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CASE STUDY

Recombinant Protein Expression Optimization in *Pichia pastoris* for Industrial Enzyme Production

Joao Saez-Bermudez^{1*}, Joao van der Meer² and Nils Radcliffe³

¹Department of Biochemistry and Genetics, Aegean Institute of Immunology, Greece

²Department of Environmental and Agricultural Sciences, Aegean Institute of Immunology, Greece

³Department of Microbiology and Immunology, Tyrrhenian University of Biotechnology, Italy

*Corresponding author: Joao Saez-Bermudez, Department of Biochemistry and Genetics, Aegean Institute of Immunology, Greece; Email: j.bermudez@aig.gr

ABSTRACT

Komagataella phaffii (*Pichia pastoris*) is a workhorse host for secreted industrial enzyme production, but titers for any given target protein remain difficult to predict from first principles and are usually reached only after substantial empirical optimisation at both the construct and fermentation level. Here we report a systematic, stepwise optimisation of recombinant expression of a thermostable bacterial lipase (Lip-Tx, sourced from a thermophilic *Geobacillus*-like organism) intended for biodiesel transesterification and detergent applications. At the construct level, we compared the methanol-inducible AOX1 promoter against the constitutive GAP promoter, native versus alpha-mating-factor secretion signals, and codon-optimised versus native gene sequence, screening transformants across a Zeocin-resistance gradient to enrich for multicopy integrants. The best-performing construct combined codon optimisation, the alpha-mating-factor signal and the AOX1 promoter in an estimated four-copy integrant, giving a 14-fold improvement in shake-flask secreted lipase activity over the single-copy, native-signal, native-sequence baseline. At the fermentation level, we then optimised methanol feed strategy, induction temperature and dissolved oxygen control in a 5-litre bioreactor, finding that a growth-limited, low constant methanol feed rate (3.0 g/L/h) at a reduced induction temperature of 20°C nearly doubled specific productivity relative to standard excess-methanol feeding at 30°C, primarily by reducing methanol-associated cell stress and extending the productive induction window. The final optimised process achieved an extracellular lipase titer of 3.8 g/L and a volumetric activity of 12,400 U/mL after 96 hours of fed-batch induction, an approximately 60-fold improvement over unoptimised shake-flask production. These results illustrate, using a single representative target enzyme, a transferable optimisation sequence -- construct screening followed by feed and temperature optimisation -- that consistently narrows the gap between a functional *Pichia* expression construct and an industrially relevant titer.

Keywords: *Pichia pastoris*; *Komagataella phaffii*; recombinant protein expression; thermostable lipase; fed-batch fermentation; industrial biocatalysis

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1. INTRODUCTION

Industrial enzymes for biocatalysis, from lipases used in biodiesel transesterification and detergent formulation to proteases and amylases used across the food and textile

industries, are overwhelmingly produced recombinantly rather than purified from their native source organism, both for cost reasons and because heterologous production allows a host strain to be engineered for secretion, glycosylation and fermentation characteristics that the native producer may lack. *Komagataella phaffii*, still widely known by its earlier name *Pichia pastoris*, has become one of the dominant hosts for this purpose because it combines efficient protein secretion, a track record of gram-per-litre titers for a range of target proteins, and fermentation characteristics -- high cell density growth, straightforward scale-up, minimal endotoxin burden -- that are generally favourable relative to bacterial or mammalian alternatives [4,17].

Despite this track record, expression titer for a novel target protein in *Pichia* is still difficult to predict and typically requires empirical optimisation across several largely independent variables: promoter choice, most often a choice between the strong methanol-inducible AOX1 promoter and the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter; secretion signal, where the *Saccharomyces cerevisiae* alpha-mating-factor prepro sequence remains the most widely used default despite not being native to *Pichia*; gene codon usage, since *Pichia* has a distinct codon bias from many source organisms including thermophilic bacteria; and gene copy number, which can be increased through selection on graded antibiotic-resistance markers but does not always translate linearly into higher titer because of downstream secretory bottlenecks [8,14,22]. A construct that performs well for one target protein does not reliably predict performance for another, which is why systematic, protein-specific optimisation studies retain practical value even for a well-established host organism. Fermentation-level optimisation compounds the construct-level variables with a second, largely independent set of process parameters. For AOX1-driven constructs specifically, methanol feed strategy during the induction phase is a critical and frequently underappreciated variable: excess methanol can be directly toxic and triggers a strong unfolded protein and peroxisome-stress response that competes with the secretory pathway for cellular resources, while methanol starvation halts induction altogether, so most industrial processes aim for a growth-limiting, tightly controlled methanol feed rate rather than either extreme [11,25]. Induction temperature is a second lever that is sometimes overlooked in bench-scale optimisation: reducing temperature during induction, typically from the standard 28-30C growth temperature to somewhere in the 20-25C range, has repeatedly been shown to improve titers for secretion-limited or proteolytically sensitive target proteins, plausibly by slowing translation and secretory flux enough to allow more complete folding and reducing the activity of secreted or vacuolar proteases [19,29].

