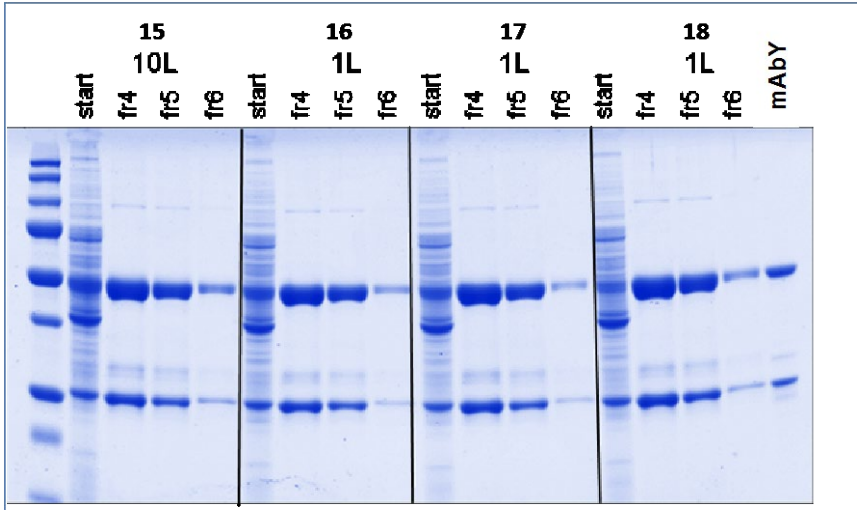
RT	Glycoform	Area (Abundance)	Relative Abun (%)
10,48	M3	259,86	3,00
12,46	M3GlcNac	248,09	2,86
14,20	**G0**	8162,84	**94,14**
	Sum	8670,79	100,00

- Strains were made where two different flippase variants were expressed in addition to GNT1 and GNT2 in *alg3-alg11* deletion background
- The total glycosylation level (sum of peaks) increased ~4x (flippase 1) or ~9x (flippase 2)
- The glycan pattern remained good with 93-95% G0 glycans
- Glycoengineering in C1 continues to e.g. galactosylation and fucosylation on target proteins like Mabs



Production of Biologics in C1

Fermentations for mAbY production

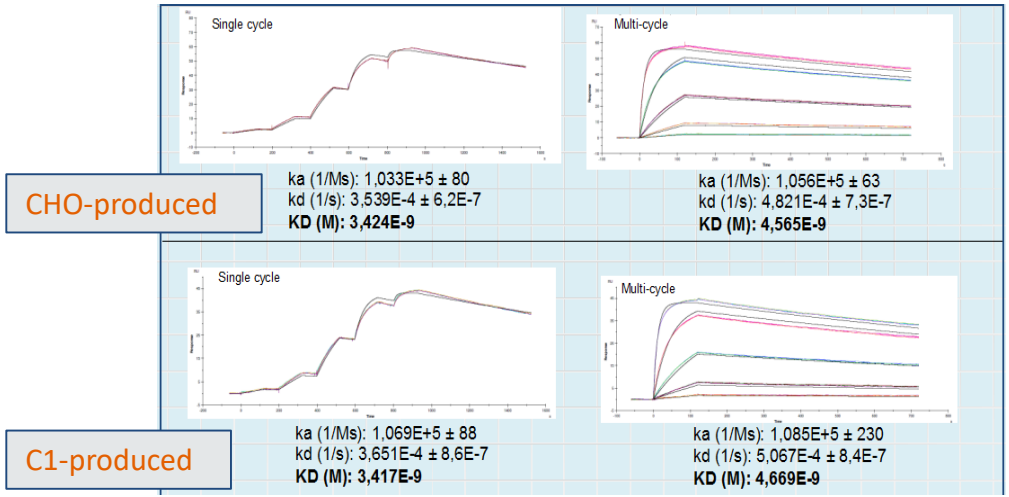


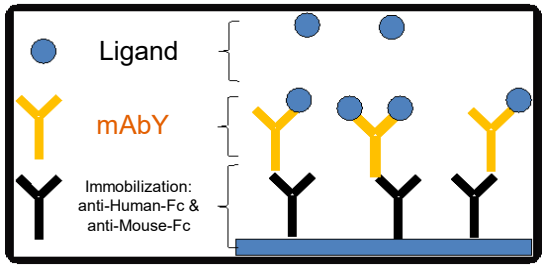
Fermentation #	Vessel volume (l)	Initial (final) culture volume (l)	Antibody titre (g/l)
15	10	8.0 (10.5)	8.0
16	1	0.8 (1.1)	6.3
17	1	0.8 (1.1)	6.5
18	1	0.8 (1.1)	7.9

