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Enzyme-Assisted Fermentation of Lactose for the Recombinant Brazzein Production in Pichia pastoris

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Abstract

Sweet proteins are attractive candidates for reduced-sugar foods because they provide intense sweetness at very low use levels. Brazzein is a particularly promising sweet protein due to its high sweetness potency, thermal and pH stability, and sucrose-like taste profile. Although recombinant production of brazzein has been demonstrated in several heterologous systems, improving the economic and environmental feasibility of microbial production requires attention to the carbon sources used for fermentation. Here, we develop an enzyme-assisted fermentation strategy to produce brazzein from lactose-containing substrates using engineered *Pichia pastoris*. Because *P. pastoris* does not efficiently utilize lactose, we supplement cultures with β-galactosidase to hydrolyze lactose *in situ* into fermentable monosaccharides that support yeast growth and recombinant protein production. We first compare three secretion signals and identify the α-mating factor pre–pro signal as the most effective of those tested for extracellular brazzein production. We then show that lactose alone does not support substantial *P. pastoris* growth, whereas β-galactosidase supplementation enables growth in lactose-containing medium. Finally, we evaluate lactose concentration and methanol induction strategy and find that brazzein production depends on the balance between lactose-derived carbon availability and methanol-driven expression. The highest specific yield observed in this study is approximately 1847 μg/g biomass under sequential methanol induction with 30 g/L lactose, corresponding to a volumetric yield of approximately 9.5 mg/L. These results indicate that enzyme-assisted lactose hydrolysis can extend the range of carbon sources usable by *P. pastoris* without additional lactose-metabolism engineering. All experiments were performed with analytical-grade lactose in defined medium and in shake flasks without pH or dissolved-oxygen control; the approach therefore remains a laboratory-scale proof of concept that requires validation with industrial whey permeate.

Key points

• β-galactosidase lets Pichia pastoris grow on lactose without pathway engineering

• Sequential methanol induction at 30 g/L lactose gave 1847 μg brazzein per g biomass

• Volumetric yield *9.5 mg/L* is comparable to reported glucose-fed brazzein production

Introduction

Sweet proteins are a class of naturally occurring proteins that have an intense sweet taste while contributing minimal caloric value (Novik et al. 2023). As interest grows in natural alternatives to sucrose and artificial sweeteners, sweet proteins have attracted increasing attention as next-generation ingredients for reduced-sugar foods and beverages. Among them, brazzein is a particularly attractive candidate because of its high sweetness potency, favorable thermal and pH stability, and sucrose-like taste profile (Ming and Hellekant 1994; Poirier et al. 2012). Originally isolated from the fruit of *Pentadiplandra brazzeana*, brazzein is present only in limited amounts in its natural source, making direct extraction unsuitable for commercial production. To overcome this limitation, recombinant expression has been explored in several heterologous systems, including microbial hosts such as *Escherichia coli* and *Lactococcus lactis* (Berlec et al. 2006), *Pichia pastoris* (Poirier et al. 2012), and *Kluyveromyces lactis* (Yun et al. 2016), as well as transgenic animals (Yan et al. 2013) and plants (Lamphear et al. 2005; Choi et al. 2022). Although transgenic animal and plant systems demonstrate the feasibility of producing brazzein outside its native source, microbial fermentation offers a more practical route for industrial production because of its shorter production cycle, controlled cultivation conditions, established scalability, and compatibility with existing biomanufacturing infrastructure. Among the microbial hosts, *P. pastoris* has emerged as a widely used platform for recombinant protein production because it supports high-cell-density cultivation, efficient secretion of heterologous proteins, and scalable fermentation processes (Li et al. 2022).

Conventional microbial fermentation processes commonly use readily metabolized sugars, such as glucose or sucrose, because these substrates support robust microbial growth and are compatible with established production hosts (Singh et al. 2017). However, when these sugars are derived from primary agricultural crops such as corn, sugarcane, or sugar beet, they can contribute substantially to raw material costs and create competition with crop-based supply chains already used for food and animal feed production (Banat et al. 2014; Zhang et al. 2022; Augustin et al. 2024). Consequently, food-processing by-products and agro-industrial residues are increasingly being explored as alternative carbon sources for microbial fermentation to support the production of biofuels, commodity chemicals, enzymes, microbial biomass, and other value-added bioproducts (Diaz et al. 2018; Sadh et al. 2018; Cuadrado-Osorio et al. 2022).

Among potential alternative feedstocks, dairy whey permeate is particularly attractive because it is generated in large quantities during cheese and casein manufacturing, resulting in millions of tons of whey permeate produced annually worldwide (Buchanan et al. 2023). Whey permeate is rich in lactose, which typically accounts for 70–75% of the total solids (Ahmad et al. 2019; Tsermoula et al. 2021), making it a potentially low-cost and abundant carbon source for biomanufacturing applications. However, lactose is not readily metabolized by many industrial production hosts, including *P. pastoris*, limiting the direct utilization of lactose-rich whey streams for recombinant protein production. To address this challenge, previous studies have engineered lactose-utilization pathways in industrial microorganisms through heterologous expression of lactose transporters and β-galactosidase enzymes (Rubio-Texeira 2006; Zotta et al. 2020; Cunha et al. 2021). While effective, these approaches

can increase strain-construction complexity and impose additional metabolic burdens on the production host (Wu et al. 2016; Ceroni et al. 2018).