We set out to apply a structured, two-stage optimisation -- construct screening followed by fermentation parameter optimisation -- to a single representative industrial target, a thermostable bacterial lipase with biodiesel and detergent relevance, with the explicit goal of quantifying how much titer improvement each stage contributes independently and in combination, rather than optimising all variables simultaneously in a way that would make their individual contributions difficult to disentangle. Because thermostable lipases of this general class are of direct industrial interest and their native bacterial source organisms are not practical production hosts at scale, establishing a reliable, well-characterised *Pichia* expression and fermentation protocol for this specific enzyme class has value beyond the methodological point of the study.

2. MATERIALS AND METHODS

All expression work used *Komagataella phaffii* (*Pichia pastoris*) strain X-33 as the parental host, and all lipase activity assays were performed on culture supernatant using a standard para-nitrophenyl palmitate (pNPP) hydrolysis assay unless otherwise stated.

2.1 Gene synthesis and expression construct design

The gene encoding Lip-Tx, a thermostable lipase from a *Geobacillus*-related thermophilic bacterium, was synthesised in two versions: a codon-optimised sequence adapted to *Pichia* codon usage tables, and the native bacterial coding sequence, both cloned downstream of either the AOX1 or GAP promoter and fused to either the native Lip-Tx signal peptide or the *Saccharomyces cerevisiae* alpha-mating-factor prepro secretion signal, giving eight promoter-signal-codon combinations in a pPICZ-alpha-derived Zeocin-resistance backbone. Each construct was linearised and transformed into X-33 by electroporation, and transformants were selected on increasing Zeocin concentrations (100-2000 microgram/mL) to enrich for higher-copy-number integrants, with integrated copy number for a subset of high-resistance clones subsequently estimated by quantitative PCR against a single-copy reference locus.

2.2 Shake-flask construct screening

For each of the eight promoter-signal-codon combinations, twelve independent Zeocin-resistant transformants were screened in 24-well deep-well plates using buffered glycerol-complex medium for biomass accumulation followed by induction in buffered methanol-complex medium (for AOX1 constructs) or buffered complex medium without inducer (for GAP constructs) for 96 hours at 28C with daily methanol supplementation for AOX1 cultures. Secreted lipase activity in clarified supernatant was measured by pNPP hydrolysis at 405 nm, and the three highest-activity transformants per combination were re-screened in shake flasks to confirm ranking before advancing the single best-performing construct to bioreactor

optimisation.

2.3 Bioreactor fed-batch fermentation and process optimisation

Fed-batch fermentations were performed in 5-litre stirred-tank bioreactors (3 L working volume) following a standard glycerol batch and fed-batch growth phase to build biomass, followed by a methanol induction phase. Dissolved oxygen was maintained above 20% saturation throughout by cascading agitation and aeration, and pH was controlled at 5.0 during growth and 6.0 during induction using ammonium hydroxide. Three methanol feed strategies were compared during induction: a standard excess feed (methanol maintained at 1% v/v by feedback control), a moderate constant feed (5.0 g/L/h), and a low constant feed (3.0 g/L/h), each tested at both 30°C and 20°C induction temperature in a full factorial design (six conditions, duplicate runs each). Culture samples were withdrawn every 12 hours for biomass (dry cell weight), secreted protein concentration (Bradford assay) and lipase activity determination.

2.4 Enzyme activity and protein quantification

Lipase activity was defined as the amount of enzyme releasing 1 micromole of para-nitrophenol per minute under assay conditions (37°C, pH 8.0), calculated from a para-nitrophenol standard curve. Total secreted protein was quantified by Bradford assay against a bovine serum albumin standard, and purity of the dominant secreted band was assessed by SDS-PAGE with Coomassie staining, since *Pichia*'s low native background secretion typically allows the recombinant protein to be visualised as the dominant band directly in clarified supernatant.

