Title

Characterization of the exopolysaccharides produced by the industrial yeast Komagataella phaffii.

Authors

Fischer, Amanda

Castagna, Vanessa

Omer, Shafraz

et al.

Publication Date

2024-01-09

DOI

10.1093/jimb/kuae046

Peer reviewed

Journal of Industrial Microbiology and Biotechnology, 2024, 51, kuae046
https://doi.org/10.1093/jimb/kuae046
Advance access publication date: 22 November 2024
Genetics and Molecular Biology of Industrial Organisms – Short Communication

Characterization of the exopolysaccharides produced by the industrial yeast *Komagataella phaffii*

Amanda Fischer[1], Vanessa Castagna[1], Shafraz Omer[1], Micah Marmorstein[1], Junqi Wu[1], Shannon Ceballos[1], Emma Skoog[1], Carlito B. Lebrilla[2], Chris Suarez[2], Aletta Schnitzler[1]

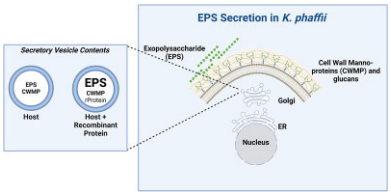
[1]TurtleTree, 1100 Main Street, Suite 300, Woodland, CA 95695, USA
[2]Department of Chemistry, University of California, Davis, CA 95616, USA
Correspondence should be addressed to: Amanda Fischer, 1100 Main St, Suite 300, Woodland, CA, USA 95695. E-mail: amanda@turtletree.com

Abstract: The yeast *Komagataella phaffii* has become a popular host strain among biotechnology start-up companies for producing recombinant proteins for food and adult nutrition applications. *Komagataella phaffii* is a host of choice due to its long history of safe use, open access to protocols and strains, a secretome free of host proteins and proteases, and contract manufacturing organizations with deep knowledge in bioprocess scale-up. However, a recent publication highlighted the abundance of an unknown polysaccharide that accumulates in the supernatant during fermentation. This poses a significant challenge in using *K. phaffii* as a production host. This polysaccharide leads to difficulties in achieving high purity products and requires specialized and costly downstream processing steps for removal. In this study, we describe the use of the common *K. phaffii* host strain YB-4290 for production of the bioactive milk protein lactoferrin. Upon purification of lactoferrin using membrane-based separation methods, significant amounts of carbohydrate were copurified with the protein. It was determined that the carbohydrate is mostly composed of mannose residues with minor amounts of glucose and glucosamine. The polysaccharide fraction has an average molecular weight of 50 kDa and consists mainly of mannan, galactomannan, and amylose. In addition, a large fraction of the carbohydrate has an unknown structure likely composed of oligosaccharides. Additional strains were tested in fermentation to further understand the source of the carbohydrates. The commonly used industrial hosts, BG10 and YB-4290, produce a basal level of exopolysaccharide; YB-4290 producing slightly more than BG10. Overexpression of recombinant protein stimulates exopolysaccharide production well above levels produced by the host strains alone. Overall, this study aims to provide a foundation for developing methods to improve the economics of recombinant protein production using *K. phaffii* as a production host.

One-Sentence Summary: Overexpression of recombinant protein stimulates the hyperproduction of high-molecular-weight, mannose-based, exopolysaccharides by the industrial yeast *Komagataella phaffii*.

Keywords: Exopolysaccharide (EPS), Recombinant protein production, Precision fermentation, *Komagataella phaffii (K. phaffii)*, *Pichia pastoris (P. pastoris)*

Graphical abstract



List of Abbreviations

EPS:	exopolysaccharide
K. phaffii :	*Komagataella phaffii*
rbLf:	recombinant bovine lactoferrin
GPC:	gel permeation chromatography
MS:	mass spectrometry
DSP:	downstream processing
DCW:	dry cell weight
Cut:	cutinase

Introduction

The industrial yeast, *Komagataella phaffii (K. phaffii)*, is renowned for its application in developing recombinant proteins across diverse product categories from industrial enzymes to therapeutic antibodies. More recently, biotechnology start-up companies have successfully employed *K. phaffii* for the production of recombinant proteins with a range of applications in food from texture, flavor, smell, nutritional content, and health benefits (Barone et al., 2023). Examples of some of these products and companies

Received: September 19, 2024. **Accepted:** November 21, 2024.

include heme proteins (Motif FoodWorks and Impossible Foods), egg white proteins (The Every Company), milk proteins (Perfect Day and Remilk), and sweet proteins (Oobli) (Supplemental Table 3).