- SDS gel analysis of the mAbY antibody purified from the fermentations by protein A affinity chromatography
- 'start' depicts the sample loaded to the protein A column, fr4-fr6 are the elution fractions obtained from the chromatography
- Sample of CHO-produced mAbY is shown as control
- Mass spectrometry analysis showed that both chains were intact

mAbY Binding assay by Biacore T200

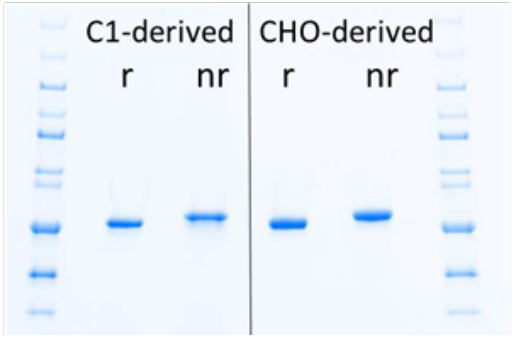
Studying the interaction of mAbs in real time



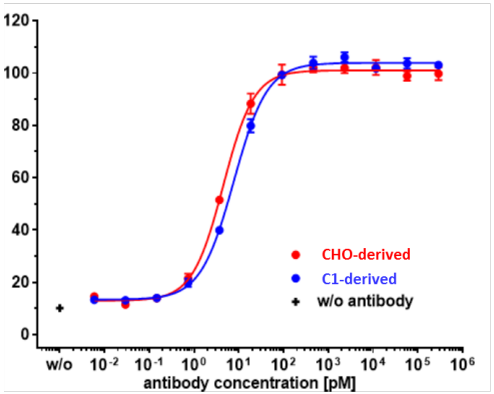


- The binding properties of C1 produced mAbY were compared to the CHO produced mAbY in a Biacore T200 assay
- **The C1-produced mAbY and CHO-produced mAbY showed virtually indistinguishable binding kinetics**
- **Similar results were obtained with other mAbs**

Success in Bispecific mAb expression



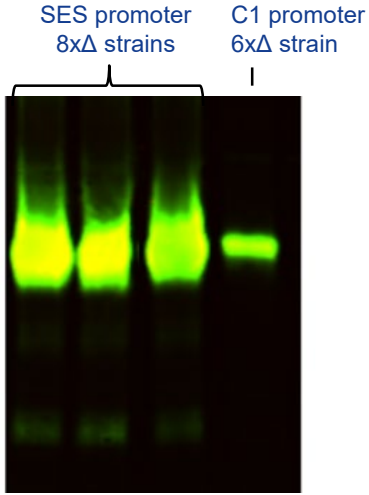
r: reduced conditions
nr: non-reduced conditions

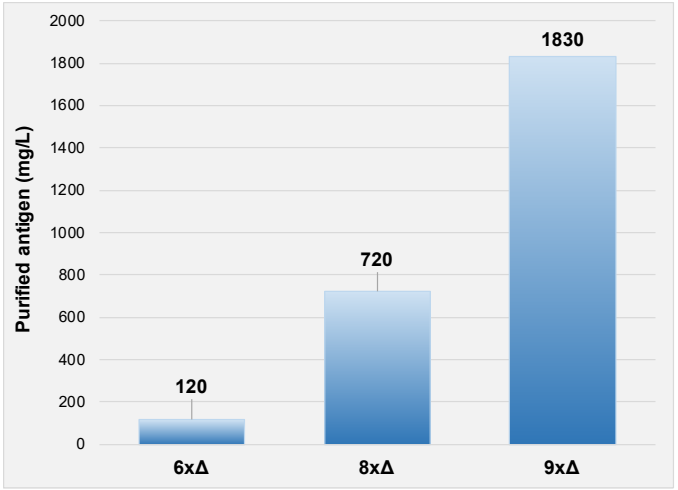


- **Production level 1 g/L in a 6-day fermentation process**
- Difficult to express in several hosts
- The function of C1-produced mAb was compared to the CHO-produced control in a bioassay
  - C1-produced purified using only single chromatography step
  - CHO-produced fully purified
- **Potency of C1-produced mAb in the bioassay is comparable to the CHO-produced control**