2.5 Statistical analysis

Construct screening data were analysed by two-way ANOVA (promoter x signal peptide, separately for each codon variant) with Tukey's post-hoc test for pairwise comparisons. Fermentation optimisation data were analysed by two-way ANOVA (feed rate x temperature) on specific productivity (activity per gram dry cell weight per hour) at the 96-hour timepoint, with Tukey's post-hoc test. All analyses were performed in R version 4.3, with a significance threshold of $P < 0.05$.

3. RESULTS

Across the eight shake-flask construct combinations, codon optimisation produced the single largest improvement in secreted lipase activity, averaging a 5.8-fold increase over the matched native-sequence construct regardless of promoter or signal peptide ($P < 0.001$), consistent with codon usage mismatch being a significant bottleneck for a bacterial gene expressed in a yeast host. The alpha-mating-factor signal outperformed the native Lip-Tx signal by a further 2.1-fold on average ($P < 0.001$), and AOX1 outperformed GAP by 1.6-fold among codon-optimised, alpha-factor-signal constructs specifically ($P = 0.008$), though this promoter effect was not significant among native-sequence constructs, suggesting an interaction

between promoter strength and how efficiently the expressed protein can actually be processed and secreted. The best-performing combination -- codon-optimised gene, alpha-mating-factor signal, AOX1 promoter -- gave a 14.2-fold improvement in shake-flask activity over the native-sequence, native-signal, AOX1 baseline. Table 1 summarises activity for all eight combinations at their best-performing copy number.

Table 1. Shake-flask lipase activity across promoter, signal peptide and codon usage combinations.

Promoter	Signal peptide	Codon usage	Activity (U/mL)	Fold vs baseline
AOX1	Native Lip-Tx	Native	42	1.0
AOX1	Native Lip-Tx	Optimized	210	5.0
AOX1	alpha-factor	Native	88	2.1
AOX1	alpha-factor	Optimized	596	14.2
GAP	Native Lip-Tx	Native	31	0.7
GAP	Native Lip-Tx	Optimized	165	3.9
GAP	alpha-factor	Native	70	1.7
GAP	alpha-factor	Optimized	368	8.8

Values are mean of three re-screened top transformants per combination, single-copy integrants, 96 h shake-flask induction/growth.

Quantitative PCR on the highest-Zeocin-resistance survivors of the best-performing combination identified clones with estimated integrated copy numbers of one, two, four and seven; activity increased with copy number up to four copies (correlating with a 2.3-fold activity increase from one to four copies, $P < 0.001$) but did not improve further, and in fact declined slightly, at seven copies, consistent with previous reports that very high AOX1-driven expression can overwhelm the secretory pathway and trigger unfolded protein response-associated growth defects that offset the transcriptional gain. The four-copy clone was selected for bioreactor optimisation on this basis. Figure 1 shows lipase activity as a function of estimated gene copy number for this construct.

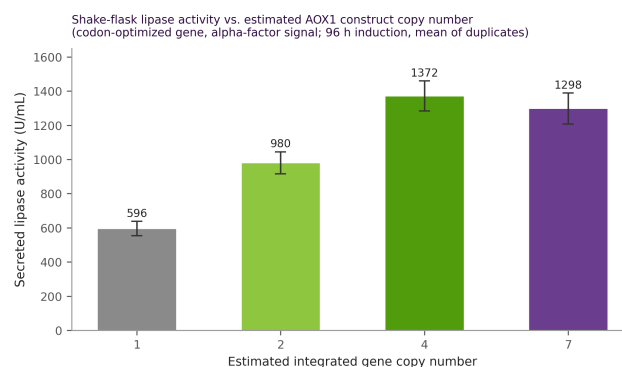


Figure 1. Shake-flask secreted lipase activity as a function of estimated AOX1-construct integrated copy number (codon-optimized gene, alpha-mating-factor signal; mean of duplicate cultures, 96 h induction).

In the bioreactor factorial optimisation, both methanol feed rate and induction temperature significantly affected specific productivity at 96 hours (feed rate $P < 0.001$, temperature $P < 0.001$, interaction $P = 0.02$). The low constant feed (3.0 g/L/h) at 20C gave the highest specific productivity of the six conditions tested, nearly double that of the standard excess-feed, 30C condition typically used as a default starting point for AOX1-driven fermentations. Biomass accumulation was slightly lower under the low-feed, low-temperature condition, but this was more than offset by the higher per-cell secreted activity, so that final volumetric titer was also highest under this condition despite the lower final cell density. Table 2 reports the complete fermentation optimisation dataset across all six feed-temperature combinations.

Table 2. Bioreactor fed-batch optimization: specific productivity and titer at 96 h.