TurtleTree is producing lactoferrin as a food ingredient in select food and beverage applications using *K. phaffii* as a production host. Lactoferrin is an iron-binding glycoprotein naturally occurring in mammalian mucosal secretions (i.e., milk and saliva) and in neutrophil granules. Apart from its main biological function, namely binding and transporting iron ions, lactoferrin also has antibacterial, antiviral, antiparasitic, anticancer, and antiallergic functions and properties (García-Montoya et al., 2012). Bovine lactoferrin is a 689-amino acid glycoprotein with a molecular weight of 80–87 kDa (Hurley et al., 1993). Native bovine lactoferrin (bLf) has five putative *N*-linked glycosylation sites and is present in bovine milk in different glycoforms, of which the most abundant glycoform has four of the sites occupied with heterogeneous glycan structures (Wei et al., 2000).

Despite the advantages of this platform host and the favorable qualities of the products it can produce, secreted proteins produced from these strains can be plagued by the presence of unwanted polysaccharides, known as exopolysaccharides (EPS) (Denton et al., 1998; Pan et al., 2022, Trimble et al., 1991). During high cell density fed-batch fermentation, *K. phaffii* strains have been known to produce EPS at concentrations up to 8.7 g/L in the supernatant (Steimann et al., 2024). To effectively remove these polysaccharides, additional downstream processing (DSP) steps, such as chromatography, are necessary (Li et al., 2018; O'Leary et al., 2004). Unfortunately, these additional DSP procedures are both costly and time-consuming for manufacturers.

The research regarding the origins of these carbohydrates and the influences of manufacturing processes remains largely unexplored. Steimann et al. (2024) summarize the existing knowledge of EPS, and emphasize that beyond the detection of EPS noted in some literature and basic structural information, there lacks a concrete understanding of this common and undesired coproduct. The industry needs a solution for increasing product purity in a cost-effective and efficient manner.

The objective of this study was to deepen our understanding of the composition and source of the EPS produced by *K. phaffii* during fermentation. Characterization of the EPS molecular weight and structure is necessary to ensure product safety and inform DSP methods to achieve higher purity products. Exploring how host lineage and the stress of recombinant protein production influence polysaccharide formation can help support strain or process engineering techniques to reduce or eliminate EPS production or accumulation. In addition, this study reports on the development and implementation of methods to monitor and track EPS levels during upstream and downstream process optimization for EPS reduction. The information provided in this study serves as a starting point for streamlining purification and manufacturing methods to achieve high purity products with low-cost DSP for *K. phaffii*.

Materials and Methods

Strains

Komagataella phaffii strains BG10, YB-4290, YB-4290.TT_bLf, BG11.2_cutinase (BG10, ΔAox1, extra copy of Hac1, cutinase), and YB-4290.2_cutinase (YB-4290, ΔAox1, extra copy of Hac1, cutinase).

Fed-Batch Fermentations

All fed-batch fermentations were carried out using 2-L glass vessels equipped with inline control of temperature, pH, feed rate, dissolved oxygen, and mixing. Depending on the strain, the carbon source, pH, and duration of the fermentation varied. For all strains, the production phase was set to target a low growth rate similar to what is described in literature (Life Technologies Invitrogen, 2002; Looser et al., 2015). Chemically defined FM22 media and PTM salts were used for all fermentations.

Total Carbohydrate Assay

The anthrone–sulfuric acid microplate assay adapted from Leyva et al. (2008) was used to determine total carbohydrate mass. The Sigma product M2069-5 G, D-(+)-mannose was used to prepare a standard curve.

Carbohydrate Linkage, Monosaccharide, and Polysaccharide Composition Analysis

The following were carried out by the Lebrilla Lab at the University of California, Davis, as described in previous publications (Bacalzo et al., 2023; Couture et al., 2024; Galermo et al., 2018; Xu et al., 2017). The carbohydrate glycosidic linkage analysis and monosaccharide analysis were performed using ultra high-performance liquid chromatography coupled with triple-quadrupole mass spectrometry (UHPLC/QqQ-MS). The polysaccharide analysis was carried out using high-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (HPLC/QTOF-MS). The following sets of standards were used for quantification and identifications. Linkage analysis standards included a library of 22 glycosidic linkages prepared using commercial oligosaccharide standards. Polysaccharide analysis standards included a set of 13 polysaccharides commonly found in food. Monosaccharide analysis standards included a pooled set of standards containing the 15 most common monosaccharides found in food.

Carbohydrate Molecular Weight Analysis

Gel permeation chromatography (GPC) was carried out by McGill University, Department of Chemistry. A Shodex LB-806 M OH pak column and a refractive index detector were used for detection. A pullulan polyS polymer was used to generate a standard curve (Shodex) (Arnling Bååth et al., 2018; Čížová et al., 2017; Verhertbruggen et al., 2019).