Success in expressing high level of ZAPI antigen

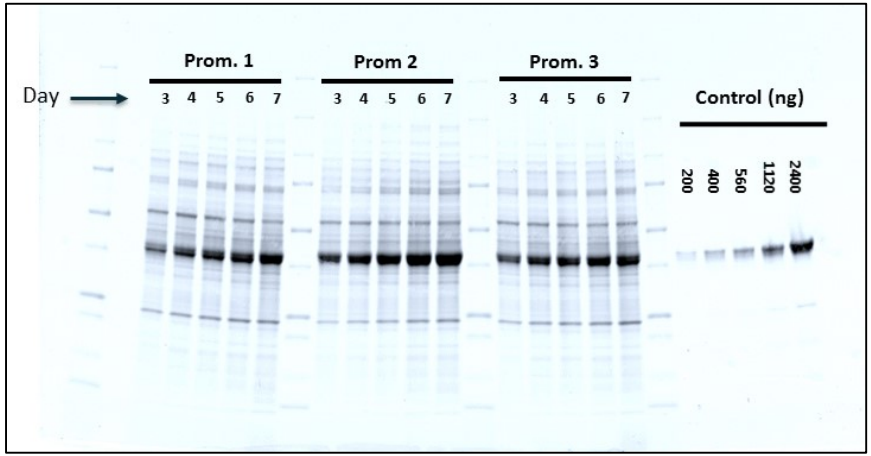
- Schmallenberg virus antigen coupled with Spytag - difficult-to-express protein in animal cells and microbial systems
- First strain had a native C1 promoter in 6xΔ protease strain
- Using synthetic promoter (SES) for expression, higher copy number and 8x protease deletion strain increased production six-fold
- Development of the protease deficient strain and process conditions increased the titer even further
- The produced protein formed nanoparticles with Spycatcher efficiently and showed good immunogenicity in cattle





- **The new strain using SES promoter system in improved protease deletion background significantly increased the production and stability of the target antigen**
- **Up to 1.8 g/L was purified by affinity chromatography**

Production of an Fc-fusion protein in C1



Strain	Time point	Fc-fusion protein concentration (g/L)
Prom. 1	Day 7	9.0
Prom. 2	Day 7	12.2
Prom. 3	Day 7	7.2
Optimized Prom. 2	Day 7	13.2

- The Fc-fusion protein was expressed with three synthetic promoters with different strengths
- The strains were grown in fermentors at 1 litre scale
  - Growth and total protein production was similar between the strains
  - The Fc fusion protein was purified with protein A affinity chromatography
  - Best production level of 12.2 g/l on day 7 was obtained from promoter 2
  - Expression with an optimized synthetic promoter resulted in **13.2. g/l** production level

Summary

- Myceliophthora thermophila C1 is an industrialized protein production host that is now developed for therapeutic protein manufacture - with several large biopharma companies entering into collaborations.
- We have identified critical proteases to deal with for therapeutic protein production enhancement and enabled a very significant reduction of the protease load in the production strains.
- Our glycoengineering program aims at high proportions of human glycoforms G0, G2, FG0 and FG2. Excellent G0 levels have been reached through alg3-alg11 deletion strategy.
- Monoclonal antibodies have been produced in C1 with levels reaching 22 g/l and rates up to 3.1 g/l/day. The binding characteristics of the C1-produced antibodies were very similar to CHO-produced controls. Fab fragments have been expressed at levels up to 14.5 g/l and Fc-fusion proteins up to 13.2 g/l in a 7-day process.
- Difficult to express proteins have been produced in C1 at superior levels as compared with other production systems.

Product	Production level g/L
Mabs	22
Fabs	14.5
Fc-fusion proteins	13.2
Difficult-to-express	
Bispecific antibodies	1.0
Viral antigens	1.8
VLPs (extracellular prod.)	0.3
Bacterial vaccine protein	6.0

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