Development of *Myceliophthora thermophila* into a Highly Productive Biologics Production Host

Marika Vitikainen

PEGS Europe 2018
VTT – beyond the obvious

VTT is one of the leading research, development and innovation organizations in Europe. We help our customers and society to grow and renew through applied research. The business sector and the entire society get the best benefit from VTT when we solve challenges that require world-class know-how together and translate them into business opportunities.

Our vision
A brighter future is created through science-based innovations.

Our mission
Customers and society grow and renew through applied research.

Strategy
Impact through scientific and technological excellence.

Established in 1942

258 M€
Net turnover and other operating income (VTT Group 2017)

2,368
Total of personnel (VTT Group 31.12.2017)

36%
from abroad (VTT Group 2017)

27%
Doctorates and Licentiates (VTT Group 2017)

Owned by
Ministry of Economic Affairs and Employment
VTT Industrial Biotechnology Production Platforms

- Yeasts and bacteria
- Algae and cyanobacteria
- Enzymes *in vitro* & *in vivo*
- Plant cells
- Filamentous fungi

Strain engineering, synthetic biology, modelling, process development, piloting

- Biofuels
- Bioplastic precursors
- Therapeutic molecules
- Bionanomaterials
- Food and cosmetic ingredients
- Platform and high-value chemicals
- Industrial enzymes
Cell Factories for Recombinant Protein Production

Host systems

- **Prokaryotic**
  - *Escherichia coli*

- **Yeast**
  - *Saccharomyces cerevisiae*
  - *Pichia pastoris*
  - *Kluyveromyces lactis*

- **Filamentous fungus**
  - *Trichoderma reesei*
  - *Myceliophthora thermophila*

- **Higher eukaryotes**
  - Plants and plant cell cultures

**From gene to protein**

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

- 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 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 > 120g/L
- Highest production level of an individual recombinant enzyme 80g/L
Our Goal

To further develop C1 into efficient gene expression system to help speed up the development, lower production costs and improve the performance of biologic vaccines and drugs 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-glycosylation structures
Expression of two ORFs using split-marker – targeted integration

- For expressing two different ORFs or the same ORF from two copies
- Recombination of the two arms of the dual expression cassette will occur upon transformation
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

Efficient Expression

Rantasalo A et al., A universal gene expression system for fungi. Nucleic Acids Res. 2018 Oct 12;46(18)
Effect of different protease inhibitors on protease activity indicates what types of extracellular proteases are present in the culture supernatant.

Treatment of fermentation culture supernatant with different protease inhibitors followed by a protease activity assay.
Isolation and Identification of Extracellular Proteases

1. **Fermentation**
   - Culture supernatant

2. **Affinity Chromatography**
   - Analysis of fractions

3. **Protease activity assays**
   - Spiking with target proteins
   - Zymogram gels with target protein

4. **LC-ESI MS/MS**
   - Fractions with proteolytic activity

**Reducing Proteolytic Activity**
C1 Protease Library in *Pichia pastoris*

- More than 50 proteases are and will be included in the library.
- Culture supernatants of each strain expressing a C1 protease were tested in spiking experiments.
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

Reducing Proteolytic Activity
Total Extracellular Protease Activity Is Greatly Reduced in C1 Protease Deficient Strains

- Direct fluorescence-based assay
- The activity in the 8xΔ strain is reduced over 50-fold compared to the 1xΔ strain (fermentation culture)
- The activity in the 8xΔ strain is reduced 8-fold compared to the 4xΔ strain (fermentation culture)
Protease Deletion Strains are Improving Stability of Target Proteins - Difficult to Express Protein -

Vaccine antigen

9xΔ

10xΔ

Target proteins spiked into the culture supernatant of the protease deletion strains
Protease Deletions are Improving Stability and Production of a Fc-fusion Protein

<table>
<thead>
<tr>
<th>5xΔ</th>
<th>6xΔ</th>
<th>10xΔ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0h</td>
<td>4h</td>
<td>O/n</td>
</tr>
<tr>
<td>Yellow full-length</td>
<td>Red degradation product</td>
<td></td>
</tr>
</tbody>
</table>

Reducing Proteolytic Activity

- Target proteins were spiked into the culture supernatant of the different protease deletion strains

- Fc-fusion was produced in 5xΔ and 6xΔ strains in 1L fermentors at 38°C
- The protein A purification yield from day 6 was 8.1 g/l, corresponding to 1.35 g/l/day production rate
- The fermentation was not fully optimized
- This Fc-fusion protein is not produced in 10xΔ strain yet
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 low amount of hybrid glycans
  - Less engineering steps needed for C1
  - O-glycosylation was not identified in therapeutic proteins expressed in C1
  - Stable genome - defined glycan structure is stable from culture to culture and batch to batch
- The first steps of glycoengineering C1 cells have been taken and were successful

