Dyadic Netherlands

The C1-enzyme platform: A versatile fungal system for gene discovery, gene expression and protein production

Dyadic Netherlands

Research Subsidiary of Dyadic Inc, USA

- Dyadic Nederland BV, Wageningen, The Netherlands
- www.dyadic.nl

Focus

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

Tools

- Fungal molecular biology
- Genomics
- Enzymology
- Fermentation technology

Team Dyadic Netherlands

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Dyadic's Commercial Enzyme production platforms

Enzyme producing fungi currently employed by Dyadic:

Fungus	Current Market	Development
Myceliophthora thermophila C1 *(Formerly Chrysosporium lucknowense)*	*Textile*	*Biofuels, Feed/food, P&P, specialty proteins*
Trichoderma sp.	*Feed, paper&pulp, textile*	*P&P, Feed, Biofuels*

C1: An industrially relevant fungus

An efficient protein production system

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***Myceliophthora thermophila (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
- GRAS status acknowledged by the FDA (2009)

Intellectual Property

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

The United States Of America

The Director of the United States Patent and Trademark Office

Has received an application for a patent for a new and useful invention. The title and description of the invention are enclosed. The requirements of law have been complied with, and it has been determined that a patent on the invention shall be granted under the law.

therefore, this

United States Patent

Grants to the person(s) having title to this patent the right to exclude others from making, using, offering for sale, or selling the invention throughout the United States of America or importing the invention into the United States of America for the term set forth below, subject to the payment of maintenance fees as provided by law.

If this application was filed prior to June 8, 1995, the term of this patent is the longer of seventeen years from the date of grant of this patent or twenty years from the earliest effective U.S. filing date of the application, subject to any statutory extension.

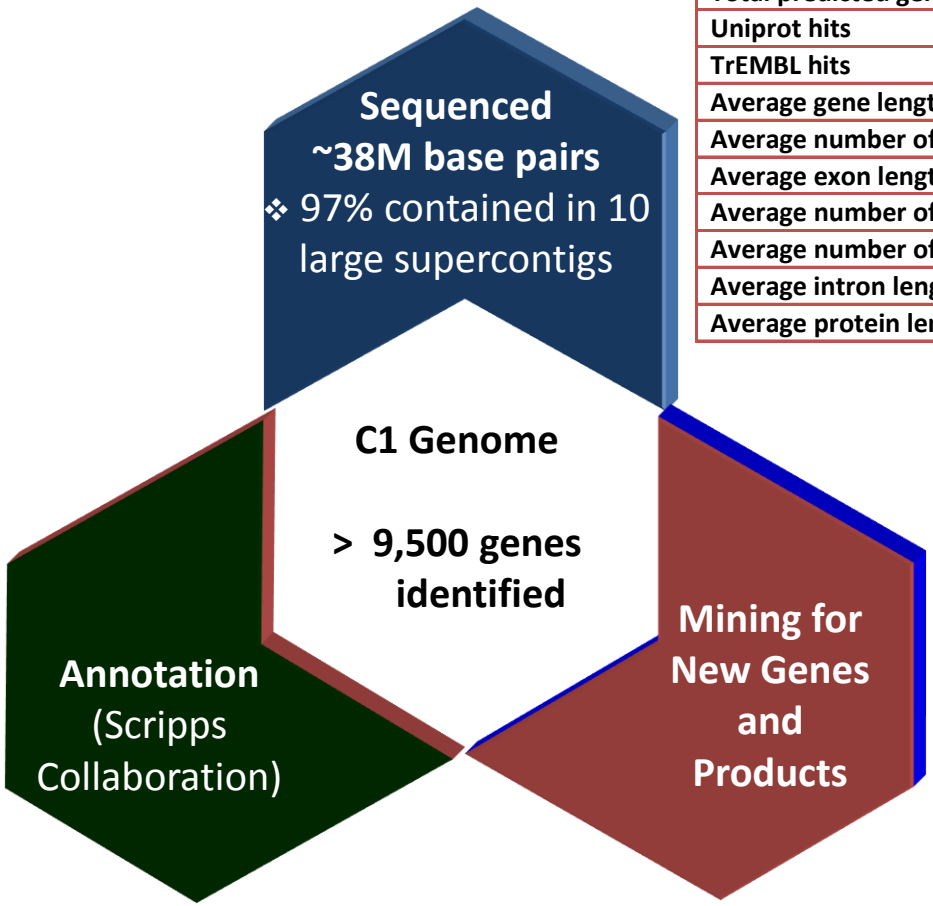
If this application was filed on or after June 8, 1995, the term of this patent is twenty years from the U.S filing date, subject to any statutory extension. If the application contains a specific reference to an earlier filed application or applications under 35 U.S.C. 120, 121 or 365(c), the term of the patent is twenty years from the date on which the earliest application was filed, subject to any statutory extensions.

