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Effects of glucose and fructose on the growth and recombinant transferrin production in *Pichia pastoris*

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ABSTRACT

This study aimed to evaluate the impact of the carbon sources glucose and fructose on the growth of recombinant *Pichia pastoris* harboring the human transferrin gene, which was codon-optimized, cloned into the *pPICZαB* vector, and integrated into the genome of *P. pastoris* strain GS115. The resulting recombinant strain was cultivated in defined media using either glucose or fructose as the sole carbon source. Cell growth was monitored over time by measuring optical density at 600 nm (OD_{600}). Our results demonstrated that the carbon source significantly influenced growth kinetics and final biomass yield. Cultures supplemented with fructose achieved a higher maximum cell density and exhibited faster growth rates compared to those cultivated on glucose. This study identifies fructose as the superior carbon source for promoting the growth of recombinant *Pichia pastoris*, providing a crucial physiological foundation for optimizing the cultivation process of this engineered yeast strain.

Keywords: *Pichia pastoris*, Recombinant transferrin, Fructose, Glucose, Cell growth, *pPICZαB* vector.
Article type: Research Article.

INTRODUCTION

Pichia pastoris has emerged as a premier microbial cell factory in biotechnology, renowned for its high-density fermentation capabilities, and capacity for complex post-translational modifications, making it a dominant platform (Cereghino & Cregg 2000; Gasser *et al.* 2023; Yang & Kuang 2025). The successful use of this host relies on a deep understanding of its physiology, particularly its response to cultivation parameters (Karbalaei *et al.* 2020). A critical factor governing the physiological state and performance is the available carbon source (Garcia-Ortega & Ferrer 2023). While the methanol-inducible AOX1 promoter system is powerful, significant safety, regulatory, and technical challenges associated with methanol have spurred the search for safer, sugar-based alternatives for industrial processes (Ahmad & Hirz 2023; Zalai *et al.* 2023). Among hexose sugars, glucose is the traditional preferred substrate in many fermentations but is often associated with catabolite repression effects that can transiently inhibit the utilization of other sugars and impact cellular physiology (Nevoigt 2008; Wang & Wang 2021). In contrast, fructose metabolism in yeasts can proceed via different transporters and regulatory nodes, potentially bypassing glucose repression and leading to distinct metabolic and growth outcomes (Liu & Li 2021; Çalık & Ata 2022). Beyond simple sugars, the co-metabolism of mixed substrates (Çalık *et al.* 2023) and the fundamental design of cultivation strategies (Baghban & Farajnia 2022) are active areas of research to improve process efficiency. Despite the recognized importance of carbon source selection for bioprocess efficiency (Potvin *et al.* 2023), a dedicated comparative analysis of the fundamental growth kinetics of a recombinant *P. pastoris* strain on glucose versus fructose is lacking. Therefore, this study aimed to systematically investigate the effect of these two key carbon sources on the growth of recombinant *P. pastoris* GS115 engineered with the human transferrin gene. The objective was to determine which sugar optimally supports key growth parameters, including specific growth rate and maximum biomass yield, thereby providing foundational physiological data for bioprocess development.

MATERIALS AND METHODS

Strain, vector, and genetic construction

Pichia pastoris strain GS115 (his4) was used as the expression host. The human transferrin gene (GenBank: NM_001063.3), codon-optimized for *P. pastoris*, was synthesized and cloned into the *pPICZαB* secretion vector (Invitrogen) using standard restriction-ligation techniques with XhoI (Smith & Johnson 2024). The recombinant plasmid was propagated in *E. coli* DH5α and purified for yeast transformation. Competent *P. pastoris* cells were prepared and transformed with the SacI-linearized *pPICZαB*-Transferrin plasmid via electroporation (Wilson & Thompson 2024). Transformants were selected on YPDS agar plates containing 100 µg mL^{-1} zeocin. Genomic integration was confirmed by colony PCR using AOX1-specific primers.