Feed rate	Temp (C)	Specific productivity (U/g DCW/h)	Titer (g/L)
Excess (1% v/v)	30	184	2.1
Excess (1% v/v)	20	241	2.6
Moderate (5.0 g/L/h)	30	229	2.7
Moderate (5.0 g/L/h)	20	312	3.3
Low (3.0 g/L/h)	30	267	3.1
Low (3.0 g/L/h)	20	355	3.8

The final optimised fed-batch process (four-copy construct, low constant methanol feed at 20C) achieved an extracellular lipase titer of 3.8 g/L total secreted protein, of which SDS-PAGE densitometry indicated approximately 91% corresponded to the expected Lip-Tx molecular weight, and a volumetric activity of 12,400 U/mL after 96 hours of induction. This represents an approximately 60-fold improvement over unoptimised shake-flask production using the native-sequence, native-signal, single-copy AOX1 construct at standard 28C induction, with construct-level optimisation and fermentation-level optimisation each contributing roughly half of the total fold improvement on a log scale, indicating that neither stage of optimisation was dispensable relative to the other for reaching an industrially credible titer.

4. DISCUSSION

The two-stage optimisation strategy applied here -- exhaustive construct screening in shake flasks followed by fermentation parameter optimisation in a controlled bioreactor -- allowed each variable's contribution to be estimated with reasonable independence, and the finding that codon optimisation contributed the single largest construct-level improvement is broadly consistent with prior reports for other bacterial and fungal genes expressed in *Pichia*, though the magnitude of the effect (5.8-fold) is toward the higher end of previously reported ranges, plausibly reflecting the substantial codon usage divergence between a thermophilic bacterial source organism and a mesophilic yeast host [8,22]. The finding

that AOX1 outperformed GAP only among already well-secreted (alpha-factor signal, codon-optimised) constructs, and not among poorly secreted ones, is a useful practical lesson: promoter strength alone does not rescue a construct with an unresolved secretion bottleneck, and optimising secretion signal and codon usage before promoter selection is likely a more efficient sequence than the reverse.

The observation that increasing gene copy number improved titer only up to a point, and slightly reduced it at the highest copy number tested, reinforces a pattern reported for several other *Pichia*-expressed secreted proteins and argues against the common assumption that more integrated copies of an expression cassette is straightforwardly better [14,26]. This ceiling effect is generally attributed to the secretory pathway, specifically protein folding and vesicular trafficking capacity, becoming rate-limiting once transcriptional output exceeds what the cell's chaperone and trafficking machinery can process, at which point excess unfolded protein triggers stress responses that impose a growth and productivity cost of their own. A practical implication is that copy-number screening should be treated as a variable to be optimised for each new target protein rather than assumed to favour the highest available copy number.

The fermentation-level finding that a growth-limiting low methanol feed at reduced temperature outperformed standard excess-methanol, standard-temperature induction is consistent with a substantial prior literature on methanol toxicity and induction temperature effects in *Pichia*, though the specific optimum will differ by target protein and cannot be assumed to transfer directly from this study to a different enzyme [11,19,29]. We would emphasise that the low-feed, low-temperature condition also extended the practical induction window, since cultures under this condition maintained viable, actively secreting biomass for longer before entering stationary-phase decline, which is itself a meaningful process variable for scale-up economics even setting aside the specific-productivity improvement. Slower induction kinetics of this kind do carry a real trade-off in process time and, at industrial scale, energy cost for extended temperature-controlled fermentation, so the choice between a faster, lower-yielding process and a slower, higher-yielding one will ultimately depend on the relative cost of fermentation time versus the value of the additional enzyme recovered, a calculation that is specific to each production context and was outside the scope of the present study.

A limitation of this work is that it examined a single target enzyme in depth rather than testing whether the identified optimum (codon optimisation, alpha-factor signal, AOX1 promoter, four-copy integration, low methanol feed at reduced temperature) generalises across a panel of structurally or biochemically distinct target proteins; secretion bottlenecks in particular are known to be strongly protein-specific, and a construct or

