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# *Active Recovery and Stability Engineering of Brazzein for Sustainable Production*

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***Abstract.*** Brazzein is a compact, disulfide-rich sweet protein whose manufacturing value depends on the recovery of a precisely processed and functionally active product rather than on expression titre alone. This review integrates evidence on structure-sweetness relationships, recombinant hosts, fusion-assisted recovery, process stability, safety, and the use of spent brewer's yeast as a circular input. Yeast and fungal systems now reach high reported titres, while soluble fusion and engineered-aggregation routes provide distinct solutions to intracellular recovery. However, studies quantify different constructs and fractions, and analytical evidence of mass or folding does not by itself demonstrate sweetness. A six-stage evaluation framework is proposed that begins with a fixed product specification and compares host performance, primary recovery, cleavage, purification, functional activity, process retention, and circular-resource burden within one mass balance. The preferred endpoint is sequence-verified, tag-free, functionally active Brazzein recovered per litre of culture after a defined treatment. Applying this endpoint would enable fair head-to-head comparison of production routes and connect molecular engineering to food performance, techno-economics, and life-cycle assessment.

***Keywords:*** Brazzein, active inclusion bodies, protein engineering, precision fermentation, spent brewer's yeast

## 1. Introduction

Reduction of free-sugar intake has intensified interest in high-intensity sweeteners that deliver sweetness at low use levels. Brazzein, first isolated from the fruit of Pentadiplandra brazzeana, is a 54-residue protein with a molecular mass of approximately 6.4 kDa and a reported potency of roughly 500-2,000 times that of sucrose on a mass basis, depending on the comparison conditions [1]. Solution, synthetic, disulfide-mapping, and crystallographic studies describe a compact fold containing one alpha helix and three antiparallel beta strands constrained by four disulfide bonds [2-5]. This structure contributes to its unusual heat and pH tolerance, but the same small, disulfide-rich architecture makes terminal processing, oxidative folding, and impurity control central manufacturing issues.

Brazzein activates the human T1R2-T1R3 sweet receptor [6], and its response is distributed across multiple surface regions rather than determined by a single epitope. Mutagenesis and receptor studies implicate the N- and C-terminal regions, loop residues, and charged surface positions [7-9].

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The terminal disulfide bond contributes directly to structural integrity and sweetness [10], while removal of an adventitious N-terminal methionine can increase the sweetness of recombinant material [11]. Therefore, a band at the expected electrophoretic size or a high protein concentration cannot substitute for evidence of terminal identity, correct disulfide organization, and biological response.

Recombinant production began with soluble Escherichia coli expression [12], expanded to Lactococcus lactis and Saccharomyces cerevisiae [13, 14], and advanced through secretory yeast, filamentous fungal, and plant platforms [15-18]. Downstream strategies now include engineered active inclusion bodies based on L6KD-SUMO [19] and a soluble gnd_v2-TEV route that connects cleavage and purification with sensory testing [20]. These advances are promising, but titres are not directly comparable when one study measures total fusion protein, another measures secreted immunoreactive material, and another reports purified target.

This review therefore addresses Brazzein as an integrated product-process problem. It first evaluates molecular and production evidence, including circular proposals based on compositionally variable spent brewer's yeast [21, 22]. It then distinguishes food-processing stability from gastrointestinal digestion [23] and interprets toxicological evidence as product- and specification-specific [24, 25]. Finally, it develops a six-stage framework in which all routes are compared by the mass of sequence-verified, tag-free, functionally active Brazzein recovered per litre after a defined treatment. This common endpoint provides the basis for comparing molecular design, host selection, downstream recovery, food performance, and circular-resource claims within one manufacturing framework.

## 2. Literature review

### 2.1. Molecular structure and product identity

Native Brazzein exists as closely related sequence forms, and recombinant constructs often differ at the N terminus. The major natural form contains pyroglutamate, whereas a commonly studied 53-residue form lacks the initial residue; bacterial expression may also leave an initiator methionine. Synthetic and recombinant studies show that these apparently minor differences can change sweetness and analytical behaviour. The four disulfide bonds are not merely generic stabilizers: they constrain the receptor-facing surface, and disruption of the terminal bond changes both local dynamics and sensory function. Consequently, the term Brazzein should always be accompanied by a stated mature sequence, terminal form, and processing history.