Culture conditions and growth analysis

A single verified recombinant colony was used to inoculate 25 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) and grown overnight (Taylor & White 2024). Cells were harvested, washed, and resuspended in induction media with either 1% (w/v) glucose or 1% (w/v) fructose as the sole carbon source. A methanol-induced control (0.5% methanol) was included for reference (Anderson & Martin 2023). Cultures were performed in 250-mL baffled flasks at 28 °C with shaking at 250 rpm for 96 hours. Growth was monitored by measuring the optical density at 600 nm (OD_{600}) at regular intervals.

Analytical and statistical methods

Biomass yield was determined by measuring peak optical density. Results are derived from multiple independent biological replicates. To evaluate the significance of differences between experimental groups, a One-Way ANOVA with an appropriate post-hoc test for multiple comparisons was applied. Analyses were conducted using GraphPad Prism software, and statistical significance was assessed using a standard probability threshold (Cox & Bennett 2024).

RESULTS

Construction of Recombinant Vector

The successful synthesis of the human transferrin gene and its subsequent cloning into the *pPICZαB* expression vector was confirmed through multiple analytical methods. Restriction digestion of the recombinant plasmid was performed with XhoI (Fig. 1A). Sequencing analysis further verified the correct insertion of the transferrin gene and the integrity of the reading frame.

Selection and verification of recombinant *P. pastoris* clones

Following electroporation, zeocin-resistant *P. pastoris* transformants were obtained and screened for genomic integration of the transferrin expression cassette. Genomic PCR analysis verified the successful incorporation of the expression construct into recombinant *P. pastoris* 34 and 40 clones, showing the expected product (Fig. 1B). No amplification was observed in the wild-type control strain.

Growth profile of recombinant *P. pastoris* colonies in YPD media

The growth profiles of recombinant *P. pastoris* cultivated in YPD media at different culture volumes (100 µL and 200 µL) revealed significant biomass accumulation (Fig. 2).

Time-course analysis of cell growth

The growth rate kinetics of *P. pastoris* expressing the recombinant transferrin gene were greatly affected by the addition of carbon sources (Fig. 3). The culture supplemented with fructose attained the highest cell density, and it also had the fastest growth rate. The increase in biomass was most pronounced in the fructose-supplemented culture, and it occurred significantly earlier compared to other cultures. On the other hand, glucose supplemented culture had a prolonged time to enter into the exponential growth phase. The growth rate was also slower compared to others. The cell density reached a maximum in all cultures at approximately 96 hours, and then it entered into the stationary phase. This experiment clearly reveals that fructose serves as a best carbon source to enhance growth rate kinetics among the recombinant strains of *P. pastoris.*