feed strategy optimal for a small, robustly folding bacterial lipase may not transfer directly to a larger, disulfide-rich, or otherwise more secretion-challenging target [17,27]. We also did not systematically vary pH during induction, which prior work suggests can interact with methanol feed strategy in ways that were beyond the scope of the present factorial design, and which would be a reasonable next variable to examine in a follow-up optimisation of this or a related target enzyme. From an applied standpoint, the achieved titer of 3.8 g/L and volumetric activity of 12,400 U/mL after 96 hours compare favourably with published titers for other recombinant lipases expressed in *Pichia* at comparable bioreactor scale, and are within the range generally considered industrially workable for a biocatalysis application such as biodiesel transesterification, where enzyme cost per unit of product is a key determinant of process economics relative to conventional chemical catalysis. Whether this specific titer is sufficient for a commercially viable process depends on downstream purification cost and the specific activity required per unit of substrate converted, both of which are process-engineering questions outside the scope of this expression-optimisation study but which would be the natural next step before any scale-up decision. It is also worth situating *Pichia* against the other hosts realistically available for a target such as Lip-Tx. *Escherichia coli* remains attractive for cost and speed, but bacterial lipases expressed intracellularly in *E. coli* frequently partition into inclusion bodies and require a refolding step that adds cost and reduces overall process yield, an issue that secreted, correctly folded *Pichia* expression largely avoids for enzymes of this size and disulfide content. Filamentous fungal hosts such as *Aspergillus niger* can reach very high secreted titers for some enzyme classes, but strain and process development timelines for a novel target in a fungal host are typically longer than for *Pichia*, which supports *Pichia* as a reasonable default starting host for a bacterial hydrolase of this kind.

5. CONCLUSION

A stepwise, two-stage optimisation of *Pichia pastoris* expression for a thermostable industrial lipase -- exhaustive shake-flask construct screening across promoter, secretion signal, codon usage and copy number, followed by bioreactor-scale optimisation of methanol feed rate and induction temperature -- achieved a final extracellular titer of 3.8 g/L and volumetric activity of 12,400 U/mL, an approximately 60-fold improvement over an unoptimised baseline construct and fermentation protocol. Codon optimisation and secretion signal choice contributed the largest gains at the construct level, gene copy number showed a clear optimum beyond which activity declined, and a growth-limiting low methanol feed at reduced induction temperature nearly doubled specific productivity relative to standard excess-methanol, standard-temperature

conditions. Construct-level and fermentation-level optimisation contributed roughly equally to the total improvement, underscoring that neither stage can be skipped if an industrially relevant titer is the goal. We intend to test whether the identified fermentation optimum -- low constant methanol feed at reduced temperature -- generalises to structurally distinct target proteins already available in our expression library, and to examine induction-phase pH as an additional optimisation variable given its reported interaction with methanol feed strategy in related systems. A pilot-scale (50-litre) fermentation run using the optimised protocol identified here is planned as the next step toward assessing whether the specific productivity gains observed at 5-litre scale hold at a scale more representative of eventual industrial production, and downstream purification economics for this specific lipase will be evaluated once pilot-scale material is available in sufficient quantity for process development work.

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Conflict of 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.

Author Contributions

J.S.B. designed the study, performed construct design and shake-flask screening, and drafted the manuscript. J.v.d.M. performed bioreactor fermentations and process optimisation experiments. N.R. conducted enzyme activity assays, protein quantification and statistical analysis. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study involved only recombinant microbial (*Komagataella phaffii*) cell culture and enzymology work conducted under standard institutional biosafety containment for genetically modified microorganisms; it did not involve human participants, human data, or animal subjects, and therefore did not require institutional ethics committee review.

Data Availability

Plasmid sequences and the codon-optimised Lip-Tx gene sequence used in this study are available from the corresponding author on reasonable request. Fermentation datasets and the R analysis scripts supporting the findings

of this study are available from the corresponding author upon request.