Structural verification also requires more than a single assay. Intact mass can establish gross sequence and processing consistency but cannot prove disulfide connectivity. Reversed-phase chromatography can reveal changes in hydrophobic surface exposure but is not a direct measure of native structure. Circular dichroism, nuclear magnetic resonance, disulfide mapping, or another orthogonal conformational method should therefore be combined with intact-mass and terminal analysis. Functional testing then closes the evidence chain. This layered specification is especially important for fusion-derived material because cleavage may be efficient while leaving a non-native terminal residue or a low-abundance uncleaved species [9-11].

### 2.2. Sweetness determinants and stability engineering

Brazzein engineering operates under a coupled constraint: residues that improve conformational stability may also participate in receptor recognition. Heat-dependent hydrogen-deuterium exchange

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identified residues 6-14 and 36-45 as locally sensitive regions [26], while structure-based calculations proposed substitutions that could improve thermal stability [27]. These studies prioritize candidate positions, but computational scores and localized exchange changes are hypotheses until the purified variant is tested after the intended processing treatment.

Multi-site mutation studies show that favourable single substitutions need not combine additively [28]. Crystal-structure-informed docking further supports a broad receptor-contact surface [29], which makes epistasis and altered binding orientation plausible. More recently, protein language models have been used to explore Brazzein homologues with predicted or experimentally assessed sweetness gains [30]. Such models can expand sequence space efficiently, yet their outputs must be filtered through manufacturability: secretion, disulfide formation, protease susceptibility, solubility, matrix stability, and receptor activation should be measured on the same material. E9K and the methionine-free construct are useful positive comparators because sensory evidence exists; unvalidated stability candidates should be labelled as candidates rather than established improvements.

## 2.3. Production hosts and titre comparability

Host choice determines the chemical environment in which Brazzein folds and the downstream matrix from which it must be recovered. E. coli provides short development cycles and straightforward intracellular screening, but oxidative folding and aggregation can limit recovery. L. lactis demonstrated an alternative microbial context, although early expression was low and codon optimization did not necessarily improve production. Plant expression in maize reached substantial accumulation in seed tissue and demonstrated application potential, but extraction, crop containment, batch time, and agricultural variability create a different manufacturing model from fermentation [13, 18].

Kluyveromyces lactis has supported secretory production [31], and subsequent work developed defined-medium cultivation and improved quantification [32, 33]. In Pichia pastoris, now K. phaffii, signal-peptide selection affects secretion efficiency [34]. Systematic engineering of promoter strength, signal peptides, gene copy number, translation, folding, and degradation produced a reported 639.11 mg/L in a 5 L fed-batch process, while a zeocin-based screening strategy identified a separate 61.5 mg/L multicopy clone [35]. These studies demonstrate the value of host engineering, but they do not create a universal ranking because constructs, scales, quantification methods, and purification endpoints differ.

T. reesei provides a particularly instructive case. A reported titre of approximately 1.3 g/L was accompanied by analytical evidence for expected mass, four disulfide bonds, N-terminal pyroglutamate, and native-like folding. However, sweetness was not directly established by receptor or sensory testing. The result is therefore strong evidence of high-level production and structural quality, not yet a complete demonstration of functional product yield. Across hosts, minimum comparable reporting should include culture volume, biomass or cell density, mature sequence, total target, soluble or secreted fraction, purified recovery, purity, and functional activity.

## 2.4. Recovery and cleavage architectures

Aggregation need not imply complete protein inactivation [36]. Active inclusion bodies can retain native-like structure or activity and provide a dense, centrifugable intermediate. The L6KD-SUMO architecture exploits this principle: the L6KD peptide promotes self-association, SUMO supports folding and provides a specific protease junction, and aggregate capture reduces the soluble impurity

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load. SUMO technology is well established for difficult targets [37], and the L6KD-SUMO study directly included a 53-residue Brazzein construct. Released Brz53 was compared by reversed-phase chromatography with highly purified yeast-secreted material, supporting isolation and cleavage. It did not, however, directly establish receptor activation or perceived sweetness.