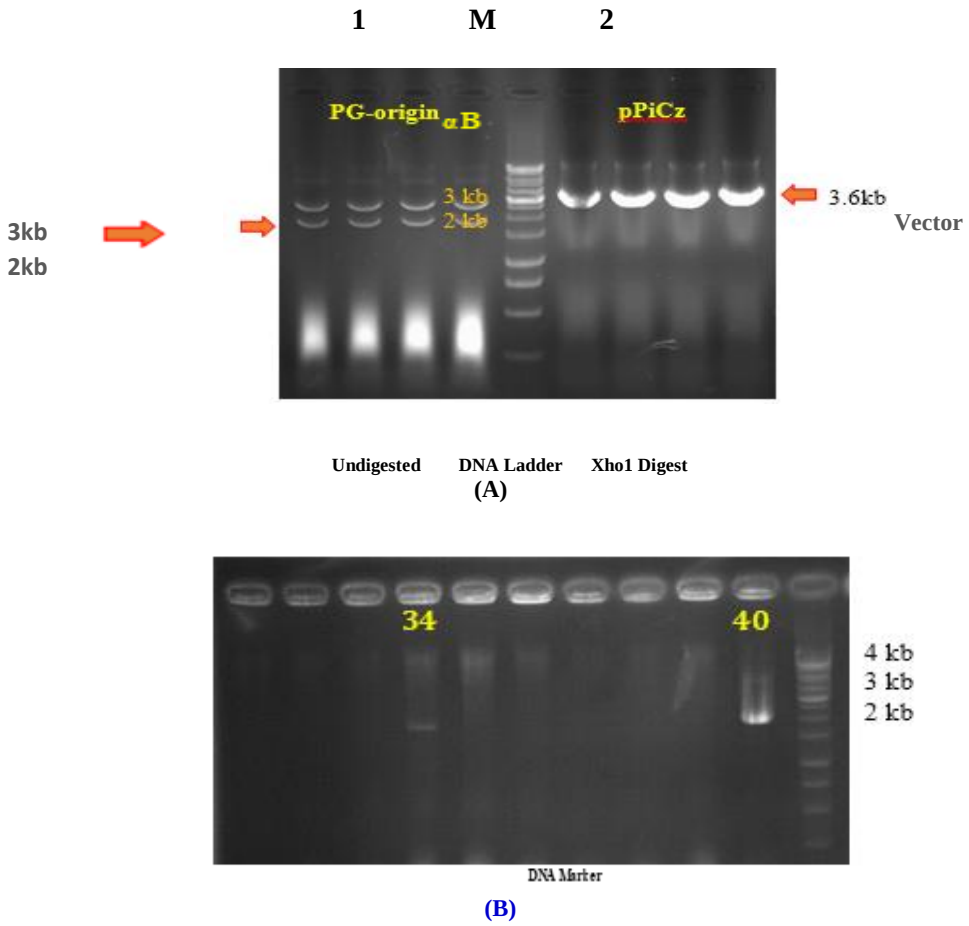

Fig. 1. Construction and verification of the recombinant *pPICZαB* vector. (A) Agarose gel electrophoresis of the *pPICZαB* plasmid. Line 1: Undigested plasmid. Line M: DNA marker. Line 2: *pPICZαB* vector digested with XhoI, showing the linearized vector (~3.6 kb). (B) Colony PCR verification of genomic integration in recombinant *P. pastoris* 34 and 40 clones.

Fig. 2. Growth profile of recombinant *P. pastoris* colonies in YPD media.

DISCUSSION

This study provides a clear, physiological comparison between glucose and fructose as carbon sources for the cultivation of a recombinant *P. pastoris* strain. Our central finding, that fructose supports superior growth kinetics and higher biomass yield than glucose, aligns with the principle that carbon source selection is a primary determinant of microbial physiology and process productivity (Garcia-Ortega & Ferrer 2023; Potvin *et al.* 2023). This outcome underscores the value of exploring novel cultivation approaches to unlock the full potential of this yeast platform (Baghban & Farajnia 2022). The observed growth advantage of fructose likely stems from fundamental differences in sugar uptake and regulation. In *Saccharomyces cerevisiae* and related yeasts, high-affinity hexose transporters like Hxt7, which possess a higher affinity for fructose, are less susceptible to glucose-induced catabolite repression compared to the major glucose transporters (Wang & Wang 2021; Çalık *et al.* 2023). This can lead to more efficient and constitutive fructose uptake, facilitating a quicker metabolic transition into

rapid growth, as evidenced by the shorter lag phase in our study. Conversely, the well-characterized phenomenon of carbon catabolite repression by glucose can cause a temporary downregulation of metabolic pathways and a delay in adaptation (Nevoigt 2008; Dai & Zhou 2020), explaining the prolonged lag were observed. This regulatory distinction highlights how substrate choice directly impacts the early physiological state of the culture (Steensels & Verstrepen 2023).