Here, we develop an enzyme-assisted fermentation strategy to produce the sweet protein brazzein from lactose-containing substrates using engineered *P. pastoris*. We first engineer *P. pastoris* to secrete brazzein by integrating a codon-optimized brazzein gene fused to different secretion signals into the AOX1 locus, enabling methanol-inducible expression and extracellular secretion. We supplement cultures with β-galactosidase to hydrolyze lactose *in situ* into glucose and galactose, which are readily metabolized by *P. pastoris*. We compare secretion signals, evaluate how different carbon-source conditions affect yeast growth and protein yield, and test whether enzymatic hydrolysis of lactose enables utilization of lactose-containing substrates for recombinant brazzein production. As an initial proof of concept, all fermentation experiments use analytical-grade lactose dissolved in defined medium rather than unprocessed whey permeate; validation with real dairy-derived whey streams is identified as essential future work.

Materials and methods

Media and reagents

To prepare 1 L buffered glycerol complex medium (BMGY) and 1 L buffered methanol complex medium (BMMY), we first dissolve 10 g of yeast extract and 20 g of peptone in 700 mL and 795 mL milliQ water, respectively. We autoclave the mixtures for 20 minutes on liquid cycle, cool to room temperature, and add the following and mix well: 100 mL 1 M potassium phosphate buffer, pH 6.0; 100 mL 10× YNB (yeast nitrogen base, stock concentration 13.4%); 2 mL 500× biotin (stock concentration 0.2 mg/mL); and 100 mL 10× glycerol (stock concentration 7.5%) to make the final BMGY. For BMMY, we add 100% methanol to a final concentration of 1% methanol. We store the media at 4°C and use them within two months.

To prepare simulated whey substrate, we use BMY as the base medium with 25 g/L glucose, 50 g/L lactose, or 25 g/L glucose plus 25 g/L galactose. β-galactosidase (from *Aspergillus oryzae*, MP Biomedicals, Irvine, CA) is used for lactose hydrolysis. Lactose used in this study is analytical grade and is used to simulate lactose-rich substrates. Materials are purchased from VWR unless otherwise noted.

Plasmid construction

We construct the plasmids with pJAG vectors from BioGrammatics. Briefly, we obtain the genes encoding the sweet protein from Twist Biosciences. We clone the genes into pJAG vectors and test three secretion signals: s1 (α-mating factor pre–pro secretion signal), s3 (Ost1p/α-mating factor fusion secretion signal), and s5 (Aga2p signal). The plasmids are named pJAG-s1-bBRZN, pJAG-s3-bBRZN, and pJAG-s5-bBRZN (Table S1). We verify the sequence of the entire ORF and the cloning junctions via Genewiz. The expressed protein carries a Tobacco Etch Virus (TEV) protease-cleavable site with a 6×His tag. The complete amino acid sequence of the target protein is as follows:

HHHHHHENLYFQGSDKCKKVYENYPVSKCQLANQCNYDCKLDKHARSGECFYDEKRNLQCICDYCEY

The full nucleotide sequence of the codon-optimized brazzein gene (165 bp), together with the sequences of the three secretion signals and the three complete expression cassettes (471 bp for s1, 480 bp for s3 and 258 bp for s5), is provided in Table S2. The brazzein-coding sequence is identical in all three constructs, which differ only in the secretion signal (Table S1).

Transformation of Pichia pastoris

Electrocompetent *P. pastoris* BG10 cells (PS20010, BioGrammatics) are transformed by electroporation. To perform a restriction digest, we treat 10 µg of plasmid DNA with FastDigest MlsI (FD1214, Thermo Fisher Scientific, MA, USA). After confirming complete linearization by agarose gel electrophoresis, we heat-inactivate the reaction, extract the vectors once with phenol/chloroform, and precipitate with ethanol using 1/10 volume 3 M sodium acetate and 2.5 volumes of 100% ethanol. We centrifuge the solution to pellet the DNA, wash the pellet with 80% ethanol, air-dry, and resuspend in 10 µL sterile deionized water. We store the digested vectors at − 20°C until use.

To transform the vector into *P. pastoris* by electroporation, we mix 80 µL of electrocompetent cells with 200 ng of linearized DNA (5 µL) and transfer the mixture to an ice-cold (0°C) 0.2 cm electroporation cuvette. We remove the electrocompetent cells from the − 80°C freezer and warm the tube rapidly by hand to preserve viability. We gently mix the DNA and cells, transfer the entire sample to a sterile ice-cold 1 mm cuvette, and ensure the sample is positioned between the metal plates. We then place the cuvette between the electrodes of the electroporation device (Bio-Rad GenePulser I) and discharge; the whole process is completed within 5 seconds. The settings are 25 µF, 200 Ω, and 1150 V. After discharge, we add 1 mL Pichia Electroporation Recovery Solution (PERS, a 1:1 mixture of YPD and 1 M sorbitol) to the cuvette and mix with the cells, then transfer the sample to a tube and incubate at room temperature with shaking at 100 rpm for 3 hours. We spread the cells onto YPD agar plates containing 1000 µg/mL G418 for selection and incubate at 30°C for 2 days. We test three plating volumes (10, 30,

and 100 µL) to identify optimal colony formation. We pick single colonies from the original selection plates and transfer them to a second selection plate to ensure that any co-picked non-transformed cells will not grow. We prepare glycerol stocks of selected clones from the second selective plate by mixing an overnight YPD culture to a final concentration of 30% v/v glycerol.