The gnd_v2-TEV system takes the opposite route by increasing soluble fusion expression. An engineered TEV protease improved cleavage at a difficult glutamine junction, and ultrafiltration was used to separate components. Sensory testing placed the recovered product at approximately 2,500-fold the sweetness of sucrose on a mass basis. This route therefore has a stronger link between recovery and function, although a complete culture-to-final-product balance was not reported. The two platforms should not be ranked by isolated headline measurements: L6KD-SUMO may simplify capture, whereas gnd_v2-TEV may avoid solubilization and shows direct sensory evidence.

pH-responsive inteins offer a third, self-cleaving architecture [38]. Removing an externally added protease could lower consumable and polishing burdens, but platform evidence cannot be transferred automatically to Brazzein. Premature cleavage during expression, incomplete conversion, pH-induced structural change, junction scars, and residual intein must all be measured. A fair head-to-head comparison should hold the mature sequence, strain background, culture scale, and final specification constant, then calculate recovery after capture, cleavage, clarification, polishing, and functional release.

## 2.5. Food processing stability digestion and safety

Thermal stability is conditional rather than absolute. Early work reported retained sweetness after heating, while recent structural analysis revealed localized changes before global unfolding [1, 26]. Temperature, residence time, pH, ionic strength, water activity, matrix composition, and cooling history should therefore be reported together. Soluble protein remaining after treatment is not necessarily receptor-active, and results obtained in purified buffer cannot be assumed to predict performance in an acidic beverage, dairy-like emulsion, or baked matrix.

Food-process stability and gastrointestinal persistence answer different questions [23]. An acid-only challenge tests chemical and conformational stress, whereas INFOGEST introduces sequential oral, gastric, and intestinal conditions with enzymes, bile salts, and changing ionic environments. Digestion experiments should quantify disappearance of intact Brazzein, peptide products, and residual activity rather than treating acid resistance as a proxy. For intended food use, a matrix panel should be selected from the actual application and paired with blinded sensory or cell-based receptor assays.

Safety conclusions must also follow the tested product. The recent toxicological evaluation of K. phaffii-derived Brazzein [24] strengthens the evidence base, but it does not automatically cover a different sequence, host, impurity profile, or cleavage route. Safety assessment should define the production strain, genetic construct, purification process, residual host proteins and DNA, aggregation state, sequence variants, intended exposure, and allergenicity strategy. Review literature supports promising food applications, but regulatory readiness depends on a reproducible manufacturing specification rather than on the protein name alone [25].

## 2.6. Spent brewer yeast as a circular input

Spent brewer's yeast is rich in protein, amino acids, vitamins, minerals, and cell-wall components, but it is not a uniform raw material. Brewery strain, wort composition, fermentation stage, harvest point, hop exposure, contamination, storage, and stabilization all change its composition. Recent

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work on lysis and fraction recovery strengthens the technical basis for valorization, yet collection, debittering, sterilization, hydrolysis, solids removal, analytical testing, and wastewater treatment remain process inputs rather than free benefits [21, 22].

Two routes should be distinguished. Recovered viable brewing cells could become a production host only after strain identity, purity, transformability, genetic stability, and reproducible secretion or intracellular expression are shown. The current literature does not establish unsorted spent brewing biomass as a controlled Brazzein host. A more defensible near-term route converts the biomass into a nutrient fraction for an independently validated production strain.

Hydrothermal processing can release proteins and amino acids from yeast cells [39], and spent brewer's yeast hydrolysate has served as a nitrogen source in succinic-acid fermentation [40]. These findings justify a Brazzein-specific medium experiment but do not prove equal performance. Hydrolysate should be standardized by available nitrogen, amino-acid profile, carbon contribution, ash, inhibitors, bioburden, and lot variability, then compared with commercial yeast extract and a defined nitrogen control. The outcome should be functional Brazzein yield per litre and per unit input burden, not biomass growth alone.