Director of the United States Patent and Trademark Office

→ Large freedom to operate

C1: Genome sequencing project

Automated gene prediction and annotation



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

Molecular phylogeny

```
C1                  CAGAGCTGCAAAACTCCCTAAACCATCGTGAACGCTACCTAGACCGTTGCTTCGGCGGGC
M. thermophila      -AGAGCTGCAAAACTCCCTAAACCATCGTGAACGCTACCTAGACCGTTGCTTCGGCGGGC
                     ************************************************************

C1                  GGCGCCCTCGCGCGCCCCCCCCTGGGGCCCGCACCGCGGGCGCCCGCCGGAGGTACACCAA
M. thermophila      GGCGCCCTCGCGCGCCCCCCCCTGGGGCCCGCACCGCGGGCGCCCGCCGGAG-TACACCAA
                    ************************************************** ********

C1                  ACTCTTGATATGTTATGGCCACTCTGAGTCTCCTGTACTGAATAAGTCA
M. thermophila      ACTCTTGATATGTTATGGCCACTCTGAGTCTCCTGTACTGAATAAGTC-
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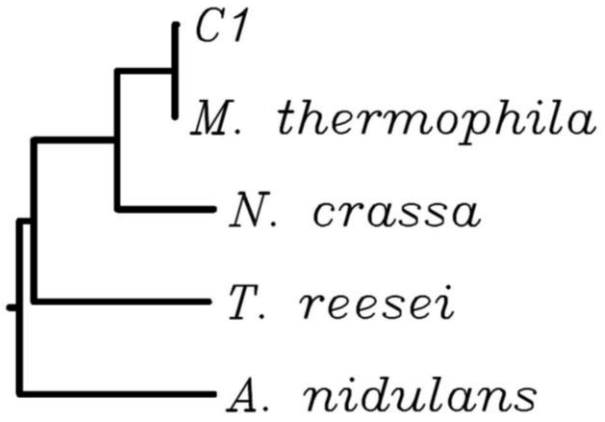
ITS1 sequence alignment.



Phylogenetic tree (ClustalW)
Accession numbers: Mt, see JGI genome project strain ATCC 42464. Nc, FJ360521. Tr, Z48932. An, AF138289.