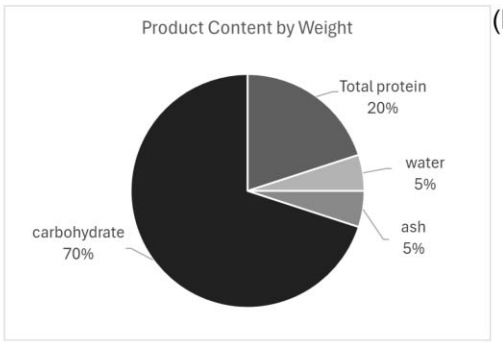
Total Composition Analysis of Low Purity rbLf Sample

Eurofins performed the following assays on the rbLf sample: ash (Eurofins method code FS044-1), total protein (Eurofins method code FSO4U-3), and bovine lactoferrin (Eurofins method code FS313-1). Moisture content was determined by drying and weighing. In this case, the total percent carbohydrate by weight was calculated by subtracting the mass of moisture, ash, and total protein from the total weight and then dividing by total dry weight.

Bovine Lactoferrin Quantification By HPLC

An Agilent 1260 HPLC, equipped with a Agilent Poroshell 300SB-C8, 5-um column, and an Agilent Multi Wavelength Detector (220 nm), was used for rbLf sample resolution and detection. Bovine lactoferrin derived from milk (Sigma L047) was used to prepare a standard curve.

(a)



(b)

Carbohydrate Type	% of sample	Content
Total Monosaccharide, hydrolyzed	41	Mannose 91%, Glucosamine 5%, glucose 3%
Free monosaccharide	<0.02	N-Acetylglucosamine 49%, Glucose 37%
Polysaccharide	5.7	Mannan 46%, Amylose 17%, Galactomannan 15%
Other carbohydrates	35.3	Uncharacterized

(c)

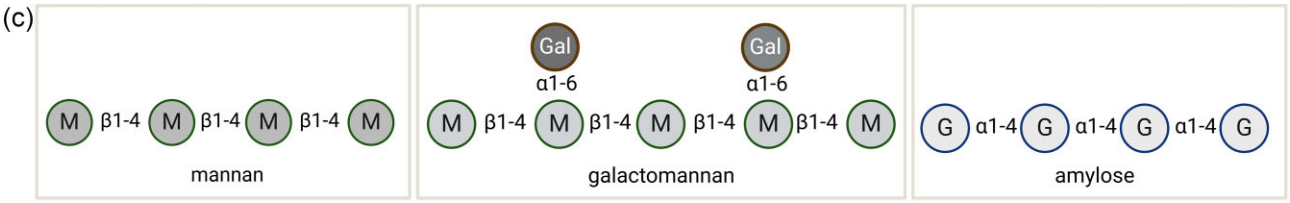


Fig. 1. Composition and abundance of the carbohydrates in the low purity recombinant bovine lactoferrin (rbLf) sample. (a) Approximate composition by weight of the low purity rbLf sample as determined by the assays performed by Eurofins, (b) analysis of the carbohydrate type, abundance, and composition, and (c) structure of the major polysaccharides found in the sample. *Note.* G = glucose; Gal = galactose; M = mannose.

Results

Komagataella Phaffii Produces High-Molecular-Weight, Mannose Exopolysaccharides

In this study, the *K. phaffii* host strain YB-4290 was used to overexpress and secrete rbLf (YB-4290.TT_bLf). When the rbLf is purified from the fermentation broth using standard filtration-based DSP methods followed by drying, the protein content is predominantly full-length rbL; however, percent total protein by weight is low. As much as 70% of the dry preparation by weight is carbohydrates with only minor portions of moisture and ash (Fig. 1). The low purity rbLf powder sample was analyzed with mass spectrometry (MS) to characterize the carbohydrate content (Bacalzo et al., 2023; Xu et al., 2017). The MS method indicated less carbohydrate (41%) than the batch record calculation (70%), although both methods revealed a high proportion of carbohydrate in the product (Fig. 1). While the MS method is accurate to analyze the ratio of different structures, it may include loss of total product in the sample preparation steps leading up to MS analysis or lack of standards. The carbohydrates were analyzed with or without hydrolysis to characterize either the total or free monosaccharide composition, respectively. The results show a very small amount of free monosaccharides (<0.02%), while some of the carbohydrate is present as polysaccharides (5.7%) (Fig. 1). The identities of the polysaccharides were determined by comparing their fingerprint chromatograms to that of 13 known polysaccharide standards. The major polysaccharides in the rbLf sample are mannan, galactomannan, and amylose (Fig. 1). About one third of the total carbohydrate mass could not be classified as either free monosaccharides or polysaccharides. This fraction is called the "other carbohydrates" in Fig. 1 and likely consists of mannose-based oligosaccharides with a range in degree of polymerization.

The molecular weight of the polysaccharides in the rbLf sample was analyzed using GPC, size-exclusion chromatography, which indicates their average molecular weight is 50 kDa (Fig. 2). Because the polysaccharide fraction is similar in molecular weight to the lactoferrin, the two remain together during standard membrane-based DSP filtration steps designed to target the 84-kDa lactoferrin protein.