Culture conditions for strain evaluation

To perform methanol induction of AOX1 promoter expression in transformed cells, we first inoculate 2 mL of sterile BMGY containing 0.75% glycerol in a 14 mL round-bottom tube with individual *P. pastoris* clones. We grow the cells overnight at 30°C and 200 rpm with good aeration for approximately 20 hours, then measure OD600. We pellet the cells by centrifugation in a 2 mL sterile tube at 8000 rpm for 30 seconds, resuspend in 2 mL sterile BMMY containing 1% methanol, and incubate at 30°C and 200 rpm. At 24 hours post-induction we measure OD600; if it is similar to or slightly higher than the pre-induction value, we add 200 µL BMMY containing 10% methanol to maintain methanol at 0.1% or 0.5% throughout the incubation. We continue incubation and feed the cells every 24 hours, harvesting supernatant at every feed until 120 hours post-induction. We separate cells from supernatant by centrifugation in 1.5 mL tubes at 14,100 rpm for 3 minutes, draw off the supernatant without disturbing the pellet, repeat the centrifugation for 1 minute to remove residual cells, and store the supernatant at − 80°C until analysis.

Protein purification and SDS-PAGE analysis

To purify recombinant brazzein, we thaw the supernatants at room temperature and capture the protein using Pierce High Capacity Ni-IMAC MagBeads (A50589, Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. We determine brazzein concentration using the bicinchoninic acid assay (BCA, 23225, Thermo Scientific, USA); samples are incubated at 37°C for one hour before absorbance at 562 nm is measured in a plate reader. We prepare denatured brazzein by mixing with 4× Laemmli sample buffer containing 50 mM DTT and boiling at 95°C for 10 min. We then perform SDS-PAGE using precast NuPAGE (4–12%) Bis-Tris gels with MES SDS running buffer in a Bio-Rad gel box, following the method of Schägger and von Jagow, with polypeptide SDS-PAGE standards. The voltage is held constant at 200 V. We stain the proteins with Bio-Safe colloidal Coomassie Brilliant Blue R using a 50 mL staining buffer containing 50 mg Coomassie Brilliant Blue R, 5 mL acetic acid, 25 mL methanol, and 20 mL water; the destaining buffer contains 5 mL acetic acid, 20 mL methanol, and 25 mL water.

Shake-flask production of recombinant brazzein

For larger-scale production of brazzein, we first grow the cells in 5 mL BMGY containing 0.75% glycerol for 20 hours, pellet 4 mL of the culture at 8000 g for 30 s, and resuspend in 40 mL BMMY containing 1% methanol in a 250 mL baffled flask (Biologix, CA, USA) to induce expression. We monitor OD600 and feed the cells with BMMY containing 10% methanol every 24 hours. All experiments are conducted in shake flasks without active pH or dissolved-oxygen control.

Culture conditions for β-galactosidase and Pichia pastoris in lactose-containing medium

To assess the combined performance of β-galactosidase and *P. pastoris*, we culture separate batches in 14 mL tubes containing 2 mL BMY-Glu20 medium for 24 h. We harvest the cells by centrifugation at 8000 rpm and wash with sterile water, then inoculate at an OD600 of 0.05 into 250 mL baffled flasks with a 30 mL working volume. The synthetic media used in these shake-flask cultures are either BMY-Lac50 or BMY-Glu25Gal25, comprising 1.7 g/L BMY and 50 g/L lactose or a mixture of 25 g/L glucose and 25 g/L galactose, respectively. We incubate the flasks at 30°C and 200 rpm, and sample approximately 2.5 mL of cell suspension at 0, 24, 48, 72, and 120 h.

β-galactosidase enzyme activity measurement

To measure the specific activity of β-galactosidase, we use o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate, which generates a yellow signal in the presence of β-galactosidase. To perform the assay in 96-well microplates, we mix 10 µL enzyme solution, 50 µL of 50 mM Tris buffer (pH 7.5), 17 µL of 4 mg/mL ONPG, and 125 µL distilled water. We initiate the reaction at 37°C and record absorbance at 420 nm every 30 seconds for 90 minutes. We determine the amount of enzyme in the reagent using the BCA method (Smith et al. 1985). One unit of enzyme is defined as the amount catalyzing the hydrolysis of 1 µmol of ONPG per minute at 37°C and pH 7.5. Specific activity is calculated as (µmol ONP)/[reaction time (min) × total enzyme amount (mg)]. The concentration of ONP is calculated using an extinction coefficient of 4.5 mM^{-1} cm^{-1} at 420 nm, corrected for the microplate path length, giving a corrected coefficient of 0.08216 µmol/OD.

Cell dry weight calibration

To convert OD600 measurements to cell dry weight (CDW), we establish an empirical calibration factor. We collect three independent culture samples at OD600 values of approximately 15 from a single representative batch, pellet each sample by centrifugation at 8000 rpm for 5

minutes, wash the pellets once with sterile deionized water, transfer them to pre-weighed 50 mL tubes, and freeze-dry overnight. We calculate CDW concentration (mg/mL) by dividing the measured dry mass by the sampled volume, then regress CDW against the corresponding OD600 value. This yields a conversion factor of 0.283 mg CDW per mL per OD600 unit (i.e. CDW (mg/mL) = OD600 × 0.283), which we apply to all batches in this study to calculate specific brazzein yield (μg brazzein per g CDW). The calibration is performed at high cell density (OD600 ≈ 15), and the OD-to-CDW relationship is assumed to be linear across the range of cell densities encountered in this study. Volumetric yield (μg/L) is calculated directly from the measured brazzein concentration in the culture supernatant and does not require the CDW factor.