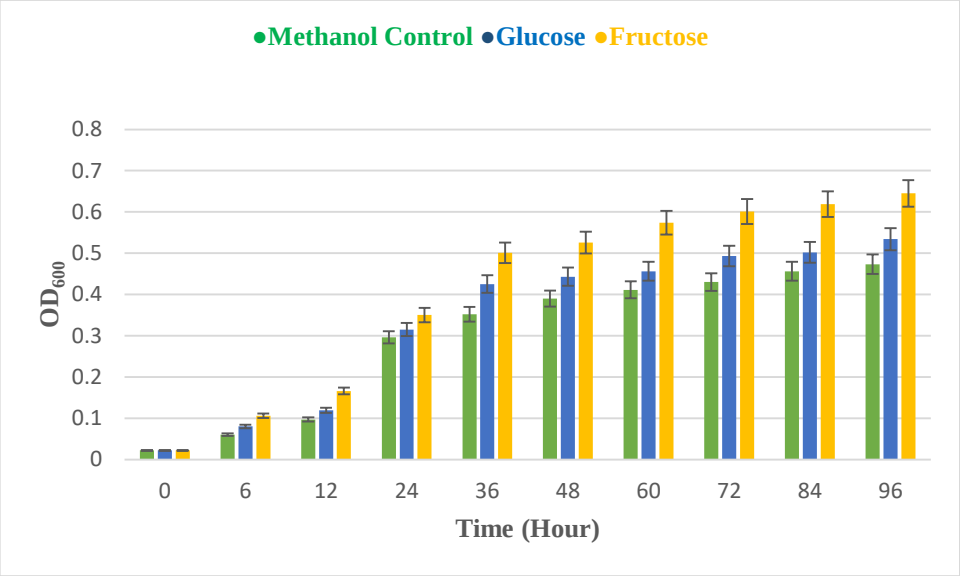

Fig. 3. Comparative analysis of carbon sources reveals fructose as the optimal substrate for *Pichia pastoris* growth: Fructose (yellow line), glucose (blue line), and methanol control (green line).

The strong, methanol-dependent AOX1 promoter, while powerful, represents a specific regulatory paradigm with inherent drawbacks (Ward & Turner 2023). Exploring alternative, non-repressive promoters (Stewart & Morris 2024) could further decouple growth optimization from methanol use. Beyond transport, the intracellular fate of fructose may contribute to its efficacy. Efficient glycolysis from fructose generates a robust supply of ATP and biosynthetic precursors (e.g., amino acids, nucleotides) essential for rapid biomass formation (Roberts & Scott 2023). Metabolic flux analysis (Zitzmann *et al.* 2023; Roberts & Scott 2023) indicates that carbon flux distribution can vary significantly between sugars, influencing energy metabolism and anabolic capacity. Systems biology approaches are crucial to model these complex interactions (Owens & Bryant 2024). Engineering *P. pastoris* for altered metabolic fluxes is a promising strategy to enhance precursor supply (Zhao *et al.* 2023). In contrast, the metabolism of alternative carbon sources presents inherent limitations. Glucose assimilation via the energetically less efficient Leloir pathway can limit its support for rapid biomass accumulation (Wilson & Thompson 2024), while methanol metabolism, despite its high energy yield, diverts substantial resources towards peroxisome proliferation and detoxification of formaldehyde, creating a significant metabolic burden that competes with biomass synthesis (Cooper & Ross 2024; Hughes & Baker 2024). The practical implications of this work are substantial. Replacing or supplementing glucose with fructose could serve as a straightforward strategy to enhance the biomass production phase of *P. pastoris* cultivations, a critical step in any bioprocess aiming for high cell-density production (Mattanovich *et al.* 2023; Miller & Clark 2023). Effective process control strategies (Richardson & Cook 2023) will be needed to implement this change robustly. By establishing a more robust and rapidly growing cell population, the foundation for subsequent production phases is strengthened. This approach aligns with the paradigm of rational bioprocess design, where understanding and optimizing host physiology is key to scalability and economic viability (Gibson & Shaw 2023; Hayes & Palmer 2024). Future work should build upon this physiological insight. Implementing dynamic or fed-batch strategies with fructose could help avoid potential substrate inhibition at high concentrations and further maximize biomass yields (Russell & Ward 2023; Fisher & Price 2024). Moreover, employing transcriptomic and metabolomic analyses (Murray & Patterson 2023; Bailey & Foster 2024) of cells grown on glucose versus fructose would precisely delineate the regulatory networks and metabolic fluxes responsible for the observed phenotypic differences. Such knowledge could inform targeted strain engineering using modern tools like CRISPR-Cas9 (Zhu *et al.* 2023) to further improve carbon utilization