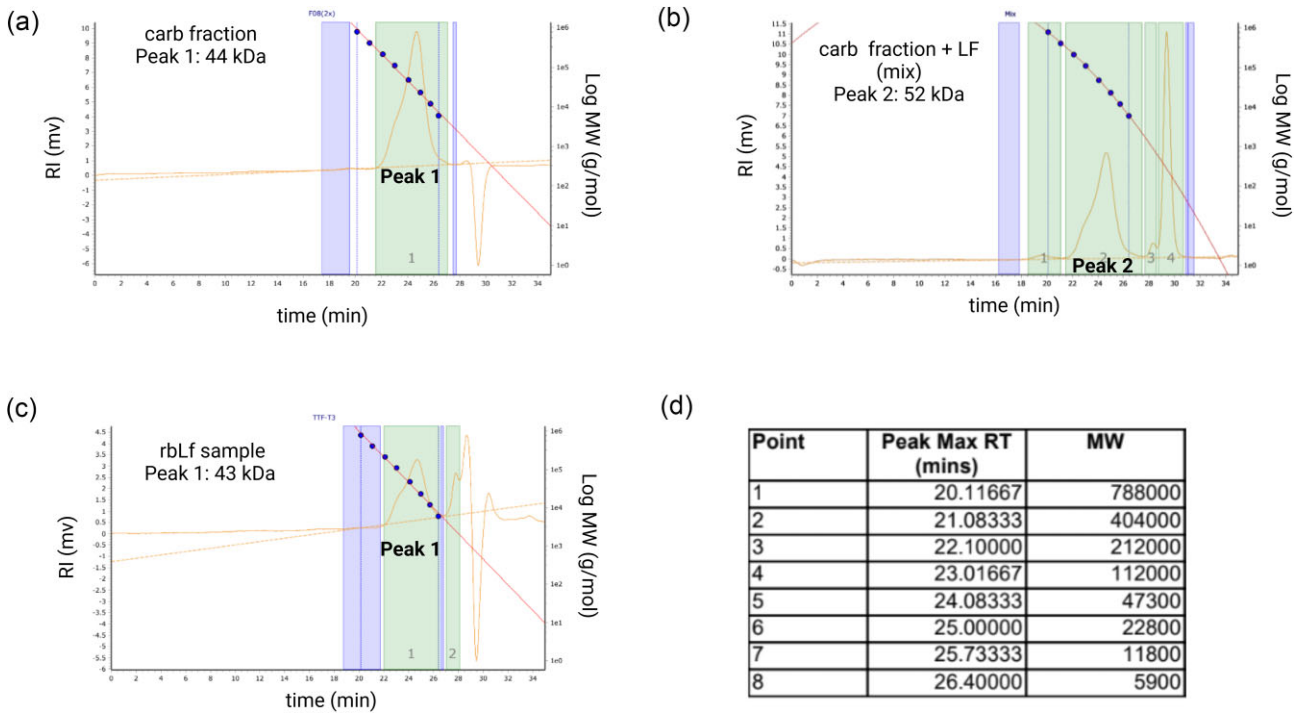
Exopolysaccharide Production Trends in Fermentation From the Recombinant Bovine Lactoferrin Strain

The strain YB-4290.TT_bLf was cultivated in 2-L fed-batch fermentation using glucose as a carbon source and a pH shift from 4.8 to 5.2 on day 3. Under these conditions, the dry cell weight (DCW) peaks near day 3 when the production phase starts and remains relatively level throughout the rest of the fermentation (Fig. 3). In contrast, the recombinant protein titer increases at a near linear rate during the production phase. The EPS production dynamics appear to mirror recombinant protein production and not biomass accumulation (Fig. 3). No obvious signs of lysis were observed during fermentation, and glycans from the rbLf itself would expect to make a minor contribution to the total carbohydrates measured. In some cases, the titer of carbohydrates was six times greater than the amount of recombinant lactoferrin produced.

Exopolysaccharide Production Trends in Fermentation From Different Host Strains Producing Cutinase

Additional strains were built in order to test the impact of host strain lineage and expression of a different, non-*N*-linked glycosylated, recombinant protein on EPS production. For this study, the well-expressed reporter protein *Fusarium solani* cutinase (cut) (Uniprot ID Q99174) was expressed in the two most commonly used lineages of *K. phaffii*, BG10 (closely related to Y-11430) and YB-4290 (closely related to Y-7556) (Table 1). This also serves as a control for the bovine lactoferrin strain built using the YB-4290 host. In these strains, cutinase expression is controlled using the native methanol inducible promoter from the *Aox1* gene.

The host strains BG10 and YB-4290 were grown using glucose as a carbon source. The strain YB-4290 consistently produced



Point	Peak Max RT (mins)	MW
1	20.11667	788000
2	21.08333	404000
3	22.10000	212000
4	23.01667	112000
5	24.08333	47300
6	25.00000	22800
7	25.73333	11800
8	26.40000	5900

Fig. 2. Gel permeation chromatography (GPC) was used to separate the protein and carbohydrate fractions of the recombinant bovine lactoferrin (rbLf) samples and determine the average molecular weight of the carbohydrate component. GPC distribution analysis reports for (a) purified carbohydrate fraction from rbLf sample, (B) an artificial mixture of purified rbLf protein and purified carbohydrate fraction, (c) the low purity rbLf sample (20% protein and 70% carbohydrate by mass), and (d) standards derived from a pullulan polyS polymer were used to create standard curve dots seen on each chromatogram.