Statistical analysis

All experiments are performed in triplicate unless otherwise stated. Data are presented as mean ± standard deviation of three biological replicates. No formal hypothesis testing was applied; comparisons between conditions are descriptive.

Results

Design of an enzyme-assisted fermentation workflow

To enable the use of lactose-containing substrates by *P. pastoris*, we implement the enzyme-assisted fermentation strategy illustrated in Fig. 1. In this approach, β-galactosidase-mediated hydrolysis of lactose generates glucose and galactose during cultivation, providing accessible carbon sources for yeast growth and recombinant brazzein production.

Secretion signal affects extracellular brazzein yield

We generate three constructs differing only in the secretion signal peptide: the α-mating factor pre–pro signal (s1), an Ost1p/α-factor fusion signal (s3), and the Aga2p secretion signal (s5). Following methanol induction, we collect culture supernatants and purify the recombinant proteins by Ni-affinity chromatography. Brazzein has a reported molecular weight of approximately 6.5 kDa, and the His-tagged construct used here is expected to migrate in this low-molecular-weight range. SDS-PAGE analysis of Ni-affinity-purified culture supernatants reveals a dominant low-molecular-weight band consistent with the expected size of His-tagged brazzein (Fig. 2a) for all three constructs.

We next compare brazzein production among the three constructs during methanol-induced expression (Fig. 2b). Over the 8-day induction period, strains carrying the s1 secretion signal produce consistently higher brazzein yields than those carrying s3 or s5. We therefore select the s1 strain for all subsequent experiments evaluating fermentation on lactose-derived substrates. Because only a single clone per construct was characterized, and gene copy number was not determined, these yield differences are attributed to the secretion signal only provisionally.

Enzymatic lactose hydrolysis enables growth in lactose-containing medium

To evaluate whether *P. pastoris* can utilize lactose as a carbon source, we compare yeast growth in BMY medium containing glucose or lactose, with or without β-galactosidase, monitoring optical density at 600 nm over 24 h (Fig. 3).

Cultures supplied with glucose show robust growth, reaching an OD600 of approximately 0.30 after 24 h. When lactose is provided as the sole carbon source without enzyme supplementation, negligible cell growth is observed. Supplementation with β-galactosidase at 0.18 U/mL supports measurable cell growth, and a higher enzyme concentration (1.8 U/mL) further improves growth kinetics and final cell density, approaching that of the glucose control.

Lactose concentration and methanol induction strategy jointly influence specific brazzein yield

We next examine how lactose concentration and methanol induction strategy influence brazzein production. We grow cultures in media containing lactose at 15, 30, or 50 g/L with β-galactosidase-mediated hydrolysis, and induce recombinant protein expression using three methanol addition strategies: direct addition of 0.1% methanol at 0 h, direct addition of 0.5% methanol at 0 h, or sequential induction with 0.1% methanol at 0 h followed by 0.5% methanol after 48 h (Fig. 4a–c).

Under low methanol induction (0.1%), brazzein production increases steadily during the induction period, with the highest specific yield in cultures containing 15 g/L lactose (Fig. 4a). Higher lactose concentrations do not consistently improve specific brazzein yield.

When methanol is added directly at 0.5% (Fig. 4b), the response is strongly dependent on lactose concentration. At 30 g/L lactose, brazzein remains below the detection limit of the assay throughout the entire cultivation period. At 15 g/L lactose, brazzein production is delayed relative to the corresponding condition in Fig. 4a, with no detectable yield before approximately 45 h; once production begins, yields continue to rise and exceed those observed under all Fig. 4a conditions at the final timepoint.

Under sequential methanol induction (Fig. 4c), the highest brazzein yield is observed at 30 g/L lactose, reaching approximately 1847 µg/g biomass after 144 h. This corresponds to a volumetric yield of approximately 9.5 mg/L. Yields at 50 g/L lactose remain low under all three induction strategies. The lactose concentration giving the highest specific yield shifts from 15 g/L under low methanol induction (Fig. 4a) to 30 g/L under sequential induction (Fig. 4c).

Discussion

The results presented here establish an enzyme-assisted strategy for producing recombinant brazzein from lactose-containing substrates using engineered *P. pastoris*. Among the three secretion signals tested, the α-mating factor pre–pro signal gave the highest extracellular brazzein yield. This is consistent with the established role of the α-MF pre–pro signal in the *P. pastoris* secretory pathway (Brake et al. 1984): the signal directs the preprotein into the endoplasmic reticulum, after which the Kex2 protease cleaves the pro-region to release the mature protein. This proteolytic maturation step may improve folding fidelity and secretion efficiency for small, disulfide-rich proteins such as brazzein. We note, however, that differences in secretion efficiency among signal peptides are frequently confounded by clone-to-clone variation in integration site and gene copy number, which we did not control for; the ranking reported here should therefore be regarded as an empirical selection for this study rather than a general conclusion about signal peptide performance.

The three signals tested differ in how the mature protein is released. The α-MF pre–pro signal (s1) and the Ost1p/α-MF fusion (s3) both terminate in the Kex2 recognition sequence, so the pro-region is removed in the late secretory pathway to expose the 6×His tag at the N-terminus of the secreted product. The Aga2p signal (s5) is an 18-residue pre-signal removed by signal peptidase in the endoplasmic reticulum. Signal peptidase cleavage of heterologous fusions in *P. pastoris* can be less efficient than Kex2-mediated maturation, and incomplete processing would reduce the amount of correctly matured protein recovered from the supernatant. This offers a possible explanation for the lower yields obtained with s5, but we did not determine the N-terminus of the secreted product by Edman degradation or mass spectrometry, so processing efficiency was not measured directly.

