Development of Modular Strategies for Enhancing Biomanufacturing in *Komagataella phaffii*

By
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The landscape of protein therapeutics is advancing towards more complex modalities that offer greater medical potential but present significant challenges to existing biomanufacturing frameworks. Alongside these technical challenges, socio-economic factors drive an urgent need for accelerated, cost-effective biomanufacturing to increase rapid response capabilities, ensure equitable access to advanced treatments, and alleviate the economic strain on global healthcare systems. This creates a significant and unresolved gap: the cost-effective production of increasingly complex protein therapeutics under an accelerated timeline. *Komagataella phaffii* holds great potential to fill this gap, as it is a eukaryotic microorganism well-established for the rapid and cost-effective expression of recombinant proteins. However, realizing this potential requires advanced engineering strategies to extend *K. phaffii*'s strengths to these complex modalities. This thesis presents the development of modular strategies to enhance the biomanufacturing of such complex therapeutics in *K. phaffii*.

The first part of this thesis focuses on providing alternative manufacturing strategies for VLP vaccines, a promising next-generation vaccine platform whose adoption has been limited by manufacturing challenges. To streamline VLP vaccine development and manufacturing, a modular production framework was developed. This framework's core strategy involves the secretory production of VLP scaffold subunits in *K. phaffii* followed by their in vitro assembly, which then allows for flexible "plug-and-display" antigen attachment to the pre-formed scaffold. This approach can be adopted for versatile antigen adaptation and can be stockpiled for rapid response. More importantly, this framework circumvents the manufacturing challenges associated with traditional intracellular VLP production, such as complex downstream purification that leads to increased production cost and decreased yield and quality. This transition not only makes each modular step more suited for current available operational units, especially downstream processing, significantly increasing scalability and reducing cost, but also holds promise for integration into future continuous manufacturing frameworks. A specific implementation and key outcome of this approach was the development of the SpyCatcher::I53-50 modular VLP scaffold, which was then validated by demonstrating the high-fidelity display of a SpyTag-fused HIV Env trimer antigen with preservation of critical neutralizing epitopes, showcasing its potential as a promising modular VLP vaccine platform. Enabling the secretory production of the scaffold's multimeric subunits required overcoming significant manufacturability challenges. For instance, proteolytic degradation of the SpyCatcher-153-50A fusion was resolved via protein engineering, while secretion of the aggregation-prone 153-50B subunit was achieved through a multi-pronged approach combining process optimization, host engineering, and a novel pseudo-chaperone strategy. These rational engineering efforts were not only crucial for producing these key building blocks but also serve as a practical roadmap for tackling other hard-to-produce targets.

The challenges encountered in efficiently optimizing VLP subunit production, even with extensive rational engineering, underscored a broader imperative for more powerful and systematic optimization tools. This realization motivated the second part of this thesis: the

development of a modular high-throughput screening (HTS) platform to accelerate the engineering of *K. phaffii* for enhanced production of complex biologics, using monoclonal antibodies (mAbs) as an industrially significant proof-of-concept. The HTS platform is built on a dual-mode yeast surface display (YSD) system. By establishing a quantitative correlation between surface display and secretion for a full-length mAb, this work unlocks the potential of YSD for reliably screening secretion phenotypes, enabling, for the first time, screening under normal cultivation conditions at an unprecedented scale. Its successful application in a genome-wide CRISPR/Cas9 knockout screen identified numerous novel host gene knockout targets. Individual validation of 20 top-ranked candidates confirmed that 15 led to statistically significant increases in mAb specific productivity, with the most effective target yielding an 8-fold improvement over the baseline strain. This represents a substantial technological advancement for *K. phaffii* engineering, offering a powerful engine to shift from empirical, low-throughput optimization towards systematic, data-driven cell line development. Designed as a modular platform, it holds promise for screening other perturbation libraries and for optimizing a wide range of protein targets beyond mAbs.

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