Dyadic Netherlands

Developing industrial enzymes and proteins

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

Management:	Wim van der Wilden
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Support:	Mary van der Leij
	Rienco van der Mooren
	Marianne Cramer
Consultant:	Jaap Visser

Dyadic's Commercial Enzyme production platforms

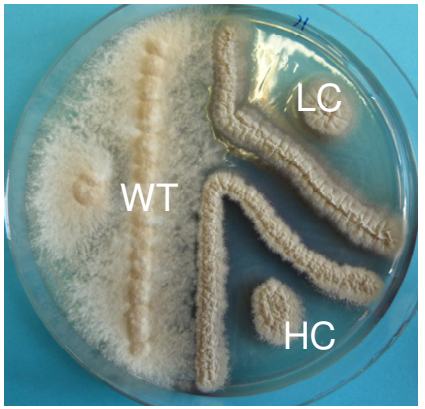
Enzyme producing fungi currently employed by Dyadic:

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

C1: developed into an efficient protein production system

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)

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

<u>Large freedom to operate</u>

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

Steps towards a mature enzyme production system

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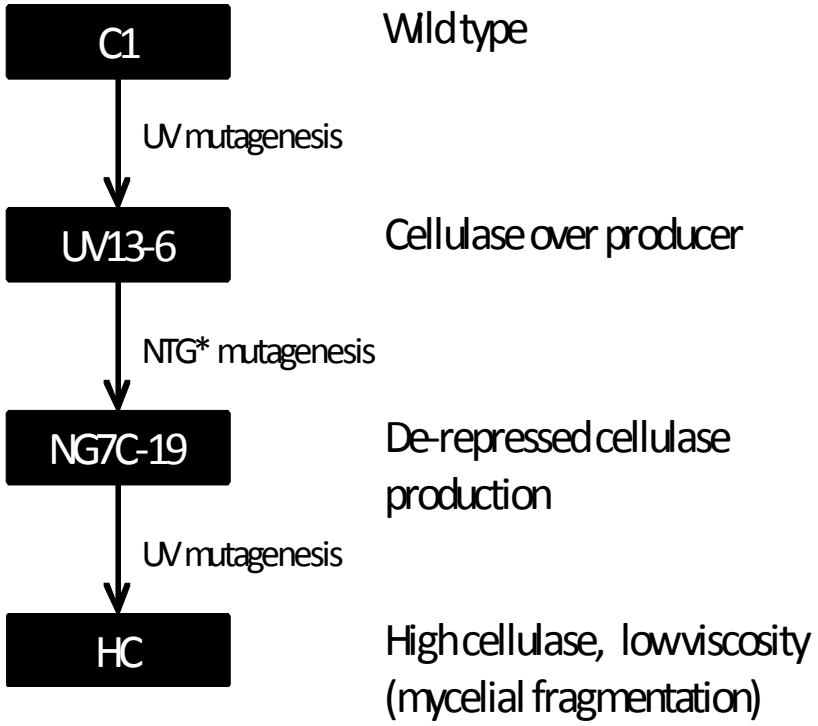
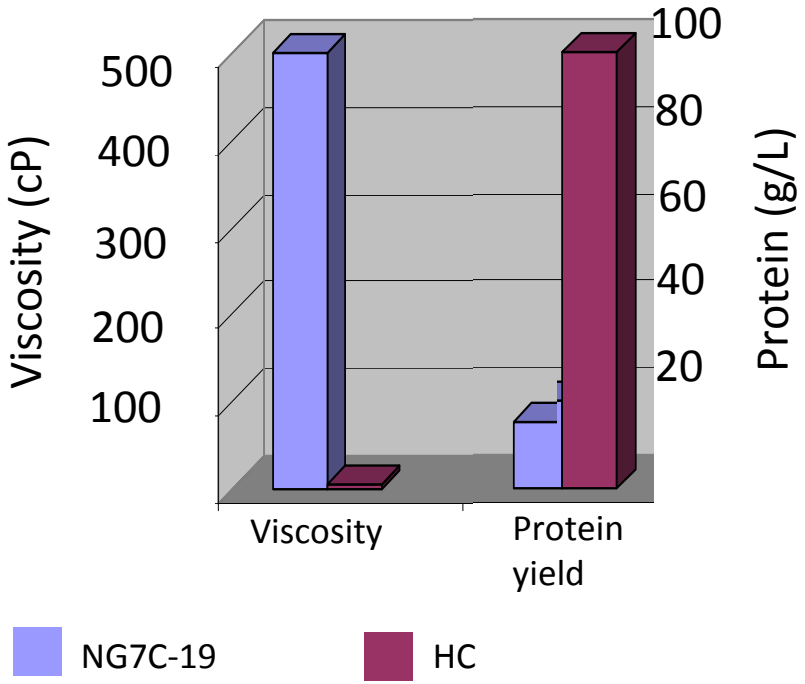
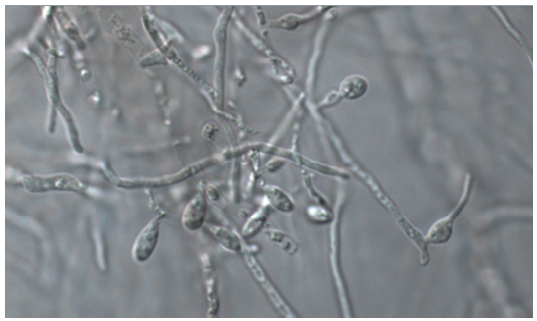
1. Development of protein hyper-producing strains
2. Development of versatile genetic tools and host strains
3. Exploration of the enzymatic potential by genomics
4. Construction of tailored strains for desired enzymes and enzyme mixtures