Table 1. Description of *K. phaffii* Strains Used in This Study

Strains Used in This Study	Source	Recombinant Protein Expressed	Genotype—Hoc1, Rsf2
YB-4290 (Y-7556, CBS 2612)	NRRL	None	Both WT
YB-4290.TT_bLf	This study	Bovine lactoferrin	Both WT
YB-4290.2_cutinase	This study	Cutinase	Both WT
BG10 (Y-11430, CBS 7435)	ATUM	None	Both truncated
BG11.2_cutinase	This study	cutinase	Both truncated

more total carbohydrate than BG10. At the end of fermentation, YB-4290 and BG10 had similar biomass DCW but YB-4290 produced significantly more total carbohydrate (Table 2 and Supplemental Fig. 4). NRRL, the Northern Regional Research Laboratory (aka National Center For Agricultural Utilization Research); ATUM, atum.bio.; WT , wildtype.

The cutinase versions of these strains, BG11.2_cutinase and YB-4290.2_cutinase, were tested in fermentation using glucose as a carbon source during the batch phase and switching to methanol during the production phase for induction of cutinase expression. The two cutinase strains from the different host backgrounds secrete significantly different levels of the recombinant protein and the YB-4290.2_cut strain produces close to double the amount of cutinase and also twice the amount of EPS as the BG11.2_cut strain (Table 2 and Supplemental Fig. 4). Although both cutinase strains have elevated EPS production compared to the host strains, seemingly stimulated by recombinant protein production, it is difficult to say whether the total amount of EPS produced is due to differences in the recombinant protein titer or differences in the host strain backgrounds. As expected, when the carbohydrate composition of these broths was characterized by electrophoresis and carbohydrate staining, the YB-4290 and YB-4290.2_cutainase strains showed much stronger staining than the BG10 and BG11.2_cutinase strains.

Discussion

EPS Characterization

This study confirms the observation that *K. phaffii* produces an abundance of EPS that accumulate during high-cell-density fed-batch fermentation and further characterizes the composition, structure, and size of these EPS (Steimann et al., 2024). In addition to what has been reported in this study, future work is needed to determine the composition of the uncharacterized carbohydrate fraction. This could include GPC separation or another size-exclusion method. These isolated fractions could then be further characterized by multiglycomic methods to confirm they are indeed mannose oligosaccharides.

EPS Linked to Recombinant Protein Production and YB-4290 Lineage

In contrast to Steimann et al., this study reports a very different effect of recombinant protein production and host lineage on the EPS production. The host strains, BG10 and YB-4290, produce a basal level of EPS that accumulates to 10–12 mg/ml by the end of fermentation; YB-4290 producing slightly more than BG10. In both host backgrounds, YB-4290 and BG10, overexpression of recombinant protein, either cutinase or rbLf, leads to significantly higher