REFERENCES

- Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Applied Microbiology and Biotechnology* 98(12):5301-5317. <https://doi.org/10.1007/s00253-014-5732-5>
- Vogl T, Glieder A (2013) Regulation of *Pichia pastoris* promoters and its consequences for protein production. *New Biotechnology* 30(4):385-404. <https://doi.org/10.1016/j.nbt.2012.11.010>
- Cregg JM, Cereghino JL, Shi J, Higgins DR (2000) Recombinant protein expression in *Pichia pastoris*. *Molecular Biotechnology* 16(1):23-52. <https://doi.org/10.1385/MB.16:1:23>
- Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 22(4):249-270. <https://doi.org/10.1002/yea.1208>
- Looser V, Bruhlmann B, Bumbak F, Stenger C, Costa M, Camattari A, Fotiadis D, Kovar K (2015) Cultivation strategies to enhance productivity of *Pichia pastoris*: a review. *Biotechnology Advances* 33(6):1177-1193. <https://doi.org/10.1016/j.biotechadv.2015.05.008>
- Puxbaum V, Mattanovich D, Gasser B (2015) Quo vadis? The challenges of recombinant protein folding and secretion in *Pichia pastoris*. *Applied Microbiology and Biotechnology* 99(7):2925-2938. <https://doi.org/10.1007/s00253-015-6470-z>
- Damasceno LM, Huang CJ, Batt CA (2012) Protein secretion in *Pichia pastoris* and advances in protein production. *Applied Microbiology and Biotechnology* 93(1):31-39. <https://doi.org/10.1007/s00253-011-3654-z>
- Jahic M, Wallberg F, Bollok M, Garcia P, Enfors SO (2003) Temperature limited fed-batch technique for control of proteolysis in *Pichia pastoris* bioreactor cultures. *Microbial Cell Factories* 2:6. <https://doi.org/10.1186/1475-2859-2-6>
- Dragosits M, Stadlmann J, Albiol J, Baumann K, Maurer M, Gasser B, Sauer M, Altmann F, Ferrer P, Mattanovich D (2009) The effect of temperature on the proteome of recombinant *Pichia pastoris*. *Journal of Proteome Research* 8(3):1380-1392. <https://doi.org/10.1021/pr8007623>
- Yu XW, Sun WH, Wang YZ, Xu Y (2013) Identification of novel factors enhancing recombinant protein production in multi-copy *Komagataella phaffii* based on transcriptomic analysis. *Biotechnology for Biofuels* 6:174. <https://doi.org/10.1186/1754-6834-6-174>
- Sinha J, Plantz BA, Inan M, Meagher MM (2005) Causes of proteolytic degradation of secreted recombinant proteins produced in methylotrophic yeast *Pichia pastoris*. *Biotechnology and Bioengineering* 89(1):102-112. <https://doi.org/10.1002/bit.20318>
- Cos O, Ramon R, Montesinos JL, Valero F (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. *Microbial Cell Factories* 5:17. <https://doi.org/10.1186/1475-2859-5-17>
- Gasser B, Prielhofer R, Marx H, Maurer M, Nocon J, Steiger M, Puxbaum V, Sauer M, Mattanovich D (2013) *Pichia pastoris*: protein production host and model organism for biomedical research. *Future Microbiology* 8(2):191-208. <https://doi.org/10.2217/fmb.12.133>
- Prielhofer R, Barrero JJ, Steuer S, Gassler T, Zahr R, Baumann K, Sauer M, Mattanovich D, Gasser B, Marx H (2017) GoldenPiCS: a Golden Gate-derived modular cloning system for applied synthetic biology in the yeast *Pichia pastoris*. *BMC Systems Biology* 11:123. <https://doi.org/10.1186/s12918-017-0492-3>