Development of protein hyper-producing strains







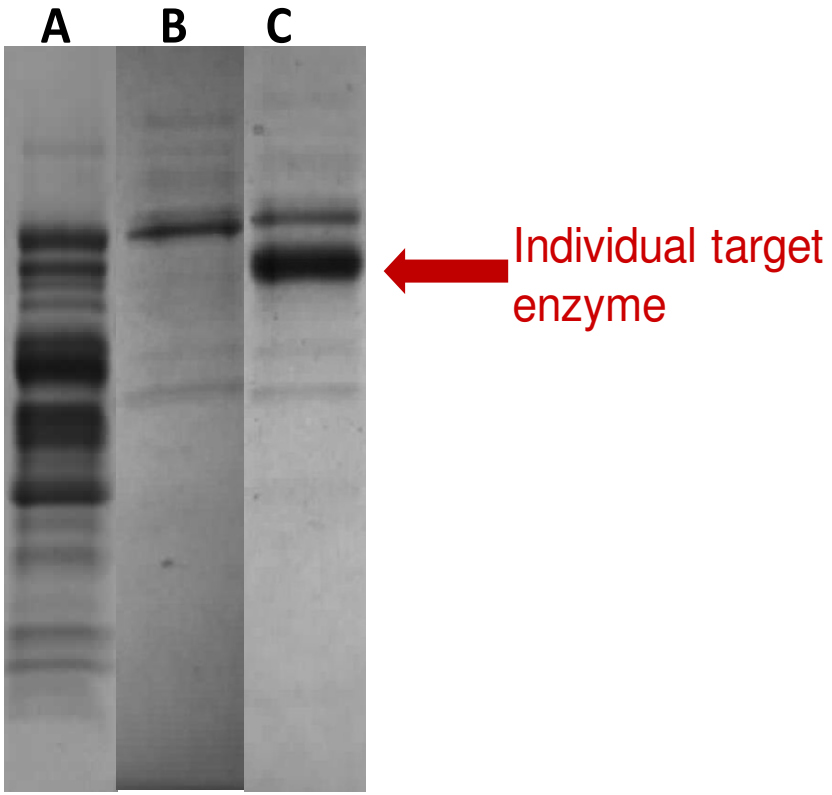
Development of versatile genetic tools and strains

