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Yarrowia lipolytica, *Komagataella phaffii* and secretory proteins: Recombinant laccase as a case study

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ABSTRACT

For decades, *Komagataella phaffii* has been the reference host for recombinant secretory protein (rsProt) production. However, secretion bottlenecks associated with the limited processing capacity of the endoplasmic reticulum restrict the secretion efficiency under high protein loads. Besides, *Yarrowia lipolytica* possesses a secretion pathway resembling that of filamentous fungi and naturally secretes large amounts of hydrolytic enzymes, making it a promising alternative host. In this study, we systematically investigated the impact of pre- and pro-sequences on the secretion of LacVader, an evolved laccase with industrial applications. In *Y. lipolytica*, nine constructs combining four pre-sequences (αPre, Yps3Pre, Lip2Pre, SoAmyPre) and two pro-sequences (αPro, Lip2Pro) were integrated at the *LIP2* locus and expressed under the constitutive P_{TEF} promoter. Comparative analysis revealed that most pre–pro constructs resulted in higher laccase specific activity compared to *K. phaffii* expressing the enzyme under the canonical P_{AOX1} promoter and the *S. cerevisiae* α-factor signal peptide. Notably, both the pre- and pro-sequences had a strong influence on laccase secretion in *Y. lipolytica*. The Lip2Pro sequence consistently enhanced secretion, with the Yps3Pre–Lip2Pro construct yielding the highest activity, eightfold greater than that obtained in *K. phaffii*. These findings highlight the crucial role of secretion signal optimization in rsProt production and confirm the superior potential of *Y. lipolytica* over *K. phaffii* as a robust host for industrial enzyme secretion.

For more than three decades, the methylotrophic yeast *Komagataella phaffii* has been a workhorse to produce recombinant secretory proteins (rsProt). The classical expression system, combining the tightly regulated promoter from alcohol oxidase 1 (P_{AOX1}) with the *Saccharomyces cerevisiae* α-factor signal peptide, remains a benchmark in industrial biotechnology (Bustos et al., 2022). Despite its advantages, including high cell density cultivation and the possibility to express recombinant genes under the control of the P_{AOX1} promoter at high level upon methanol induction, *K. phaffii* faces limitations in protein folding and secretion machinery. Under conditions of high rsProt synthesis, the endoplasmic reticulum (ER) becomes saturated, leading to protein misfolding, ER stress, and reduced secretion efficiency (Zahrl et al., 2018). More recently, *Yarrowia lipolytica* has emerged as a robust alternative host. This yeast grows efficiently on low-cost industrial byproducts, reaches high cell densities, and benefits from an expanding synthetic biology toolbox (Vandermies and Fickers, 2019, Liu and Qi, 2025). Importantly, *Y. lipolytica* naturally secretes large amounts of hydrolytic enzymes (lipases, proteases, laccases) and exhibits a filamentous fungi-like secretory pathway, which provides superior protein export capacity compared to *K. phaffii* (Nicaud, 2012, Celińska and Nicaud, 2019, Darvishi et al., 2018).

Secretion efficiency depends critically on N-terminal signal peptides (SP), which typically consist of a pre-sequence (Pre) that direct proteins into the ER and a pro-sequence (Pro) that increase protein solubility in the ER, assist protein folding, maturation, and trafficking to the Golgi compartment. The pre-sequence is cleaved in the ER by a signal peptidase, whereas the pro-sequence is removed later in the Golgi by Kex-like proteases. While the presence of a pro-sequence is not essential for protein secretion, the specific amino acid sequence of the pre-sequence is a critical determinant of rsProt productivity (Celińska et al., 2018, Celińska and Nicaud, 2019). We previously compared the production of lipase B from *Candida antarctica* (CalB) in both hosts and found that, despite a seven-fold higher mRNA level in *K. phaffii*, enzyme production was significantly higher in *Y. lipolytica*, a discrepancy attributed to the

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Table 1
Yeast strains used in this study.

Strain	Parental strain, (plasmid), genotype	Reference
RIY146	*Y. lipolytica*, Po1d, eyk1::Leu2ex, Ura$^-$	Vandermies et al. 2017
RIY667	*K. phaffii*, (pJRoC30), pPDF-αfactor-LacVader	Mateljak and Alcalde, (2021)
RIY668	RIY146, (RIP534), pTEF-αPre-LacVader-Lip2tt	This work
RIY670	RIY146, (RIP535), pTEF -Yps3Pre-LacVader-Lip2tt	This work
RIY672	RIY146, (RIP536), pTEF-Lip2Pre-LacVader-Lip2tt	This work
RIY674	RIY146, (RIP537), pTEF-SoAmyPre-LacVader-Lip2tt	This work
RIY676	RIY146, (RIP538), pTEF-αPre-αPro-LacVader-Lip2tt	This work
RIY678	RIY146, (RIP539), pTEF-Yps3Pre-αPro-LacVader-Lip2tt	This work
RIY680	RIY146, (RIP540), pTEF-Lip2Pre-αPro-LacVader-Lip2tt	This work
RIY682	RIY146, (RIP541), pTEF-SoAmyPre-αPro-LacVader-Lip2tt	This work
RIY684	RIY146, (RIP542), pTEF-αPre-Lip2Pro-LacVader-Lip2tt	This work
RIY686	RIY146, (RIP543), pTEF-Yps3Pre-Lip2Pro-LacVader-Lip2tt	This work
RIY688	RIY146, (RIP544), pTEF- Lip2Pre-Lip2Pro-LacVader-Lip2tt	This work
RIY690	RIY146, (RIP545), pTEF-SoAmy-Lip2Pro-LacVader-Lip2tt	This work