Chrysosporium lucknowense C1 re-classified as *Myceliophthora thermophila*

C1: Genome sequencing project

Genome mining for industrial enzymes

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Opportunities

1. Identification of new targets for commercialization.
   - Cellulases, xylanases, amylases, proteases, lipases, phytases, oxidoreductases
   - Metabolites

2. C1-strain improvement
   - Identification of key genes involved in expression, secretion, post-translational modification, other aspects of physiology.

 Develop novel products

C1: Genome sequencing project

Genome mining for industrial enzymes

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	C1	*A. niger* [a]	*T. reesei* [b]
Cellulases (GH3, 5, 6, 7, 12 and 45)	30	35	27
Cellulases (GH61)	24	7	9
Cellulose binding domains (CBM1)	46	8	11
Xylanases	13	5	5
Arabinofuranosidases / arabinases	14	13	3
Esterases (Axe, Fae)	13	15	2

a) From CAZy database b) from JGI genome project

C1: An efficient protein production system

Versatile genetic tools and hosts developed

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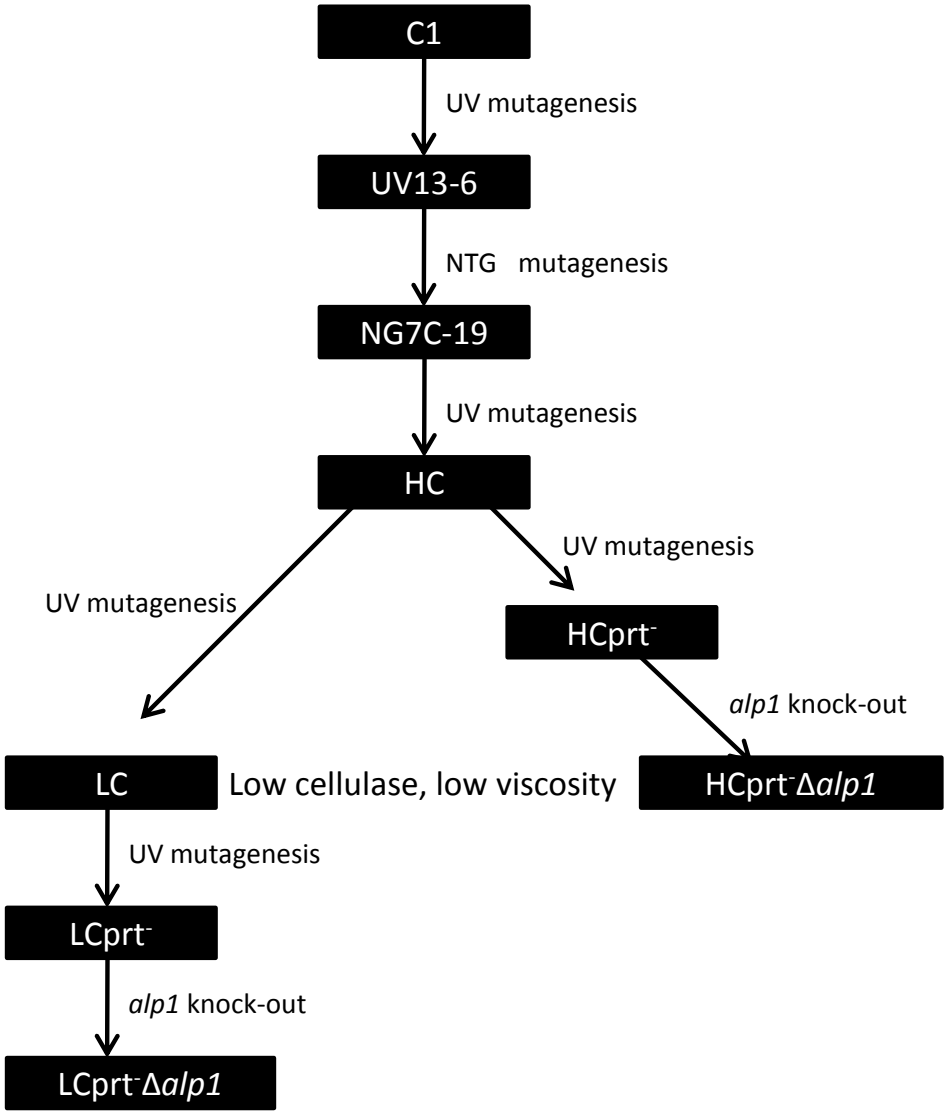