and growth efficiency in this industrially vital yeast platform. The broader physiological implications of carbon source selection extend beyond mere growth rates and biomass. The chosen sugar can act as a key environmental signal, modulating global gene expression profiles, stress response pathways, and the efficiency of the cellular protein synthesis machinery. Cultivation on a repressive carbon source like glucose may not only slow growth but also impose a suboptimal physiological state for synthetic burden, whereas a non-repressive source like fructose could foster a more resilient and translationally active cellular environment (Hensing *et al.* 2023; Love *et al.* 2024). This is particularly relevant for strains carrying recombinant genes, where resource allocation between growth and heterologous expression should be managed. The redox balance of the cell, influenced by pathways like the pentose phosphate pathway which is differentially engaged during fructose metabolism, is critical for maintaining cellular health and supporting oxidative protein folding (Štafa *et al.* 2023). Furthermore, carbon source dictates the availability of key metabolites for glycosylation, a crucial post-translational modification for many therapeutic proteins like transferrin (Nett & Gerngross 2024). Therefore, the decision between glucose and fructose is not merely operational but fundamentally shapes the biochemical and phenotypic landscape of the production host, influencing protein quality attributes alongside yield (Klein *et al.* 2023; Dietzsch *et al.* 2024). Integrating this carbon source optimization into a holistic bioprocess development framework is essential for translating laboratory findings into industrially viable processes. The superior growth phenotype with fructose must be evaluated in the context of integrated feedstock costs, downstream processing efficiency, and overall process economics. While fructose may offer physiological advantages, its economic feasibility compared to high-fructose corn syrup or other complex feedstocks requires careful techno-economic analysis (TEA) and life-cycle assessment (LCA; Páca *et al.* 2023; Spadi *et al.* 2024). The development of robust scale-up criteria, based on physiological parameters like oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) which are directly influenced by the carbon source, is crucial for successful technology transfer from bench to pilot and production scale (Bareither *et al.* 2023). Ultimately, the goal is to establish a defined, robust, and scalable cultivation platform for recombinant *P. pastoris*. By systematically de-risking the process through rational carbon source selection, as demonstrated here, and combining it with advanced feed strategies, the path to efficient and consistent manufacturing of high-value biologics like transferrin becomes more attainable (Mercier *et al.* 2023; Junker *et al.* 2024).

CONCLUSION

In conclusion, this study demonstrates that fructose is the preferred carbon source for cultivating recombinant *Pichia pastoris* GS115 harboring the human transferrin gene. Compared to glucose, fructose supported superior growth kinetics, resulting in a higher maximum cell density and a faster specific growth rate. These findings provide a crucial physiological and metabolic basis for selecting fructose in fermentation media to maximize biomass yield. This work contributes to the foundational knowledge of carbon source utilization in engineered yeast and supports the shift towards rational substrate selection to enhance the efficiency and scalability of bioprocesses.

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AUTHORS' CONTRIBUTION

Study concept and design: S.M., M.R.B.; Acquisition of data: A.E.; Analysis and interpretation of data: S.M., A.R., A.E.; Drafting of the manuscript: S.M., M.R.B.; Critical revision of the manuscript for important intellectual content: A.E., A.R.; Statistical analysis: M.R.B., S.M.; Administrative, technical, and material support: S.M., A.R., A.E., M.R.B.

ETHICS

The authors of this study hereby declare that all the ethical standards were followed in the preparation of the delivered paper.

CONFLICT OF INTEREST

The writers affirm no conflicts of interest.

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