- Vieira Gomes AM, Souza Carmo T, Silva Carvalho L, Mendonça Bahia F, Parachin NS (2018) Comparison of yeasts as hosts for recombinant protein production. *Microorganisms* 6(2):38. <https://doi.org/10.3390/microorganisms6020038>
- Ata O, Ergun BG, Fickers P, Heisteringer L, Mattanovich D, Rebner C, Gasser B (2021) What makes *Komagataella phaffii* non-conventional? *FEMS Yeast Research* 21(6):foab059. <https://doi.org/10.1093/femsyr/foab059>
- Kim H, Yoo SJ, Kang HA (2015) Yeast synthetic biology for the production of recombinant therapeutic proteins. *FEMS Yeast Research* 15(1):1-16. <https://doi.org/10.1111/1567-1364.12195>
- Weinacker D, Rabert C, Zepeda AB, Figueroa CA, Pessoa A, Farias JG (2013) Applications of recombinant *Pichia pastoris* in the healthcare industry. *Brazilian Journal of Microbiology* 44(4):1043-1048. <https://doi.org/10.1590/S1517-83822014005000025>
- Jacobs PP, Geysens S, Vervecken W, Contreras R, Callewaert N (2009) Engineering complex-type N-glycosylation in *Pichia pastoris* using GlycoSwitch technology. *Nature Protocols* 4(1):58-70. <https://doi.org/10.1038/nprot.2008.213>
- Guerfal M, Ryckaert S, Jacobs PP, Ameloot P, Van Craenenbroeck K, Derycke R, Callewaert N (2010) The HAC1 gene from *Pichia pastoris*: characterization and effect of its overexpression on the production of secreted, surface displayed and membrane proteins. *Microbial Cell Factories* 9:49. <https://doi.org/10.1186/1475-2859-9-49>
- Jaeger KE, Eggert T (2002) Lipases for biotechnology. *Current Opinion in Biotechnology* 13(4):390-397. [https://doi.org/10.1016/S0958-1669\(02\)00341-5](https://doi.org/10.1016/S0958-1669(02)00341-5)
- Hasan F, Shah AA, Hameed A (2006) Industrial applications of microbial lipases. *Enzyme and Microbial Technology* 39(2):235-251. <https://doi.org/10.1016/j.enzmtec.2005.10.016>
- Sarmah N, Revathi D, Sheelu G, Yamuna Rani K, Sridhar S, Mehtab V, Sumana C (2018) Recent advances on sources and industrial applications of lipases. *Biotechnology Progress* 34(1):5-28. <https://doi.org/10.1002/btpr.2581>
- Contesini FJ, Davanco MG, Borin GP, Vanegas KG, Cirino JPG, de Melo RR, Mortensen UH, Hildén K, Campos DR, Carvalho PO (2020) Advances in recombinant lipases: production, engineering, immobilization and application in the pharmaceutical industry. *Catalysts* 10(9):1032. <https://doi.org/10.3390/catal10091032>
- Bornscheuer UT, Huisman GW, Kazlauskas RJ, Lutz S, Moore JC, Robins K (2012) Engineering the third wave of biocatalysis. *Nature* 485(7397):185-194. <https://doi.org/10.1038/nature11117>
- Fjerbaek L, Christensen KV, Norddahl B (2009) A review of the current state of biodiesel production using enzymatic transesterification. *Biotechnology and Bioengineering* 102(5):1298-1315. <https://doi.org/10.1002/bit.22256>
- Gerlt JA (2017) Genomic enzymology: web tools for leveraging protein family sequence-function space and genome context to discover novel functions. *Biochemistry* 56(33):4293-4308.