The growth experiments show that lactose alone does not support substantial *P. pastoris* growth, whereas β-galactosidase supplementation restores proliferation in lactose-containing medium. This addresses lactose utilization at the process level rather than through additional host engineering. Previous approaches have typically introduced lactose transporters and β-galactosidases into the production strain (Rubio-Texeira 2006; Zotta et al. 2020; Cunha et al. 2021). External enzymatic hydrolysis instead decouples lactose conversion from strain construction, which may simplify process development and avoids the metabolic burden associated with additional heterologous machinery (Wu et al. 2016; Ceroni et al. 2018). *Kluyveromyces lactis* natively metabolizes lactose via its LAC gene cluster (Rubio-Texeira 2006) and is an alternative host for brazzein production (Yun et al. 2016); the approach described here retains the high-cell-density cultivation and secretion characteristics of *P. pastoris* without requiring lactose-metabolism engineering. We have not, however, performed a side-by-side comparison of the two hosts or a cost analysis of enzyme addition versus strain engineering, so the relative merits of the two strategies remain open.

The fermentation experiments indicate that brazzein yield depends on the interaction between carbon availability and induction strength rather than on carbon supply alone. Increasing lactose concentration did not consistently increase specific yield, and the strongest induction condition did not give the highest sustained production. A plausible interpretation is that methanol oxidation in *P. pastoris* generates formaldehyde and reactive oxygen species that affect cell physiology, and that this stress is exacerbated at higher substrate loads; this would be consistent with the absence of detectable brazzein at 30 g/L lactose under direct 0.5% methanol induction. We emphasize that we did not measure formaldehyde, reactive oxygen species, residual sugars, or cell viability, so this mechanism is a hypothesis suggested by the yield data rather than a demonstrated cause. Similarly, the shift in optimal lactose concentration from 15 g/L under low methanol induction to 30 g/L under sequential induction is consistent with higher cell densities being able to use more lactose-derived carbon productively, but we did not measure carbon uptake or biomass composition to test this directly.

The peak specific yield of 1847 µg brazzein per g biomass corresponds to a volumetric yield of approximately 9.5 mg/L, which is of the same order as the *9.8 mg/*L reported by Poirier et al. (2012) under optimized glucose-fed conditions. This comparison should be treated as indicative only. The two studies differ in cell density, flask geometry, induction and feeding strategy, purification workflow, and protein quantification method, and no direct glucose-fed control was run in parallel here. The present data therefore support the weaker statement that a waste-derived carbon source can sustain brazzein production in a broadly similar range, not that the two processes have equivalent productivity.

Several limitations constrain the conclusions that can be drawn from this work. First, all fermentations used analytical-grade lactose in defined medium rather than industrial whey permeate. Real whey-derived streams contain salts, minerals, residual peptides, and other components that may affect β-galactosidase activity, yeast physiology, and downstream purification, and validation with authentic whey permeate is required before any claim of dairy by-product valorization can be made. Second, all cultivations were performed in shake flasks without pH or dissolved-oxygen control, conditions under which oxygen transfer and pH drift are uncontrolled variables; performance in instrumented bioreactors may differ substantially. Third, brazzein was quantified by BCA assay on Ni-affinity-purified supernatant fractions, which measures total protein in the eluate rather than brazzein specifically; co-purifying species would inflate the reported yields, and an orthogonal quantification method such as

densitometry, ELISA, or mass spectrometry would strengthen these measurements. Fourth, the CDW calibration was established at a single high cell density (OD600 ≈ 15) and assumed to be linear over the full range of densities encountered, which introduces uncertainty into specific yields calculated at low densities. Fifth, only one clone per secretion signal construct was characterized and copy number was not determined. Sixth, the study reports three biological replicates per condition without formal statistical testing, so apparent differences between neighboring conditions should be interpreted with caution. Finally, no sensory evaluation of the purified protein was performed, so the functional sweetness of the product obtained under these conditions has not been verified.

Future work should validate the strategy with unprocessed whey permeate, move to instrumented bioreactors with controlled pH and dissolved oxygen, apply brazzein-specific quantification, and explore methanol-free or mixed-feed expression systems to improve compatibility with food-grade manufacturing. A techno-economic assessment at pilot scale would be needed to determine whether the cost advantage of a waste-derived feedstock is retained once enzyme costs and process complexity are included.

Conclusion

In this study, we developed an enzyme-assisted fermentation strategy to produce the sweet protein brazzein from lactose-containing substrates using engineered *Pichia pastoris*. β-galactosidase supplementation hydrolyzed lactose into fermentable monosaccharides, enabling lactose-derived carbon to support yeast growth and recombinant protein production. Secretion-signal comparison, lactose concentration testing, and methanol-induction optimization showed that brazzein production depends on the combined effects of secretion efficiency, carbon availability, and induction strategy. The highest specific yield observed was approximately 1847 μg/g biomass under sequential methanol induction with 30 g/L lactose, corresponding to approximately 9.5 mg/L. Within the limits of a shake-flask study using model lactose, these results indicate that enzyme-assisted lactose hydrolysis can broaden the range of carbon sources usable by *P. pastoris* without additional lactose-metabolism engineering. Validation with unprocessed whey permeate, controlled bioreactor cultivation, and techno-economic analysis at pilot scale will be required to establish whether the approach is viable for industrial production of recombinant sweet proteins.

