Development of the filamentous fungus *Myceliophthora thermophila* C1 into a next-generation therapeutic protein production system

**CONTACT INFO:** rtchelet@dyadic.com

**PURPOSE**
Filamentous fungi are naturally excellent protein producers and they are exploited in the enzyme industry. *Myceliophthora Thermophila* C1 has been developed for over 20 years for enzyme production, and the best protein production yields from this fungus exceed 120 g/l in a 6-7 day process. C1 is non-pathogenic and non-toxic and has a GRAS status for manufacture of food products granted by FDA. The purpose of this work is to utilize the superior productivity of C1 in pharma industry for production of therapeutic proteins.

**OBJECTIVES**
The objectives of this study are to:
1. Generate robust and efficient production strains and expression technology for high level production of therapeutic proteins in C1.
2. Characterize C1 proteases and reduce the protease load of the C1 production strains to enable production of the sensitive therapeutic target proteins.
3. Humanize the C1 glycosylation machinery to produce proteins with homogenous human type glycans.

**METHODS**
Genetically modified C1 strains can be conveniently generated with protoplast transformation or electroporation and the DNA construct is integrated to the desired locus with high frequency. Production strains for specific proteins can be generated within about 3 weeks from transformation. C1 is typically fermented in a fed-batch process in a chemically defined medium with glucose as the only carbon source. The process is easily scalable and performs well in a single use reactor. For characterization of C1 proteases we have used plasmids, proteomics with mass spectrometry, and biochemical methods such as inhibitor affinity chromatography, zymograms, and protease expression in Pichia pastoris. A series of multiple protease deletion strains has been created with a marker recycling transformation procedure.

**RESULTS**
Characterisation and reduction of C1 proteases
When testing the C1 system for production of protease-sensitive therapeutic target proteins such as a sensitive Fc-fusion protein (Fig 2) and a vaccine antigen (Fig 1), it became clear that the C1 protease load needs to be reduced. The secreted C1 proteases were studied with various methods. For example, we isolated proteases from C1 supernatants with inhibitor affinity chromatography and studied their activity with e.g. activity measurements, spikings and zymogram gels where the target protein is coated to the gel matrix (Fig 1). We also expressed 44 C1 protease genes in *Pichia* pastoris and studied their activity against the target proteins (Fig. 1). This has identified a few critical proteases that are a problem for many of the sensitive target proteins. Deletion of the critical protease genes was started in the project from a 4-fold deletion strain that already had a significantly lower activity than a strain with a single deletion (Fig 2A). We have created a series of protease deletion strains that has further reduced the protease activity by about 8x from the 4-fold to the 8-fold deletion strain. The latest strain has 10 protease deletions, and it shows remarkably improved stability of an extremely sensitive Fc fusion protein (Fig 2B).

Production of target therapeutic proteins in C1
Production of monoclonal antibodies in C1 is under development in this work. We utilize dual vectors with split marker technology to integrate Mab-expressing constructs to a specific locus supporting high gene expression. Mabs have been expressed in high levels reaching up to 10.9 g/l in a 7-day process. The highest production rate reached so far for Mabs is 2.4 g/d/l. An example of Mab-production is shown in Fig. 3. Mabs produced in C1 have been shown to have intact glycostructure and very similar binding kinetics to CHO-produced controls as analysed by Biacore SPR measurement device. The N-glycosylation site occupancy was shown to be lower than in CHO-produced control but this issue is addressed by glycoengineering. C1 has shown great promise for production of difficult-to-express proteins such as viral antigens, sensitive Fc fusion proteins and bispecific antibodies. As an example we show a bispecific antibody that does not produce well in CHO cells. We have produced it in C1 at about 1 g/l in 6-day process. The protein shows very similar characteristiscs to its CHO-produced control based on SDS-gel electrophoresis and activity measurement in a cell-based assay (Fig. 4). A viral antigen that is very difficult to express in other platforms including several animal cell lines has been produced in C1 at over 0.7 g/l in a 6-day process (data not shown).

Glycoengineering of C1
C1 produces naturally high mannose glycans between Man3 and Man9 with a low percentage of hybrid glycans. Glycoengineering of C1 has been started by deleting the *alp3* mannansyltransferase gene, over-expressing the native mannosidase I and expressing the animal-derived GNT1 mannoseglycan transferase GNT1 and GNT2. The results are promising and significant levels of the G0 glycans have been reached. The work will continue to optimize the G0 levels and to include fucosylation and galactosylation.

**CONCLUSIONS**
1. *Myceliophthora thermophila* C1 is an industrialized protein production host now developed for therapeutic protein manufacture.
2. 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.
3. Monoclonal antibodies have been produced in C1 with levels reaching 10.9 g/l and rates up to 2.4 g/d/l. The binding characteristics of the C1-produced antibodies were very similar to CHO-produced controls. Fab fragments have been expressed at levels up to 12 g/l in a 5-day process.
4. Difficult to express proteins (viral antigens, virus-like particles, sensitive Fc fusion proteins and bispecific antibodies) have been produced in C1 in superior levels as compared with other production systems.
5. 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 1:**

<table>
<thead>
<tr>
<th>Product</th>
<th>Production level g/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mabs</td>
<td>10.9</td>
</tr>
<tr>
<td>Fabs</td>
<td>12</td>
</tr>
<tr>
<td>Fc-fusion proteins</td>
<td>8.1</td>
</tr>
<tr>
<td>Difficult-to-express</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>