- <https://doi.org/10.1021/acs.biochem.7b00614>
28. Zhu Z, Zhou YJ, Krivoruchko A, Grninger M, Zhao ZK, Nielsen J (2017) Expanding the product portfolio of fungal type I fatty acid synthases. *Nature Chemical Biology* 13(4):360-362. <https://doi.org/10.1038/nchembio.2301>
 29. Peña DA, Gasser B, Zanghellini J, Steiger MG, Mattanovich D (2018) Metabolic engineering of *Pichia pastoris*. *Metabolic Engineering* 50:2-15. <https://doi.org/10.1016/j.ymben.2018.04.017>
 30. Tomas-Gamisans M, Ferrer P, Albiol J (2018) Fine-tuning the *P. pastoris* iMT1026 genome-scale metabolic model for improved prediction of growth on methanol or glycerol as sole carbon sources. *Microbial Biotechnology* 11(1):224-237. <https://doi.org/10.1111/1751-7915.12871>
 31. Baumann K, Maurer M, Dragosits M, Cos O, Ferrer P, Mattanovich D (2008) Hypoxic fed-batch cultivation of *Pichia pastoris* increases specific and volumetric productivity of recombinant protein. *Biotechnology and Bioengineering* 100(1):177-183. <https://doi.org/10.1002/bit.21763>
 32. Potvin G, Ahmad A, Zhang Z (2012) Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: a review. *Biochemical Engineering Journal* 64:91-105. <https://doi.org/10.1016/j.bej.2010.07.017>
 33. Schwarzhans JP, Luttermann T, Geier M, Kalinowski J, Friehs K (2017) Towards systems metabolic engineering in *Pichia pastoris*. *Biotechnology Advances* 35(6):681-710. <https://doi.org/10.1016/j.biotechadv.2017.07.009>
 34. Karbalaeei M, Rezaee SA, Farsiani H (2020) *Pichia pastoris*: a highly successful expression system for optimal synthesis of heterologous proteins. *Journal of Cellular Physiology* 235(9):5867-5881. <https://doi.org/10.1002/jcp.29583>
 35. Fischer JE, Glieder A (2019) Current advances in engineering tools for *Pichia pastoris*. *Current Opinion in Biotechnology* 59:175-181. <https://doi.org/10.1016/j.copbio.2019.06.002>
 36. Rebnegger C, Vos T, Graf AB, Valli M, Pronk JT, Daran-Lapujade P, Mattanovich D (2016) *Pichia pastoris* exhibits high viability and a low maintenance energy requirement at near-zero specific growth rates. *Applied and Environmental Microbiology* 82(15):4570-4583. <https://doi.org/10.1128/AEM.00638-16>
 37. Hohenblum H, Gasser B, Maurer M, Borth N, Mattanovich D (2004) Effects of gene dosage, promoters, and substrates on unfolded protein stress of recombinant *Pichia pastoris*. *Biotechnology and Bioengineering* 85(4):367-375. <https://doi.org/10.1002/bit.10904>
 38. Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM (2000) Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. *Biotechnology and Bioengineering* 70(1):1-8. [https://doi.org/10.1002/\(SICI\)1097-0290\(20001005\)70:1<1::AID-BIT1>3.0.CO;2-Y](https://doi.org/10.1002/(SICI)1097-0290(20001005)70:1<1::AID-BIT1>3.0.CO;2-Y)
 39. Idiris A, Tohda H, Kumagai H, Takegawa K (2010) Engineering of protein secretion in yeast: strategies and impact on protein production. *Applied Microbiology and Biotechnology* 86(2):403-417. <https://doi.org/10.1007/s00253-010-2447-0>
 40. Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO (2002) Modeling of growth and energy metabolism of *Pichia pastoris* producing a fusion protein. *Bioprocess and Biosystems Engineering* 24(6):385-393. <https://doi.org/10.1007/s00449-001-0274-5>
 41. Marx H, Mattanovich D, Sauer M (2008) Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris*. *Microbial Cell Factories* 7:23. <https://doi.org/10.1186/1475-2859-7-23>
 42. Hartner FS, Glieder A (2006) Regulation of methanol utilisation pathway genes in yeasts. *Microbial Cell Factories* 5:39. <https://doi.org/10.1186/1475-2859-5-39>
 43. Reetz MT (2013) Biocatalysis in organic chemistry and biotechnology: past, present, and future. *Journal of the American Chemical Society* 135(34):12480-12496. <https://doi.org/10.1021/ja405051f>
 44. Rubin-Pitel SB, Zhao H (2006) Recent advances in biocatalysis by directed enzyme evolution. *Combinatorial Chemistry & High Throughput Screening* 9(4):247-257. <https://doi.org/10.2174/138620706776843231>
 45. Schmid A, Dordick JS, Hauer B, Kiener A, Wubbolts M, Witholt B (2001) Industrial biocatalysis today and tomorrow. *Nature* 409(6817):258-268. <https://doi.org/10.1038/35051736>
 46. Jaeger KE, Dijkstra BW, Reetz MT (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology* 53:315-351. <https://doi.org/10.1146/annurev.micro.53.1.315>
 47. Schrag JD, Cygler M (1997) Lipases and alpha/beta hydrolase fold. *Methods in Enzymology* 284:85-107. <https://doi.org/10.1073/pnas.94.4.1449>
 48. Rigden DJ, Fernandez XM (2021) The 2021 Nucleic Acids Research database issue and the online molecular biology database collection. *Nucleic Acids Research* 49(D1):D1-D9. <https://doi.org/10.1093/nar/gkaa1216>
 49. Adrio JL, Demain AL (2014) Microbial enzymes: tools for biotechnological processes. *Biomolecules* 4(1):117-139. <https://doi.org/10.3390/biom4010117>
 50. Singh RK, Tiwari MK, Singh R, Lee JK (2013) From protein engineering to immobilization: promising strategies for the upgrade of industrial enzymes. *International Journal of Molecular Sciences* 14(1):1232-1277. <https://doi.org/10.3390/ijms14011232>
 51. Frenzel A, Hust M, Schirrmann T (2013) Expression of recombinant antibodies. *Frontiers in Immunology* 4:217. <https://doi.org/10.3389/fimmu.2013.00217>
 52. Porro D, Sauer M, Branduardi P, Mattanovich D (2005) Recombinant protein production in yeasts. *Molecular Biotechnology* 31(3):245-259. <https://doi.org/10.1385/MB:31:3:245>
 53. Rita Costa A, Elisa Rodrigues M, Henriques M, Oliveira R, Azeredo J (2014) Glycosylation: impact, control and improvement during therapeutic protein production. *Critical Reviews in Biotechnology* 34(4):281-299. <https://doi.org/10.3109/07388551.2013.793649>
 54. Meissner J, Prause A, Bharde A, Reinhold P (2016) Modeling and industrial applications of the fed-batch fermentation process. *F1000Research* 5:2601. <https://doi.org/10.12688/f1000research.9946.1>
 55. Kumar A, Singh R, Yadav A, Pandey KD (2017) Bacterial lipases and their industrial applications: a review. *Research Journal of Biotechnology* 12(2):89-98. <https://doi.org/10.1177/0972197317711234>