Declarations

Ethical approval

Not applicable. This article does not contain any studies with human participants or animals performed by any of the authors.

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

YL, DW and PS conceived and designed the study. YL constructed the expression strains, performed the fermentation and protein analysis experiments, analyzed the data, and wrote the first draft of the manuscript. XL and YW performed culture and enzyme activity experiments and contributed to data analysis. JH provided methodological guidance on strain construction and protein purification. DW and PS supervised the project and acquired funding. All authors read, revised, and approved the final manuscript.

Availability of data and materials

All data supporting the findings are provided within the article and its supplementary files, and are available from the corresponding author on reasonable request. The nucleotide sequences of the codon-optimized brazzein gene and of the three expression cassettes are provided in Table S2, and the three constructs are summarized in Table S1. A plain-text FASTA file containing the same sequences is provided as Supplementary Data. Plasmids and strains generated in this study are available from the corresponding author on request.

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Figures

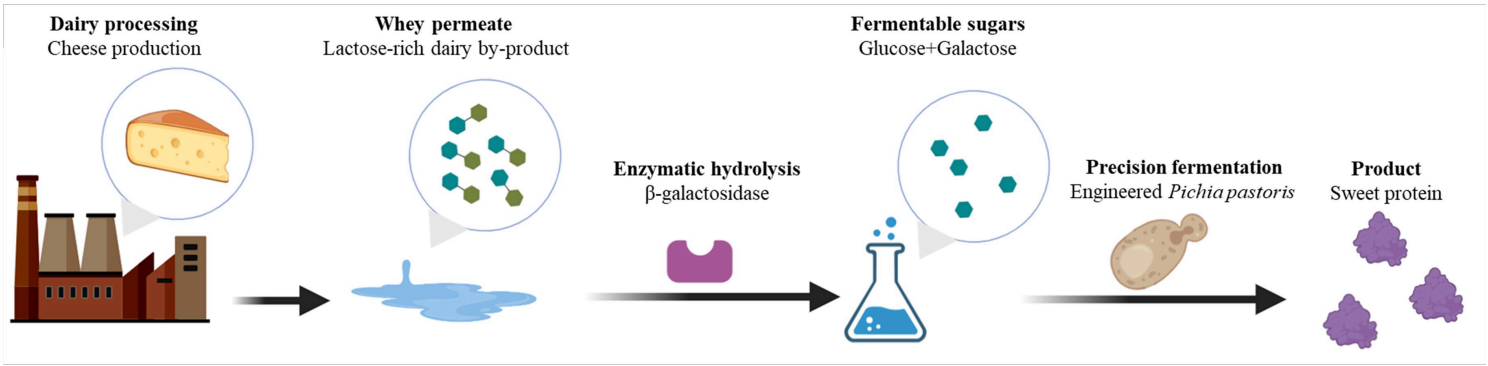

Figure 1

Schematic illustration of the enzyme-assisted fermentation strategy for the conversion of lactose into the sweet protein brazzein. Lactose present in dairy whey is hydrolyzed by β-galactosidase into glucose and galactose, which are subsequently utilized by engineered *Pichia pastoris*to produce recombinant brazzein