Genetic tools developed

- Transformation system: high efficiency, stable integration (stable progeny).
- 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 up to 90% locus specific integration.
- Based on self cloning: No foreign DNA needed

Development of versatile genetic tools and strains

- Construction of “clean” background strains (LC-strains)
- For production of relatively pure single enzyme



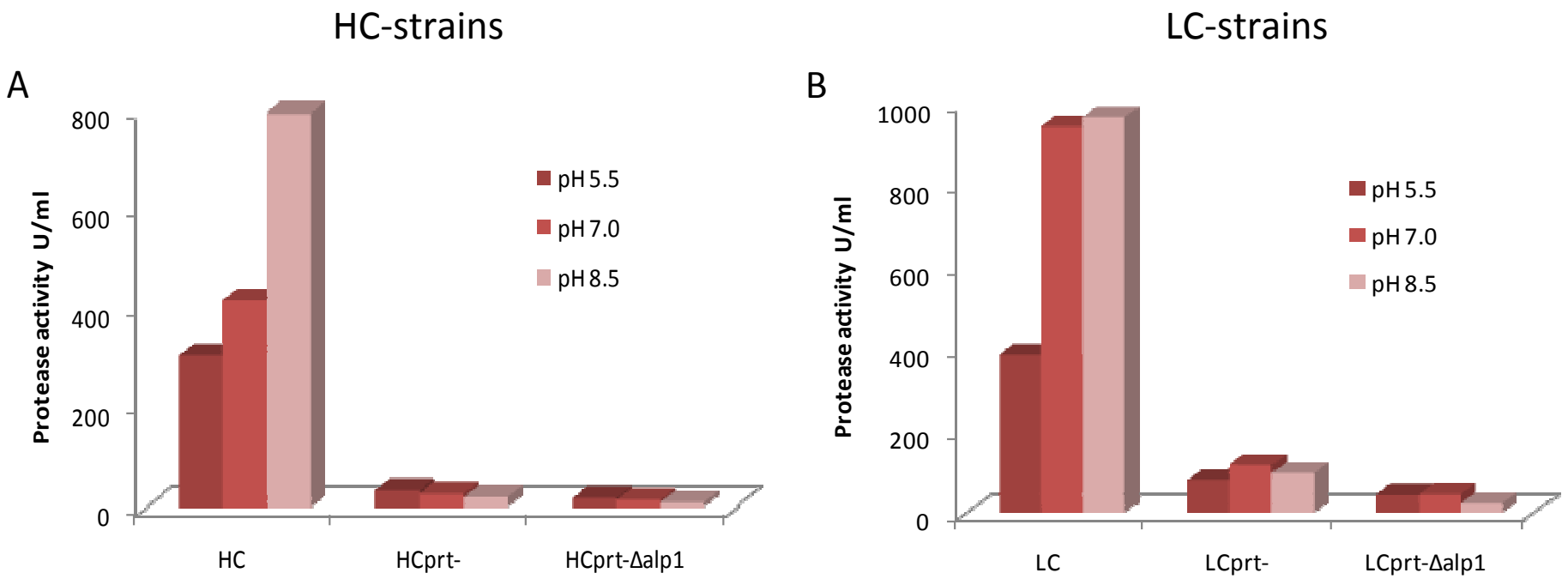
(A) Baseline C1-strain:
High cellulolytic activities
Diverse enzyme mixture
Up to 100 g/L total protein

(B) Low cellulase background strain (LC):
Almost no cellulolytic activities
Very few endogenous secreted
Suited for enzyme characterization