- Transformation system: high efficiency, stable integration
- Several genetic markers available: auxotrophic and dominant
  - Allows for multiple rounds of transformation
- Gene expression: variety of expression signals
  - Constitutive, inducible at various strengths
- Protein production: efficient secretion signals
- Targeted gene disruption: efficiency >1% up to 90%
- Variety of optimized C1-hosts
- Based on self cloning: No foreign DNA needed

Improved hosts for gene expression



Wild type

Cellulase over producer

De-repressed cellulase production

High cellulase, low viscosity (mycelial fragmentation)

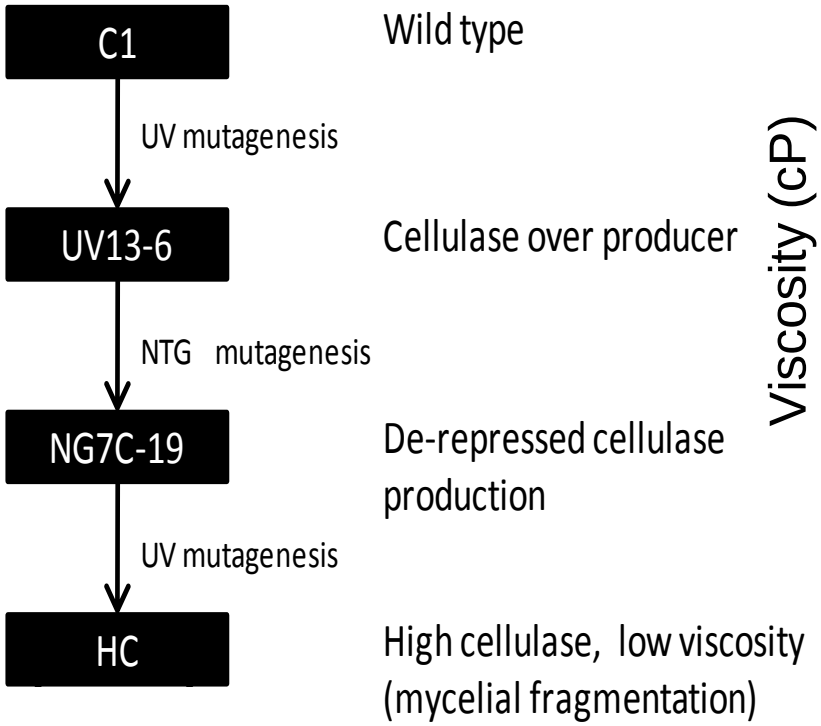
Protease-deficient

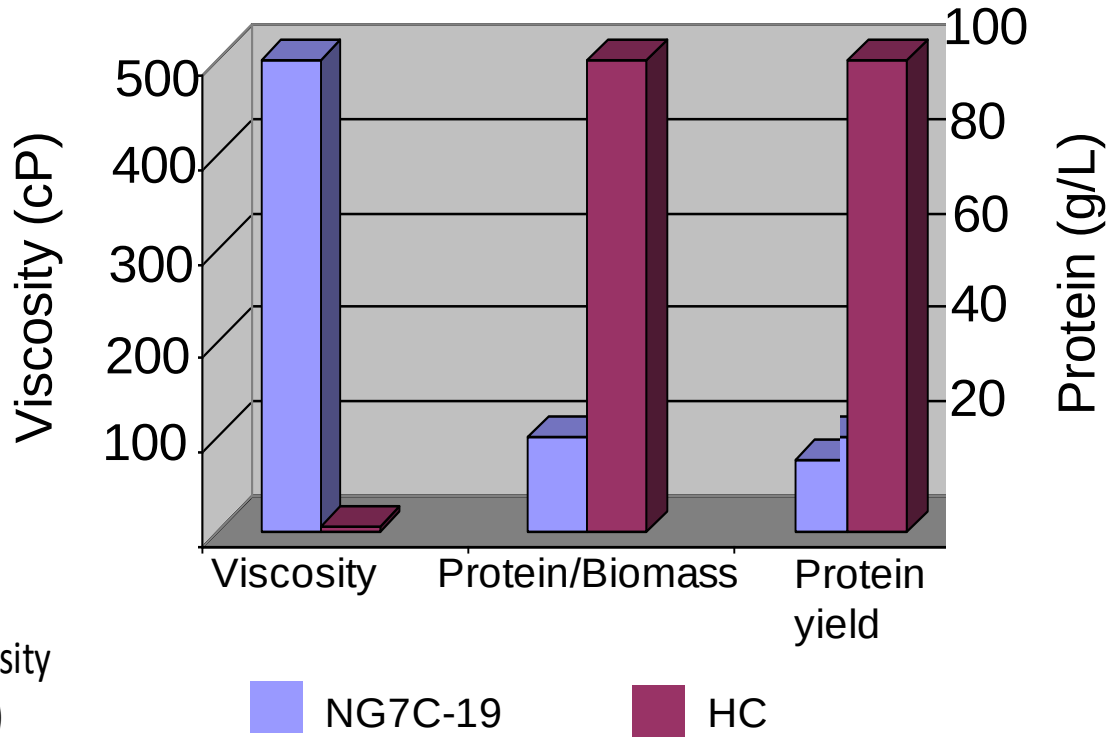
HCprt$^-$ with major protease gene *alp1* disrupted

Protease-deficient

LCprt$^-$ with major protease gene *alp1* disrupted

Development of C1 hosts strains





The HC strain shows a reduced viscosity, improved protein to biomass ratio and improved protein yields

Development of low-cellulase hosts (LC-strains)

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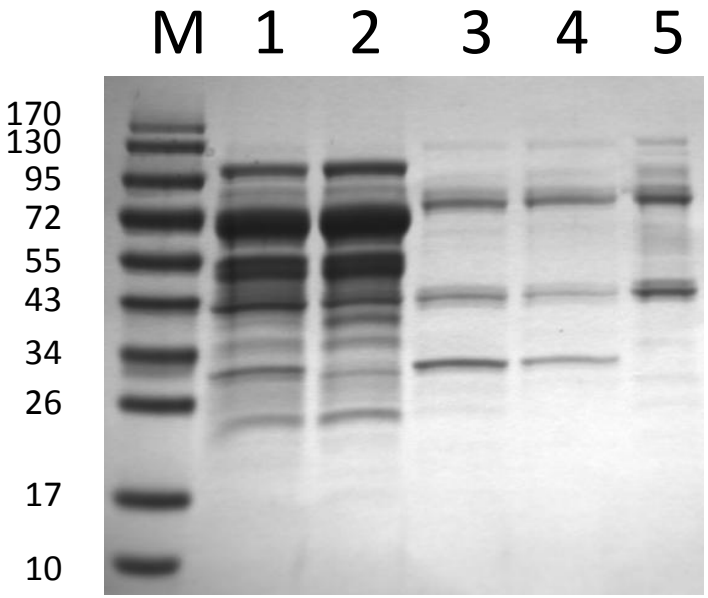