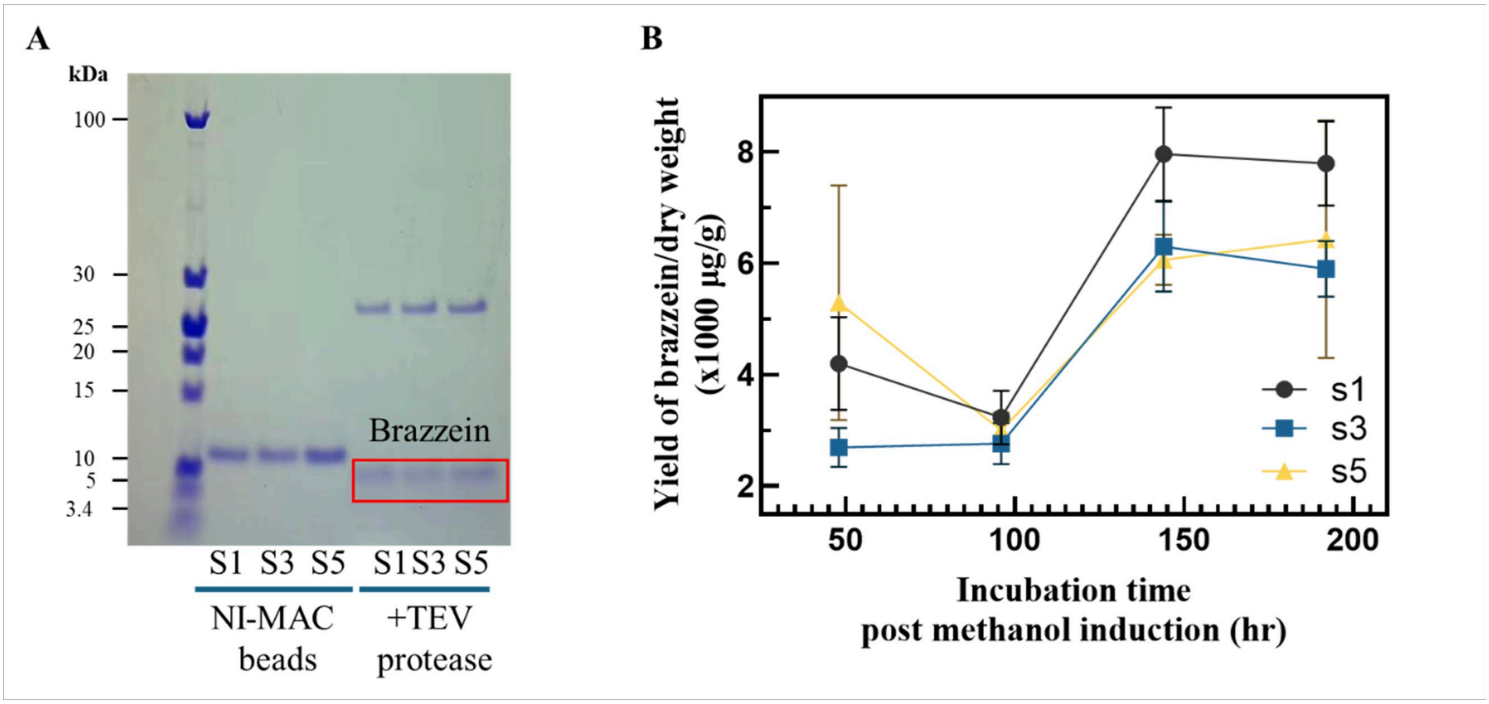

Figure 2

Optimization of brazzein secretion in *Pichia pastoris*. a Representative SDS-PAGE analysis of purified brazzein produced by recombinant *P. pastoris* strains expressing different secretion signal constructs (α-mating factor pre–pro secretion signal, s1; Ost1p/α-mating factor fusion secretion signal, s3; and Aga2p signal, s5). b Time course of brazzein production from the three strains during methanol-induced expression in 15 mL culture tubes over 8 days. Protein yield is quantified from purified culture supernatants. Data are presented as mean ± standard deviation of three biological replicates