(C) ~ Up to 80% target protein

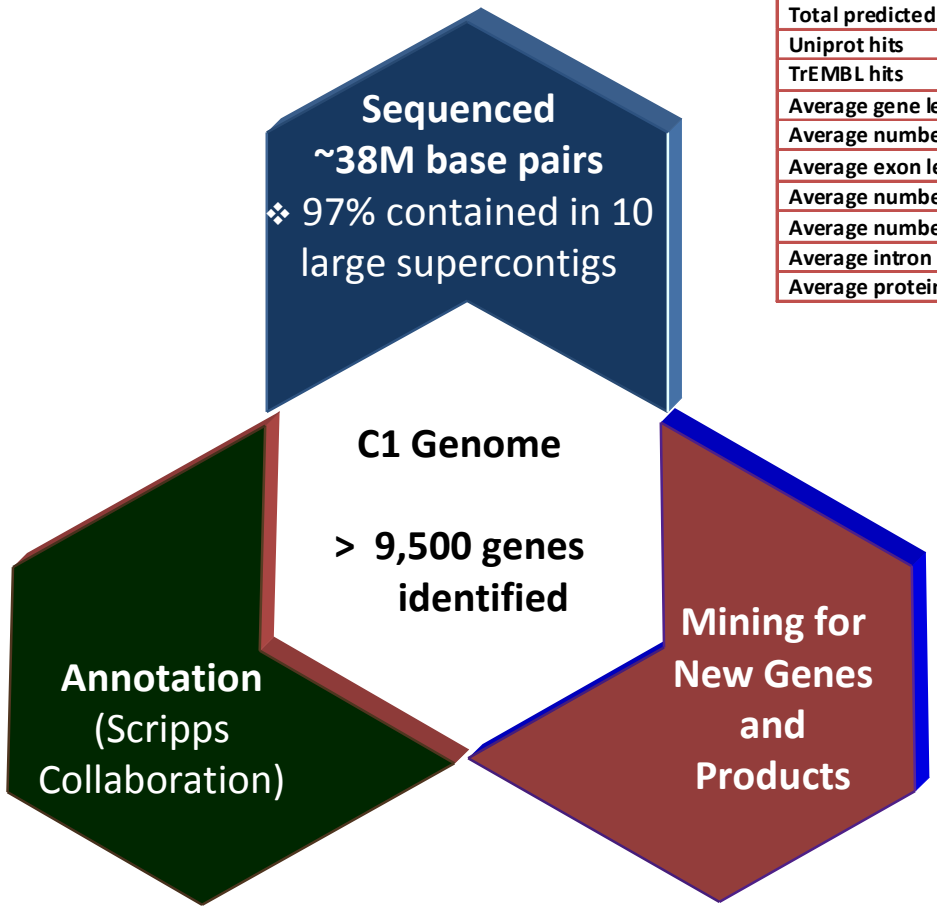
Development of versatile genetic tools and strains

Host strains with low protease activity developed



Particularly important for production of heterologous enzymes

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[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

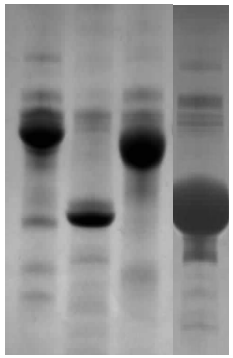
A large number of genes putatively encoding **industrially important** enzymes discovered:

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

Exploration of the enzymatic potential by genomics

Opportunities

1. Identification of new targets for commercialization.
- Cellulases, xylanases, amylases, proteases, lipases, phytases
- Via enzyme library construction and analysis