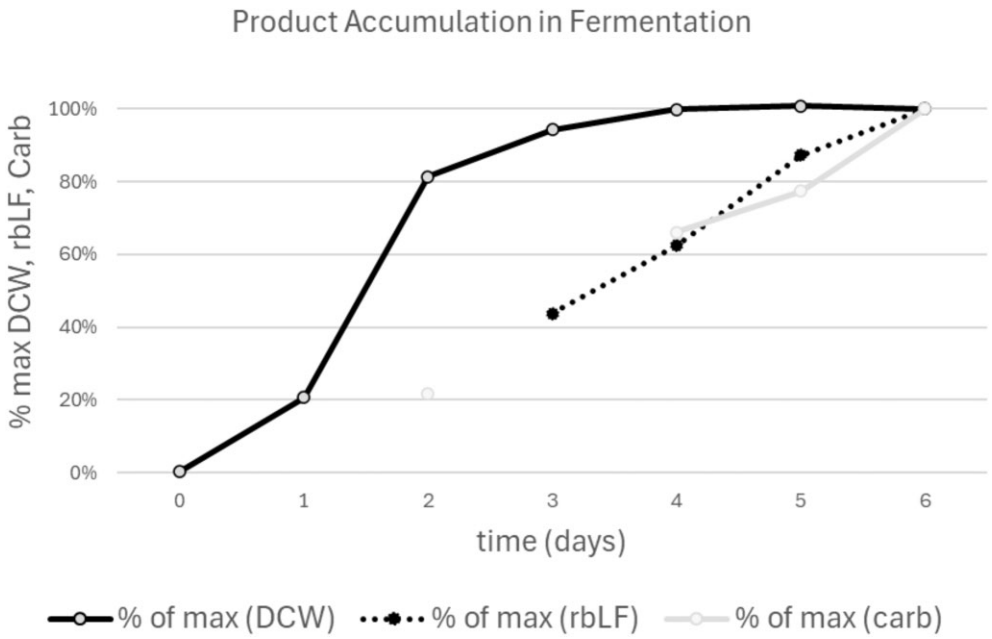


Fig. 3. Product accumulation over time from *K. phaffii* strain YB-4290.TT_bLf in fed-batch fermentation. The average dry cell weight (DCW), carbohydrate production (carb), and lactoferrin production (rbLf) are displayed as percentage of highest titer observed for the set and are shown over the course of the fermentation. This data represents the average of 12 independent fermentations (<10% coefficient of variation (CV) for each data point).

Table 2. A Comparison of the Final Amounts of Carb (Carbohydrate), DCW (Dry Cell Weight), and rPOI (Recombinant Protein of Interest, Either rbLf or Cutinase) Produced in Fed-Batch Fermentation

Strain	% of Max DCW	% of Max Carb	% of Max rPOI	% Max Carb Normalized to DCW	% Max rPOI Normalized to DCW	% Max Carb Normalized to rPOI
BG10	94%	21%	n/a	19%	n/a	n/a
YB-4290	100%	28%	n/a	23%	n/a	n/a
BG11.2_cut	80%	35%	8%	37%	8%	100%
YB-4290.2_cut	78%	60%	16%	65%	17%	87%
YB-4290.TT_bLf	83%	100%	100%	100%	100%	23%

Note. The percentage of maximum is relative to this set of data. The values are from the end of fermentation time point; for the rbLf strain this is day 6 and for all others it is day 5. For the rbLf strain this data is the average of 12 independent fermentations and for all others it is from a single fermentation.

EPS levels compared to the host strains not overexpressing recombinant protein (Table 2). In addition, EPS production dynamics appears to mirror recombinant protein production and not biomass accumulation. This interesting observation that overexpression of recombinant protein stimulates EPS production is surprising and in contrast to the findings of Steimann et al. In comparison to the rbLf strain, the cutinase strains secrete significantly less recombinant protein; however, there is no obvious correlation between recombinant protein titer and total amount of EPS produced.

Genetic Influence

Although these experiments suggest a positive correlation between recombinant protein production and EPS production, it is still unclear the impact of the host on total EPS productivity or the genetic mechanisms for EPS production. However, it is interesting to speculate that the host genotype could contribute to the EPS phenotype. The two host strain lineages used in this study were previously reported to contain single nucleotide polymorphisms (SNPs) at the Hoc1, Rsf2 and Sef1 loci; the Hoc1 and Rsf2 SNPs both result in truncations (Brady et al., 2020; Claes et al., 2024; Offei et al., 2022) (Table 1). Hoc1 is an α-1,6-mannosyltransferase involved in cell wall mannan biosynthesis, and the truncation has been correlated with the thin cell wall phenotype of the BG10 lineage (Offei et al., 2022). Rsf2 is a putative zinc finger transcription factor, responsible for controlling genes required for glycerol-based growth, respiration, cellular morphogenesis, and alcohol metabolism and cell wall remodeling (Brady et al., 2020). It is possible that one or both of these truncations in BG10 lead to loss of function, resulting in a thin cell wall phenotype and the lower levels of EPS secretion observed in this report. Conversely, the wildtype version of these proteins in the YB-4290 lineage could result in the thicker cell wall along with the hyperaccumulation of EPS observed. Future work is required to test the linkage between host genotype and the cell wall thickness phenotype.

Source of EPS

Research with the model yeast *Saccharomyces cerevisiae* demonstrated that the cell wall is primarily composed of polysaccharide (68%–75% by dry weight), and is evenly balanced between glucan and mannan structures (Baek et al., 2024; Klis et al., 2002; Kogan & Kocher, 2007; Roelofsen, 1953). The glucans are synthesized at the cell wall whereas the mannans are added to the cell wall proteins in the secretory pathway. These mannosylated proteins are then transported to the cell wall in the same secretory vesicles used for recombinant protein secretion. The cotransport

of both the EPS and recombinant protein could explain the correlation between production dynamics in fermentation as well as the significant stimulation of EPS in strains overexpressing recombinant protein. In contrast to cell wall polysaccharides enriched in glucose, the EPS are primarily mannose-based, likely originating from the cell wall mannoproteins. However, it is surprising that yeast would produce such an abundance of soluble mannans not associated with the cell wall. Stress from recombinant protein production or the unfolded protein response may disrupt the mannosylation pathway, leading to an overload of secretory vesicles with free mannans. Strains with truncations in the Hoc1 and Rsf2 may have impaired mannosylation, resulting in lower levels of secreted EPS.