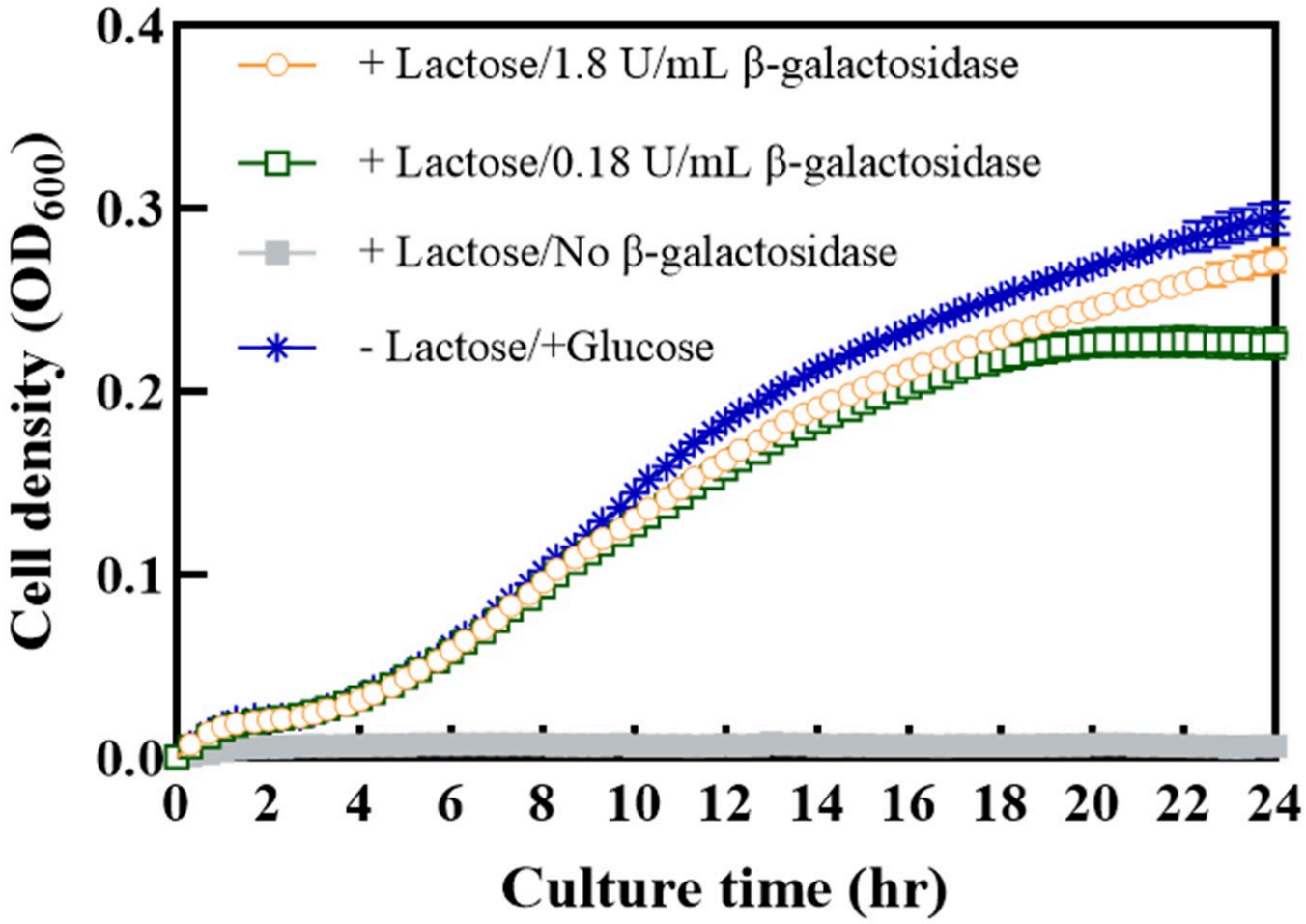

Figure 3

Growth of engineered *P. pastoris* in lactose-containing media with or without β-galactosidase. Yeast cultures are grown in BMY medium containing either 25 g/L glucose or 50 g/L lactose as the carbon source. Lactose media are supplemented with β-galactosidase at 0.18 or 1.8 U/mL. The lactose condition is carbon-normalized relative to the glucose control. Cell growth is monitored by measuring optical density at 600 nm over 24 h. Data are presented as mean ± standard deviation of three biological replicates

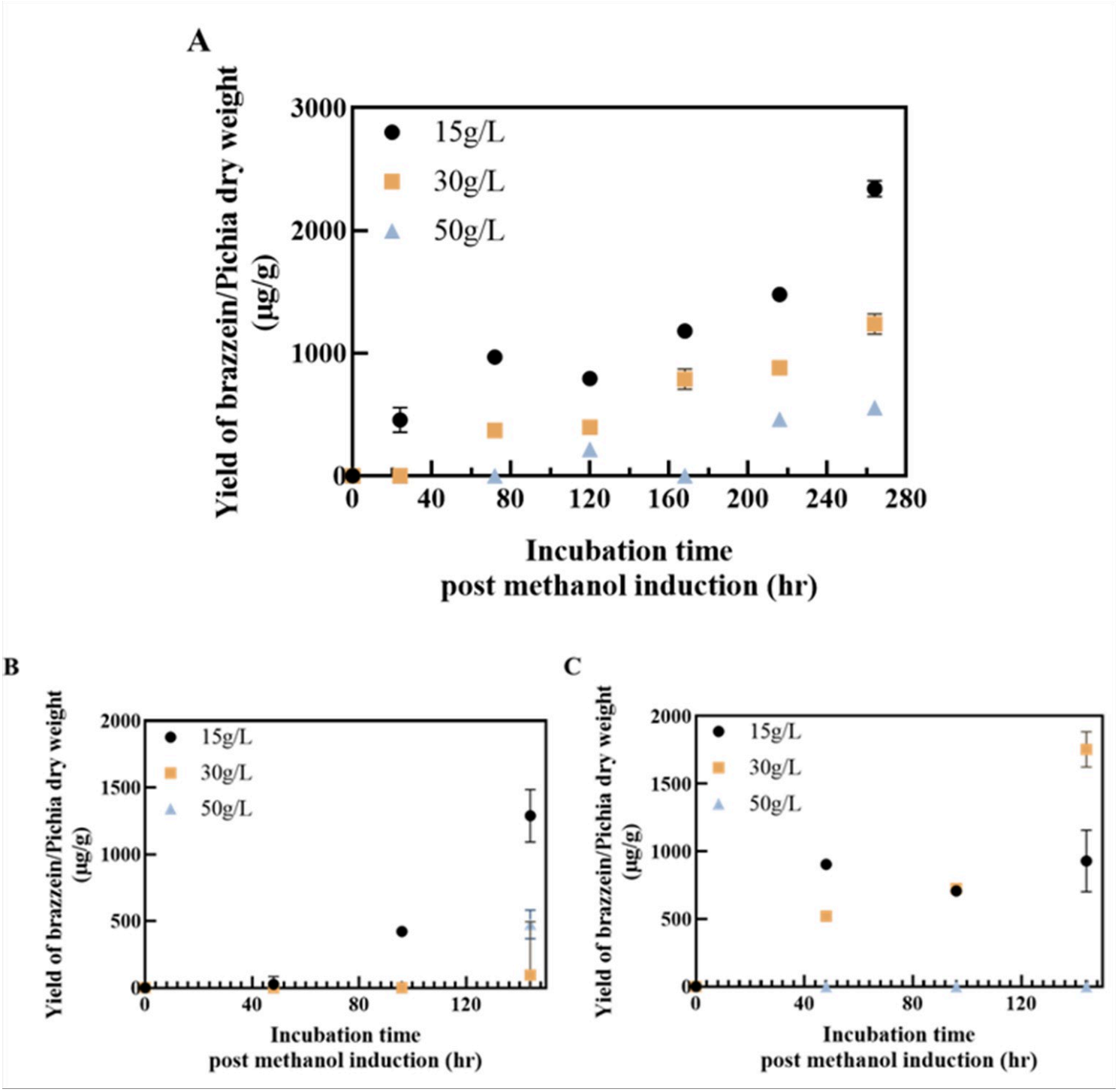

Figure 4

Effect of lactose concentration and methanol induction strategy on brazzein production by engineered *Pichia pastoris*. Cultures are grown in media containing lactose at 15, 30, or 50 g/L with β-galactosidase-mediated hydrolysis. Protein expression is induced using three methanol addition strategies: a direct addition of 0.1% methanol at 0 h; b direct addition of 0.5% methanol at 0 h; and c sequential induction with 0.1% methanol at 0 h followed by 0.5% methanol after 48 h. Brazzein yield is normalized to yeast dry weight and monitored over the induction period. Data are presented as mean ± standard deviation of three biological replicates.

Supplementary Files

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