

Genetic Modifications and Other Tools for the Enhancement and Optimization of Recombinant Protein Manufacturing in *Komagataella phaffii*

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The demand for high-quality recombinant proteins – ranging from biotherapeutics and vaccines to food ingredients and industrial enzymes – is rapidly growing worldwide. Meeting this demand, sustaining supply chains, and improving global accessibility to these proteins requires scalable and cost-efficient production platforms. Eukaryotic microorganisms are promising manufacturing hosts for meeting these manufacturing challenges due to their rapid growth rates, ability to secrete proteins into culture medium, relatively small profile of secreted host cell proteins, and overall robustness in bioprocesses. *Komagataella phaffii* (*K. phaffii*) (commercially known as “Pichia”) is one such microorganism that has been used to produce a wide array of recombinant proteins, including proteins used in food, materials, and FDA-approved biologics. Yet, for complex proteins, such as monoclonal antibodies (mAbs), space-time yields in *K. phaffii* remain below those seen from traditional manufacturing hosts (such as Chinese Hamster Ovary (CHO) cells) that have been optimized over decades through cellular and process engineering. This thesis presents several studies that narrowed this productivity gap by enhancing the volumetric productivity of *K. phaffii* through genetic engineering of the host cell and investigation of novel reactor operating strategies.

First, we explored the effects of disrupting non-essential genes on recombinant protein production in *K. phaffii*. We created a CRISPR-Cas9 screen that characterized the essentiality of every gene in the *K. phaffii* genome. We used the results from this screen in conjunction with biological inference for relevance to protein secretion to identify gene targets for genetic engineering. We disrupted 14 cell wall associated genes along with 15 vacuolar genes and observed significant improvement in the cell-specific productivity of a mAb (up to 9x) for 19 of those strains. We evaluated the performance of our best strains across multiple scales up to and including fermentation in a fed-batch reactor. We also assessed the effectiveness of combining the genetic engineering changes.

We expanded on this work by investigating whether transcriptomic data could be used in conjunction with essentiality to identify promising targets for genetic engineering. We found no correlation between gene expression level and the effectiveness of genetic edits; however, our screen did uncover an additional five genetic edits that significantly improved mAb production in *K. phaffii*.

Beyond direct genetic engineering of the host organism, we also developed a genetic engineering tool that could be used to enhance and optimize recombinant protein production in *K. phaffii*. Genomic integration can have a significant impact on volumetric productivity – specifically, the

number of copies of a heterologous transgene encoding a recombinant protein significantly influences yield and genetic stability. Current methods for determining the number of copies of a recombinant transgene have limited quantitative resolution for large constructs or constructs with high copy numbers. We developed a pipeline that uses long-read sequencing data to determine the transgene copy number for large, complex, and repetitive integrants. This pipeline can be used to characterize existing strains, evaluate the impact of copy number on productivity, support optimization of process conditions, inform strain engineering decisions, and minimize confounding variables in comparative studies.

This thesis also explored novel reactor operating conditions for engineered strains of *K. phaffii*. We developed and tested a stepped perfusion fermentation method that tested up to 11 different operating conditions within a single reactor run. We used this method to identify promising process parameters for the commercial production of NIST mAb in perfusion fermentation. We also developed a novel feeding strategy for fed-batch fermentation of trastuzumab-producing strains.

Closing the productivity gap between *K. phaffii* and traditional manufacturing hosts could unlock considerable economic and operational advantages for agile and flexible manufacturing. The work in this thesis introduced specific genetic engineering changes and operational fermentation modes that significantly improved the productivity of *K. phaffii* for specific proteins of interest. The strategies and tools developed and presented here provide a foundation for continued engineering of this organism to achieve target productivities and can be broadly applied to enhance recombinant protein production across diverse microbial systems.