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

<u>Generating leads for new/improved products</u>

Construction of tailored C1-strains for desired enzymes

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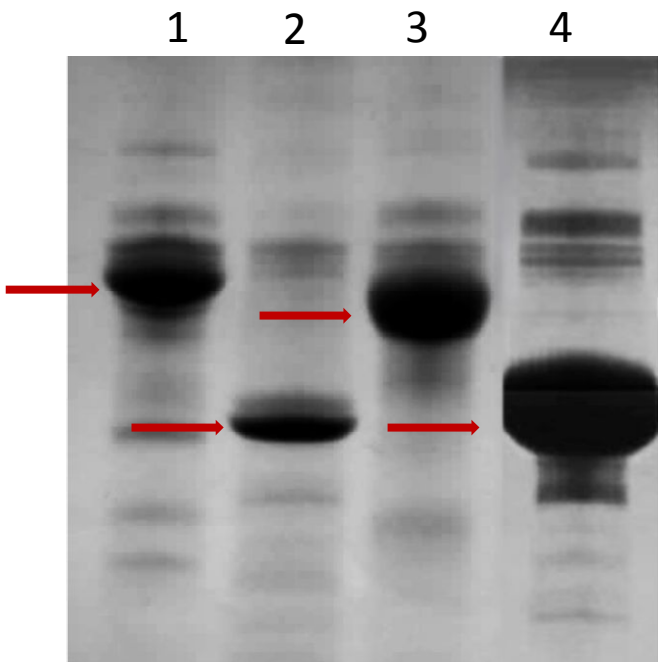
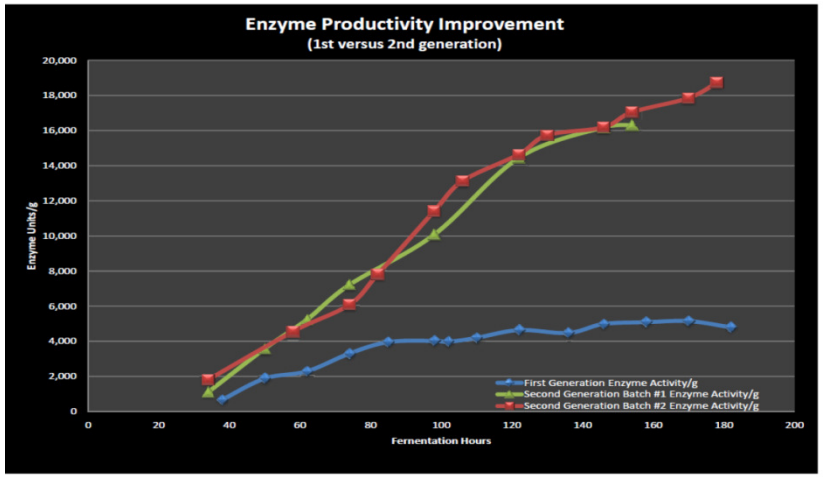
A library of single industrial enzymes produced at high levels is in place



SDS-Page analysis of end of fermentation broth of single enzyme producing LC-strains

- LC-strains routinely produce target single enzymes at the g/L scale.
- Up to 30 g/L of secreted protein has been obtained.
- 5- fold improvement within one year (commercial scale production).



Construction of tailored C1-strains for desired enzymes

Features C1-enzyme library

- Broad spectrum of xylan, cellulose, pectin and other plant fiber degrading enzymes expressed and characterized.
- Enzymes show a broad temperature and pH profile.
- Enzymes have synergistic activity and can in combinations be used for the efficient degradation of plant-derived fibers.
- Applications in biofuels/biochemicals, paper&pulp, feed, food.

<u>Several new products developed</u>

Construction of tailored C1-strains for desired enzymes

Functional heterologous enzymes/proteins produced at the g/L scale:

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

Construction of tailored C1-strains for desired enzymes

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Dedicated enzyme mixtures for lignocellulosic fuels and chemicals

- Produced by a single C1-strain in a single fermentation
- Fast liquefaction of highly viscous lignocellulosic biomass
- Fast saccharification: Ethanol process completed in 72h.
- Broad temperature range: Active between 32 ℃ and 65 ℃.
- 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

Summary C1-technology platform

- C1 is a proprietary mature enzyme production system.
- High extracellular protein/enzyme production levels obtained.
- Easy scaling, proven at the 150M^3 fermentation scale.
- Proven for heterologous enzyme and protein production.
- Versatile genetic tools and hosts developed: highly programmable.
- Genome sequenced and annotated: enzymatic potential elucidated
- Self affirmed GRAS status of a C1-product was acknowledged by the FDA (2009)
- Commercial products developed, e.g. in the bioenergy and paper&pulp space.