CalB protein proteasomal degradation before secretion in *K. phaffii* (Theron et al., 2020). To extend these findings, we evaluated herein the engineered laccase LacVader (Mateljak et al., 2021) as a model rsProt. Laccases are challenging proteins to secrete due to their size (60–100 kDa), extensive glycosylation, and the requirement for copper incorporation into multiple active sites. Nevertheless, laccases have diverse industrial applications including organic synthesis, pulp bleaching, dye decolorization, beverage stabilization, flavor enhancement, and as baking additives (Mateljak et al., 2021).

In *K. phaffii* strain RIY667 (Table 1), the LacVader coding sequence under the control of the P_{AOX1} promoter was transcriptionally fused to an evolved pre-pro-sequence from *S. cerevisiae* α-factor and integrated at the *AOX1* locus (Mate et al., 2013). In the different constructed *Y. lipolytica* strains, the LacVader expression cassettes were driven by the constitutive P_{TEF} promoter and targeted to the *LIP2* locus. Four pre-sequences were tested, namely αPre from *S. cerevisiae* α-factor, Yps3Pre from the *Y. lipolytica* GPI-anchored protease Yps3, Lip2Pre from the extracellular lipase Lip2, and SoAmyPre, a codon-optimized pre--sequence from the insect *Sitophilus oryzae* α-amylase SoAmy. These pre-sequences were selected based on their demonstrated secretory potential for heterologous proteins, namely α-amylase from *S. oryzae* and a glucoamylase from the fungus *Thermomyces lanuginosus* (Celińska et al., 2018). Two pro-sequences were also evaluated, namely the αPro from the *S. cerevisiae* α-factor and the Lip2Pro from the highly secreted *Y. lipolytica* Lip2 lipase (Celińska et al., 2018). A total of nine combinations of pre- and pro-sequences transcriptionally fused to the LacVader coding sequence were assembled using Golden Gate cloning and integrated into the *Y. lipolytica* genome at the *LIP2* locus (Fig. 1, Table 1). Biomass and laccase activity based on ABTS oxidation were measured at 60 h and 72 h, and specific activity was calculated. The methods related to plasmids and strains construction, cultures conditions and enzyme assay are detailed in supporting information A. Integration consistency check among the constructed strains led to the selection of those carrying a single copy of the LacVader expression cassette (Supporting Information B). Since all constructs were driven by the constitutive P_{TEF} promoter and integrated at the same *LIP2* locus, it was assumed that LacVader expression levels were comparable across the different *Y. lipolytica* strains. Cultivations were performed in 24-square deepwell plates under oxygen transfer conditions comparable to that of stirred tank reactors (i.e. 30–40 mmol O_2 l^{-1} h^{-1}, (Duetz et al., 2000, Duetz and Witholt, 2001).

At both sampling times, most *Y. lipolytica* constructs outperformed *K. phaffii* (Fig. 2). Only αPre, αPre-αPro, and Yps3Pre-αPro yielded lower laccase specific activities. These findings confirm our earlier observations with CalB (Theron et al., 2020) on the superior secretion capacity of *Y. lipolytica*. In *Y. lipolytica*, both pre- and pro-sequences modulated laccase specific activity, with clear evidence of synergistic effects. Specifically, laccase specific activity was minimal when the αPre sequence was used alone without a pro-sequence (NoPro, 0.3 ± 0.2 U·gCDW^{-1}, data at 72 h). Specific activity increased eight-fold upon combination with the αPro sequence (2.5 ± 0.7 U·gCDW^{-1}), and more than 110-fold when combined with the Lip2Pro sequence (34.9 ± 3.5 U·gCDW^{-1}). In contrast, the Yps3Pre sequence displayed a distinct behavior. Laccase specific activity was markedly reduced when Yps3Pre was combined with the αPro sequence compared with Yps3Pre alone (NoPro. 4.7 ± 2.2 U·gCDW^{-1} and 18.2 ± 3.1 U·gCDW^{-1} at 72 h, respectively). Conversely, a significant enhancement of laccase specific activity was observed when the Yps3Pre sequence was combined with the Lip2Pro sequence. At 72 h, the Yps3Pre-Lip2Pro construct yielded the highest laccase specific activity (58 ± 4 U·gCDW^{-1}) that is eight-fold higher than the specific laccase activity obtained for *K. phaffii* (7.3 ± 0.8 U·gCDW^{-1}). To further investigate for the differences in laccase specific activities observed for constructs based on the Yps3Pre with or without a Pro-sequence, the LacVader expression levels were quantified in strains Yps3Pre, Yps3Pre-αPro and Yps3Pre-Lip2Pro during both the exponential growth and stationary phases (i.e. at 24 h and 48 h, Fig. 3). Although