**Typical Yeast Glycan Structure**

**Dyadic C1 Glycan Structure**

**Targeted Mammalian Glycoform structures**

$\text{Man}_{30-50}$

$\text{Man}_{3-9}$

G0, G0F, G2, G2F
Improving the glycoform structure in C1 Glycoengineered strains

- Analyzed glycans from native protein samples of glycoengineered C1 strains (indicated) by permethylation + MALDI-TOF analysis
  - No fungal high mannose structures present
  - Up to 80% of Man3 structure, the important precursor for human glycoforms
- No negative effects on cell viability have been observed with any of the modifications done
- Significant amounts of G0 form have been detected
Production of Biologics in C1
Flow Diagram of C1 Expression Technology

1+ weeks
- Gene synthesis
  - The synthesis of the GOI is being done by outsourcing

2 weeks
- Plasmid construction
  - Cloning is done in Yeast or E. coli.
  - Preparation of linear fragments
  - Vectors for site directed integration

2 weeks
- Strain construction
  - Protoplast transformation
  - Colonies appears after 4-7 days
  - Streaking of colonies on selective plates
  - If needed: Removal of selection marker for re-transformation 2+ weeks

1+ weeks
- MTP screening and analysis
  - 96 or 24 well plates can be used
  - Source of inoculum can be either a frozen cell stock or mycelia from a plate.
  - Shaker incubation 4 days

1 week
- 1L scale fermentation
  - Inoculum of vegetative cells
  - 4-7 days process
  - Fed batch technology
  - Defined media with or without Yeast Extract
  - Glucose feeding
  - No Induction is needed

- Purification and analysis
  - Protein is secreted to the media
  - Biomass sedimentation
  - Protein A purification for mAbs
  - Standard purification methodology by filtration and chromatography
  - No need for virus clearance

Further fermentation development
C1 Fermentation Technology

- 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 - 42°C
- 1L to 500,000L fermentation scale

Fed-batch Process

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

From MTP to Large scale mAbs productivity
Fermentations for mAbY production

- SDS gel analysis of the mAbY antibody purified from the fermentations by protein A affinity chromatography
- Input depicts the sample loaded to the protein A column, fr4-fr6 are the elution fractions obtained from the chromatography.
- Samples of CHO-produced mAbY are shown as controls.
- Mass spec analysis showed that both chains were intact

<table>
<thead>
<tr>
<th>Fermentation #</th>
<th>Vessel volume (L)</th>
<th>Initial (final) culture volume (L)</th>
<th>Antibody titre (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>10</td>
<td>8 (10.5)</td>
<td>8.0</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0.8 (1.1)</td>
<td>6.3</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0.8 (1.1)</td>
<td>6.5</td>
</tr>
<tr>
<td>18</td>
<td>1</td>
<td>0.8 (1.1)</td>
<td>7.9</td>
</tr>
</tbody>
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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

- **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

- Difficult-to-express-protein in animal cells and microbial systems
- First strain was generated with a native C1 promoter in 5xΔ protease strain
- Synthetic promoter (SES) construct in two 8x protease deletion strains increased production several fold

- The new strain using SES promoter system significantly increased the production and stability of the target antigen
- Up to 720 mg/L was purified by affinity chromatography

![Graph showing increased production of antigen](image)
**Summary**

- *Myceliophthora thermophila* C1 is an industrialized protein production host now developed for therapeutic protein manufacture.
- Characterization of C1 proteases has identified the critical ones for therapeutic protein production and enabled great reduction of the protease load in the production strains.
- Monoclonal antibodies have been produced in C1 with levels reaching 10.9 g/l and rates up to 2.4 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 in a 7-day process.
- Difficult to express proteins have been produced in C1 in superior levels as compared with other production systems.
- The native glycan pattern of C1 gives a good starting point for humanization of the glycan pattern. The first steps of glycoengineering have been successful.

<table>
<thead>
<tr>
<th>Product</th>
<th>Production level g/L</th>
</tr>
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<tbody>
<tr>
<td>Mabs</td>
<td>10.9</td>
</tr>
<tr>
<td>Fabs</td>
<td>14.5</td>
</tr>
<tr>
<td>Fc-fusion proteins</td>
<td>8.1</td>
</tr>
<tr>
<td><strong>Difficult-to-express</strong></td>
<td></td>
</tr>
<tr>
<td>Bispecific antibodies</td>
<td>1</td>
</tr>
<tr>
<td>Viral antigens</td>
<td>0.7</td>
</tr>
<tr>
<td>VLPs (extracellular prod.)</td>
<td>0.3</td>
</tr>
</tbody>
</table>
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rtchelet@dyadic.com
marika.vitikainen@vtt.fi
markku.saloheimo@vtt.fi