Acknowledgments

We would like to thank the following individuals and groups for their contributions to this work. Jung Yi for coordinating and managing the internal and external work to characterize the carbohydrate amounts and structures present in different samples. Dr. Violeta Toader at McGill University, Department of Chemistry, for carrying out the GPC-SEC analysis for the molecular weight determination of the carbohydrates. Eric Cruz for supporting the fed-batch fermentations. Mikayla Matriano, Matthew Demer, and Mia Gaiero for carrying out the HPLC and carbohydrate assays and data analysis. Victoria Flagg and Hadeel Baker for reviewing the scientific content for accuracy and clarity.

Supplementary Material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Funding

None declared.

Conflict of Interest

Nothing to report.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

Arnling Bååth, J., Martínez-Abad, A., Berglund, J., Larsbrink, J., Vilaplana, F., & Olsson, L. (2018). Mannanase hydrolysis of spruce galactoglucomannan focusing on the influence of acetylation on enzymatic mannan degradation. *Biotechnology for Biofuels*, *11*(1), 114. https://doi.org/10.1186/s13068-018-1115-y

Bacalzo, N. P., Couture, G., Chen, Y., Castillo, J. J., Phillips, K. M., Fukagawa, N. K., & Lebrilla, C. B. (2023). Quantitative bottom-up glycomic analysis of polysaccharides in food matrices using liquid chromatography-tandem mass spectrometry. *Analytical Chemistry*, 95, 1008–1015. https://doi.org/10.1021/acs.analchem.2c03707

Baek, K.-R., Rani Ramakrishnan, S., Kim, S.-J., & Seo, S.-O. (2024). Yeast cell wall mannan structural features, biological activities, and production strategies. *Heliyon*, 10(6), e27896. https://doi.org/10.1016/j.heliyon.2024.e27896

Barone, G. D., Emmerstorfer-Augustin, A., Biundo, A., Pisano, I., Coccetti, P., Mapelli, V., & Camattari, A. (2023). Industrial production of proteins with Pichia pastoris-Komagataella phaffii. *Biomolecules*, *13*(3), 441. https://doi.org/10.3390/biom13030441

Brady, J. R., Whittaker, C. A., Tan, M. C., Kristensen, D. L., Ma, D., Dalvie, N. C., Love, K. R., & Love, J. C. (2020). Comparative genome-scale analysis of Pichia pastoris variants informs selection of an optimal base strain. *Biotechnology and Bioengineering*, *117*(2), 543–555. https://doi.org/10.1002/bit.27209

Čížová, A., Korcová, J., Farkaš, P., & Bystrický, S. (2017). Efficient separation of mannan-protein mixtures by ionic liquid aqueous two-phase system, comparison with lectin affinity purification. *International Journal of Biological Macromolecules*, *98*, 314–318. https://doi.org/10.1016/j.ijbiomac.2017.02.001

Claes, K., Van Herpe, D., Vanluchene, R., Roels, C., Van Moer, B., Wyseure, E., Vandewalle, K., Eeckhaut, H., Yilmaz, S., Vanmarcke, S., Çıtak, E., Fijalkowska, D., Grootaert, H., Lonigro, C., Meuris, L., Michielsen, G., Naessens, J., van Schie, L., De Rycke, R., & Callewaert, N. (2024). OPENPichia: Licence-free Komagataella phaffii chassis strains and toolkit for protein expression. *Nature Microbiology*, 9(3), 864–876. https://doi.org/10.1038/s41564-023-01574-w

Couture, G., Cheang, S. E., Suarez, C., Chen, Y., Bacalzo, N. P., Jiang, J., Weng, C.-Y. C., Stacy, A., Castillo, J. J., Delannoy-Bruno, O., Webber, D. M., Barratt, M. J., Gordon, J. I., Mills, D. A., German, J. B., Fukagawa, N. K., & Lebrilla, C. B. (2024). A multi-glycomic platform for the analysis of food carbohydrates. *Nature Protocols*, 19(11),3321–3359. https://doi.org/10.1038/s41596-024-01017-8

Denton, H., Smith, M., Husi, H., Uhrin, D., Barlow, P. N., Batt, C. A., & Sawyer, L. (1998). Isotopically labeled bovine beta-lactoglobulin for NMR studies expressed in Pichia pastoris. *Protein Expression Purif*, *14*(1), 97–103. https://doi.org/10.1006/prep.1998.0924

Galermo, A. G., Nandita, E., Barboza, M., Amicucci, M. J., Vo, T.-T. T., & Lebrilla, C. B. (2018). Liquid chromatography-tandem mass spectrometry approach for determining glycosidic linkages. *Analytical Chemistry*, 90(21), 13073–13080. https://doi.org/10.1021/acs.analchem.8b04124

García-Montoya, I. A., Cendón, T. S., Arévalo-Gallegos, S., & Rascón-Cruz, Q. (2012). Lactoferrin a multiple bioactive protein: An overview. *Biochimica Et Biophysica Acta*, *1820*(3), 226–236. https://doi.org/10.1016/j.bbagen.2011.06.018

