A binuclear mutant of *Pichia pastoris* was selected as an efficient recombinant protein producer.

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**Introduction**

*Pichia pastoris* (syn *Komagataella* spp) is a methylotrophic yeast which is able to metabolize several carbon sources, including methanol, glucose and glycerol. The availability of constitutive and inducible promoters and the ability to secrete the recombinant product to the culture supernatant makes *P. pastoris* a popular host for production of heterologous proteins of pharmaceutical and industrial interest. Generation of production strains routinely employs screening of a high number of transformants, and subsequent selection of the best performing clones. Such high producing clones were also chosen for thorough strain characterization by transcriptomics and proteomics.

**Selection of best production strain**

During the generation of antibody Fab fragment producing *P. pastoris* clones, more than 50 transformants were screened (Fig. 1A). The two clones with the highest titer ([#3 and [#6] were selected for fed batch cultivation, where again #3 had higher product titers despite lower biomass concentration (Fig. 1B and Tab. 1).

![Image](image1.png)

**Figure 1**: Selection of best performing clones for the production of an antibody Fab fragment in *P. pastoris* (A, left) CB7435 under control of the PcyB promoter and the S. cerevisiae alpha-mating factor promoter leader. Western Blots (anti-cH domain antibody) of culture supernatants of A) screening cultures and B) glucose limited fed batch cultivations (10-12 days).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Titer[1]</th>
<th>Fab concentration [mg L⁻¹]</th>
<th>Fab productivity[1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcyB Fab #3</td>
<td>74.1</td>
<td>104</td>
<td>0.096</td>
</tr>
<tr>
<td>PcyB Fab #6</td>
<td>101.8</td>
<td>71</td>
<td>0.027</td>
</tr>
</tbody>
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Thus PcyB-Fab#3 was chosen for as best production strain.

**The best producer has two nuclei per cell**

![Image](image2.png)

**Figure 3**: Microscopic images of PcyB-Fab#3 and CBS7435 control. A) TEM images. N, nucleus; M, mitochondria; LD, lipid droplet; size bar: 1µm. B) Fluorescence microscopy of PcyB-Fab#3 cells stained with DAPI.

In electron microscopy, cells producing the Fab had a very peculiar cell morphology. Actually, more than 90% of the analysed pictures appear to contain two nuclei. To rule out that this nuclear morphology is an artefact during preparation, fluorescence microscopy of intact cells was performed. DAPI-staining of the nucleus confirmed the binucleated phenotype of the PcyB-Fab3, which is indicative of a defect in chromosome segregation.

Transcriptomics analyses using microarrays revealed a high number of regulated genes in PcyB-Fab#3. Thereof 535 genes were up-regulated specifically in this strain, but not in other producing strains, and 680 genes were specifically downregulated.

**Chemostat cultivations**

For Omics analyses, PcyB-Fab#3 and CBS7435 wildtype as control were cultivated in biological triplicates in glucose limited chemostats at μ = 0.1 h⁻¹. The Fab-producing strain reached 85% of the biomass concentration of the control, which also corresponds to the viability measurements (Tab. 2).

<table>
<thead>
<tr>
<th>Clone</th>
<th>YDM [g L⁻¹]</th>
<th>QFab [mg L⁻¹ h⁻¹]</th>
<th>Viability [1]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcyB Fab #3</td>
<td>24.56</td>
<td>3.00</td>
<td>80 %</td>
</tr>
<tr>
<td>PcyB-Control</td>
<td>28.60</td>
<td>3.30</td>
<td>98 %</td>
</tr>
</tbody>
</table>

*Viability was determined by propidium iodide staining and flow cytometry.

Interestingly, PcyB-Fab#3 producing cells were larger and featured a higher percentage of cells with double (2C) and higher DNA content (Fig. 2). To study this phenomenon in more detail, cell morphology was analysed by electron microscopy in addition to multi-omics analyses.

**Multi-omics analyses**

Despite equal growth rate and lower biomass yield, upregulation of ribosomal biogenesis genes and genes involved in mitochondrial function and import machinery was observed in PcyB-Fab#3. On the other side, genes involved in cytokinesis etc were down-regulated (Figure 4). There was no effect on genes encoding proteins with functions in folding or secretion.

Proteomics data correlated well to the observed transcriptional changes and confirmed higher abundance of proteins associated with translation and amino acid biosynthesis (Figure 5).

There was also a high impact on intracellular metabolite levels (Fig. 6). Out of 30 measured free metabolite pools, 17 had different abundance in PcyB-Fab#3 compared to the control. Measurement of 16 intracellular amino acid pools showed that nine amino acid pools were significantly increased, in agreement with higher transcript and protein levels.

**Are two nuclei better than one? - Summary & Conclusions**

- One of the selected clones efficiently producing an antibody Fab fragment turned out to possess two nuclei. Flow cytometry analysis for DNA content and fluorescence microscopy to assess nuclear morphology confirmed the binucleated phenotype.
- Although microarray and proteomics analyses revealed a high number of differentially expressed genes and proteins and highlighted specific cellular processes that were affected by the binucelar phenotype, the underlying mutation could not be identified from these data.
- Taken together, a mutant with a binuclear phenotype that was selected as high production strain was working for production of a recombinant protein in bio reactor cultivations of *P. pastoris*.

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