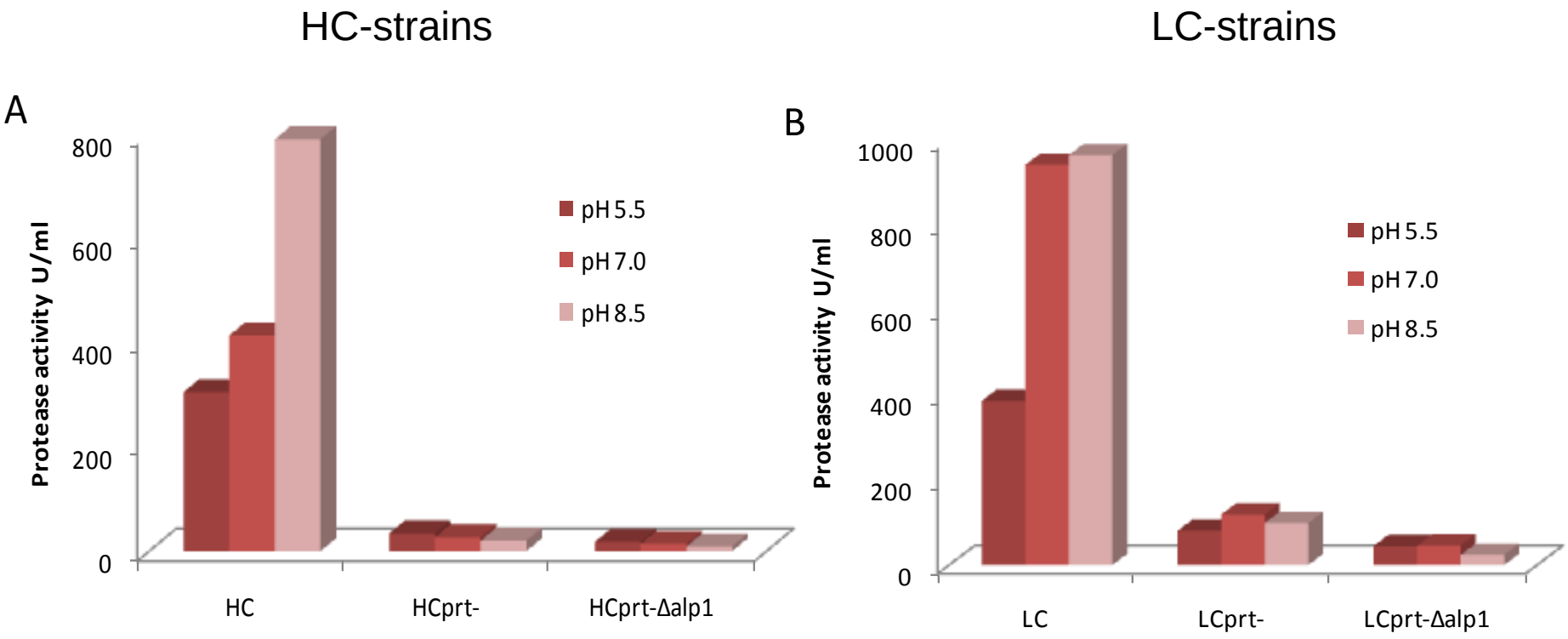
Aim: Development of a low-background host for pure protein production.



1. HC-Strain (High-cellulase):
   Non-viscous derivative of C1; Commercial fermentation; High cellulolytic activities; Diverse enzyme mixture; Up to 100 g/L total protein.
2. HCprt-
   Reduced-protease strain
3. LC
   Low-cellulase strain
4. LCprt-
   Reduced-protease strain
5. LCprt- Δ*alp1*
   Protease *alp1* knockout

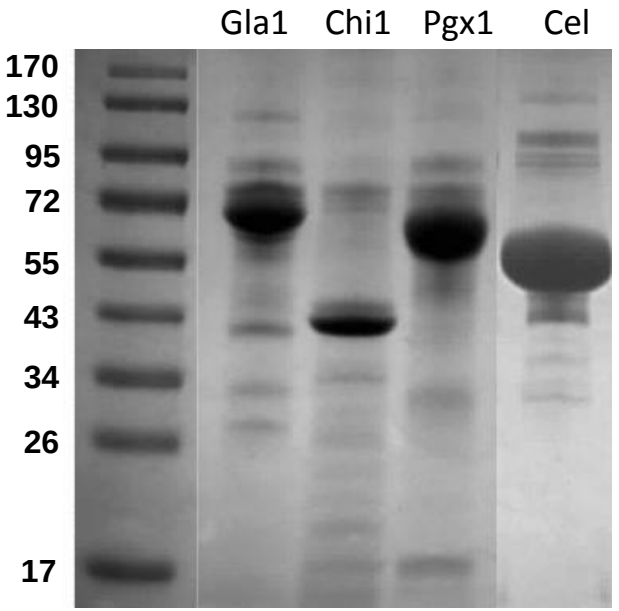
Host strains with low protease activity developed



Prerequisite for heterologous protein production

Improved hosts for gene expression

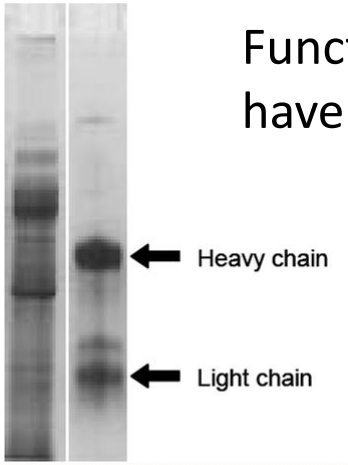
Single Enzymes produced in LC strain		
Homologous	**Number**	**Yield**
Starch-degrading enzymes	1	Gla1: 5.3 g/L
Chitin-degrading enzymes	1	Chi1: 7.5 g/L
Pectin-degrading enzymes	15	Pgx1: 7.4 g/L
Cellulose/Hemicellulose - degrading enzymes	62	Cel: 30 g/L
Proteases	1	Alp1: 3 g/L



Up to 30 g/L of the target protein produced

Improved hosts for gene expression

Source	Enzyme/protein
Fungal	Xylanases, amylase, cellulase, endo-polygalacturonase, oxidase, phytase
Bacterial	Xylosidase, Cellulase
Bacterial-directed evolution	Animal feed enzyme
Human	Immunoglobulin IgG1



Functional Heterologous enzymes/proteins from a variety of organisms have been produced in the g/L scale

recombinant human IgG antibody against TNFα

Improved hosts for gene expression

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Result of constructing more than 1000 modified C1 strains:

1. Strains with high level expression of specific enzymes (HC-strains)
   - Successfully have over expressed 7 different genes simultaneously
2. Construction of “clean” background strains (LC-strains).
   - Suitable for pure single enzyme expression
3. Strains exhibiting low protease profile
   - Suitable for heterologous enzyme expression
4. Proven scalability of fermentation from 2L to 150 m3

Acknowledgements

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- TNO Quality of Life, Zeist, The Netherlands
- Dep. Food Chemistry, Wageningen University, The Netherlands

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- Royal Nedalco, The Netherlands

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