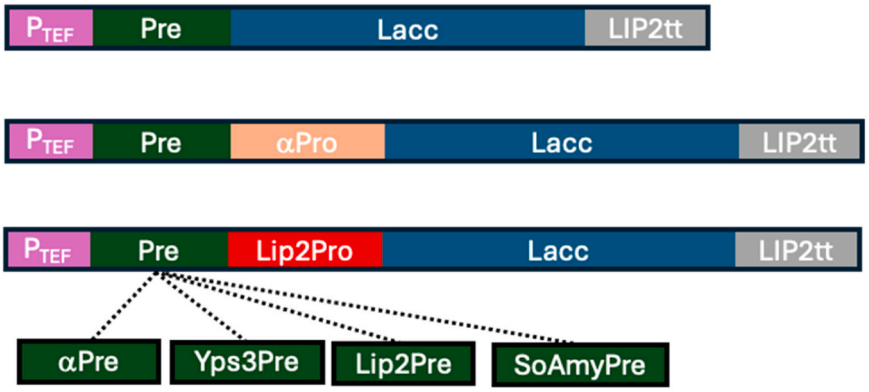

Fig. 1. Schematic representation of the constructed expression systems obtained by Golden Gate (GG) assembly (Supporting information A). Abbreviations are as follow: P_{TEF}, promoter pTEF-4UA (GGE146); Pre, presequence of α factor (αPre); Yps3 protease, (Yps3Pre); LIP2 lipase, (Lip2Pre); SoAmy α-amylase (SoAmyPre); Lacc, LacVader laccase encoding gene; LIP2tt; *LIP2* transcriptional terminator. All constructs were integrated at the *LIP2* locus of *Y. lipolytica* strain RIY146.

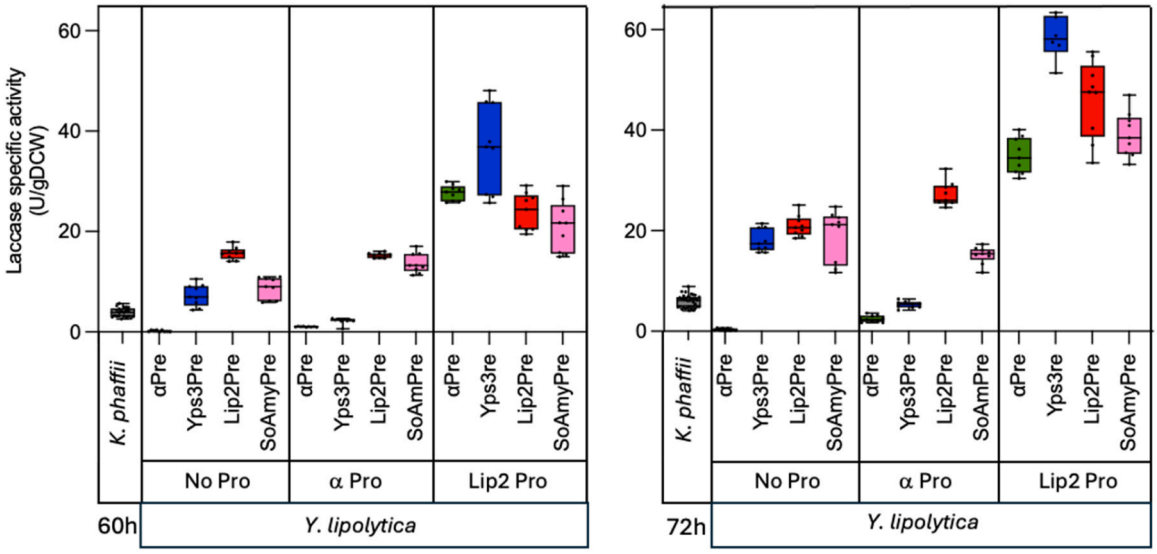

Fig. 2. Laccase specific activity after 60 h and 72 h of growth in BMD1medium for *K. phaffii* and YNBG-Cu for *Y. lipolytica* strains. Data are for biological triplicate cultures and technical duplicate of enzyme assays.