Hurley, W. L., Grieve, R. C., Magura, C. E., Hegarty, H. M., & Zou, S. (1993). Electrophoretic comparisons of lactoferrin from bovine mammary secretions, milk neutrophils, and human milk. *Journal of Dairy Science*, *76*(2), 377–387. https://doi.org/10.3168/jds.S0022-0302(93)77356-7

Klis, F. M., Mol, P., Hellingwerf, K., & Brul, S. (2002). Dynamics of cell wall structure in *Saccharomyces cerevisiae*. *Fems Microbiology Review*, *26*(3), 239–256. https://doi.org/10.1111/j.1574-6976.2002.tb00613.x

Kogan, G., & Kocher, A. (2007). Role of yeast cell wall polysaccharides in pig nutrition and health protection. *Livestock Science*, 109(1-3), 161–165. https://doi.org/10.1016/j.livsci.2007.01.134

Leyva, A., Quintana, A., Sánchez, M., Rodríguez, E. N., Cremata, J., & Sánchez, J. C. (2008). Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: Method development and validation. *Biologicals*, *36*(2), 134–141. https://doi.org/10.1016/j.biologicals.2007.09.001

Li, S., Li, L., Chen, Z., Xue, G., Jiang, L., Zheng, K., Chen, J., Li, R., Yuan, C., & Huang, M. (2018). A novel purification procedure for recombinant human serum albumin expressed in *Pichia pastoris*. *Protein Expression and Purification*, 149, 37–42. https://doi.org/10.1016/j.pep.2018.04.012

Life Technologies Invitrogen. (2002). *Pichia fermentation process guidelines (manuals & protocols)*. Invitrogen Life Technologies.

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

Offei, B., Braun-Galleani, S., Venkatesh, A., Casey, W. T., O'Connor, K. E., Byrne, K. P., & Wolfe, K. H. (2022). Identification of genetic variants of the industrial yeast *Komagataella phaffii* (*Pichia pastoris*) that contribute to increased yields of secreted heterologous proteins. *PLoS Biology*, *20*(12), e3001877. https://doi.org/10.1371/journal.pbio.3001877

O'Leary, J. M., Radcliffe, C. M., Willis, A. C., Dwek, R. A., Rudd, P. M., & Downing, A. K. (2004). Identification and removal of O-linked and non-covalently linked sugars from recombinant protein produced using Pichia pastoris. *Protein Expression and Purification*, *38*(2), 217–227. https://doi.org/10.1016/j.pep.2004.08.010

Pan, Y., Yang, J., Wu, J., Yang, L., & Fang, H. (2022). Current advances of pichia pastoris as cell factories for production of recombinant proteins. *Frontiers in Microbiology*, *13*, 1059777. https://doi.org/10.3389/fmicb.2022.1059777

Roelofsen, P. A. (1953). Yeast mannan, a cell wall constituent of baker's yeast. *Biochimica Et Biophysica Acta*, *10*, 477–478. https://doi.org/10.1016/0006-3002(53)90280-7

Steimann, T., Heite, Z., Germer, A., Blank, L. M., Büchs, J., Mann, M., & Magnus, J. B. (2024). Understanding exopolysaccharide byproduct formation in *Komagataella phaffii* fermentation processes for recombinant protein production. *Microbial cell factories*, *23*(1), 131. https://doi.org/10.1186/s12934-024-02403-3

Trimble, R. B., Atkinson, P. H., Tschopp, J. F., Townsend, R. R., & Maley, F. (1991). Structure of oligosaccharides on saccharomyces SUC2 invertase secreted by the methylotrophic yeast Pichia pastoris. *Journal of Biological Chemistry*, *266*(34), 22807–22817.

Verhertbruggen, Y., Falourd, X., Sterner, M., Guillon, F., Girousse, C., Foucat, L., Le Gall, S., Chateigner-Boutin, A.-L., & Saulnier, L. (2019). Challenging the putative structure of mannan in wheat (*Triticum aestivum*) endosperm. *Carbohydrate Polymers*, *224*, 115063. https://doi.org/10.1016/j.carbpol.2019.115063

Wei, Z., Nishimura, T., & Yoshida, S. (2000). Presence of a glycan at a potential N-glycosylation site, Asn-281, of bovine lactoferrin. *Journal of Dairy Science*, *83*(4), 683–689. https://doi.org/10.3168/jds.S0022-0302(00)74929-0

Xu, G., Amicucci, M. J., Cheng, Z., Galermo, A. G., & Lebrilla, C. B. (2017). Revisiting monosaccharide analysis—quantitation of a comprehensive set of monosaccharides using dynamic multiple reaction monitoring. *Analyst*, *143*(1), 200–207. https://doi.org/10.1039/c7an01530e

Received: September 19, 2024. **Accepted:** November 21, 2024