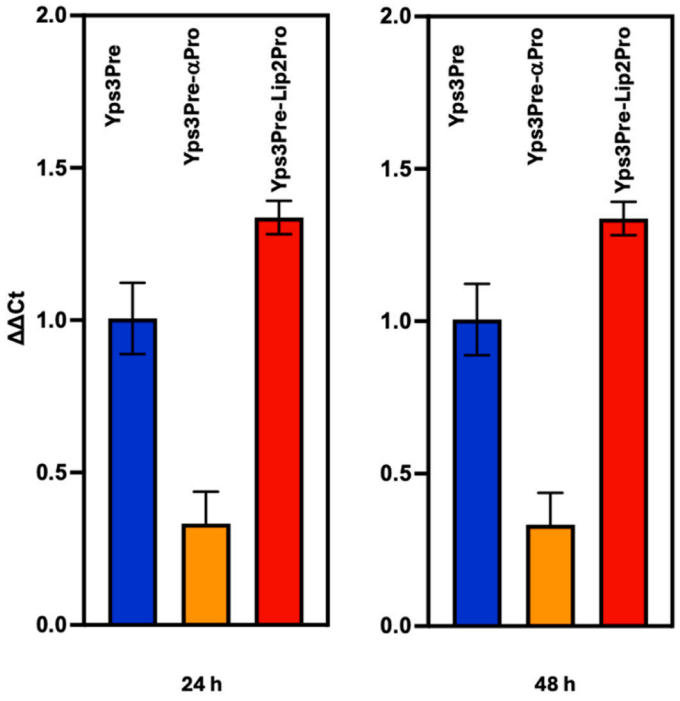

Fig. 3. LacVader expression level for *Y. lipolytica* Yps3Pre, Yps3Pre-αPro and Yps3Pre-Lip2Pro strains grown in YNBG-Cu after 24 h and 48 h. Data are means and standard deviation of triplicate cultures. Expression levels were first normalized to that of Actin (ΔCt) and to the mean expression level of Yps3Pre (ΔΔCt).

the LacVader gene was expressed under the constitutive P_{TEF} promoter, the modest variations in transcript abundance observed among the strains are most likely attributable to post-transcriptional effects rather than differences in promoter activity. In particular, the nucleotide sequence encoding the Pre- and Pro-sequence can influence mRNA secondary structure, translation efficiency, and ribosome dynamics, thereby modulating transcript stability independently of transcriptional control (Presnyak et al., 2015, Harigaya and Parker, 2016). However, the difference in LacVader mRNA relative quantification cannot support the differences observed in laccase specific activity. SoAmyPre and Lip2Pre based constructs showed no increase of laccase specific activity when combined with αPro but it significantly increases with Lip2Pro. Across all pre-sequences, Lip2Pro consistently outperformed αPro, with improvements ranging from 1.7-fold for Lip2Pre to 14-fold for αPre at 72 h.

Surprisingly, the dynamics of laccase specific activity between 60 h and 72 h were not consistent across all pre- and pro-sequence combinations. For most tested strains, the specific laccase activity increased during this interval; however, for certain constructs, namely SoAmyPre-αPro and αPre-Lip2Pro, the increase was less pronounced. In contrast, the combination of pre- and pro-sequences had no significant effect on cell growth with an average biomass of 4.6 ± 0.6 gDCW·L^{-1} at 72 h across all *Y. lipolytica* strains tested (Supporting Information C). Therefore, the nature of the pre- and pro-sequences did not appear to impose a differential metabolic burden. These findings demonstrate that the appropriate combination of pre- and pro-sequences is critical for efficient secretion, with the nature of the pro-sequence playing a decisive role in either enhancing or impairing enzyme production. Notably, the best-performing strain, Yps3Pre–Lip2Pro, achieved an eightfold higher specific activity than the *K. phaffii* reference strain. Altogether, this study shows that *Y. lipolytica* outperforms *K. phaffii* for recombinant laccase secretion and underscores the critical influence of signal peptide engineering. While *K. phaffii* remains a valuable production host, its secretory pathway is limited by folding and ER stress bottlenecks. By contrast, *Y. lipolytica* combines a naturally robust secretory machinery with flexibility in signal sequence design, enabling substantially higher enzyme yields without impairing growth. The synergistic effect of Yps3Pre-Lip2Pro highlights the potential of rational signal peptide optimization to overcome long-standing challenges in rsProt production. These findings reinforce *Y. lipolytica* as a powerful alternative host for industrial enzyme secretion and provide a framework for improving recombinant protein expression through secretion signal engineering.

Authors' statement

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CRediT authorship contribution statement

Constance Keller: Investigation, Formal analysis. **Patrick Fickers:** Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Miguel Alcalde:** Writing – review & editing. **Rocio Cozmar:** Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2026.01.015.

Data availability

Data will be made available on request.

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