## 3. Integrated manufacturing framework and discussion

The literature can be translated into six linked manufacturing decisions rather than read as separate lists of hosts, tags, mutations, and waste streams. First, the molecular product and release specification must be fixed. Second, host performance must be compared using the same mature sequence and analytical fraction. Third, capture and cleavage must be evaluated as a recovery chain. Fourth, analytical identity must be connected to receptor or sensory function. Fifth, function must be remeasured after the intended food process. Sixth, circular inputs must be assessed within the same economic and environmental boundary. Figure 1 summarizes this sequence. Each subsection below states the evidence, the decision it supports, its limitation, and the next experiment required.

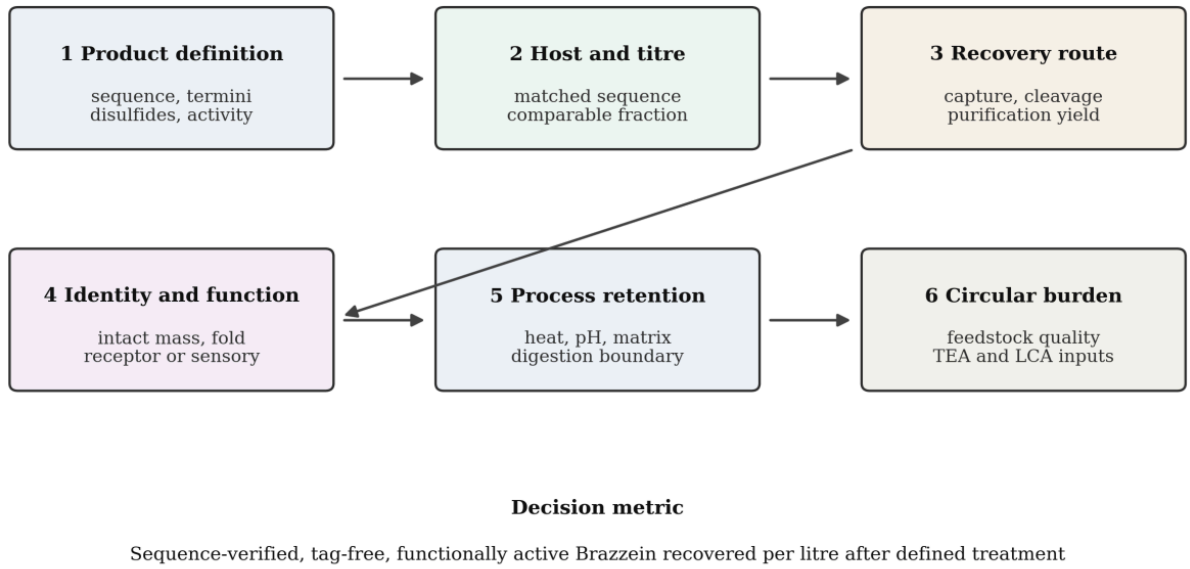



Figure 1. Six-stage decision framework for an integrated Brazzein production route

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### 3.1. Product definition and release specification

The first decision is not which host to use but which molecule is being manufactured. A defensible specification should state the mature amino-acid sequence, N- and C-terminal forms, acceptable processing variants, purity, aggregate content, intact mass, disulfide or conformational evidence, and the activity threshold. Without this definition, improvements in titre may reflect a different molecular product, and cleavage yields may include species that cannot meet the intended sweetness standard.

A tiered release strategy is practical. Tier 1 establishes identity and process control through intact mass, peptide mapping, terminal analysis, purity, and residual tag or protease. Tier 2 assesses structure through disulfide mapping and an orthogonal folding method. Tier 3 measures function using a calibrated T1R2-T1R3 cell assay and, where ethically and operationally appropriate, blinded sensory testing. Cell assays increase throughput, while sensory analysis captures temporal profile and off-notes; neither alone should be treated as a complete substitute for the other.

The immediate next step is to select one reference product, preferably a sequence with direct sweetness evidence, and prepare a traceable analytical standard. Every host and recovery construct can then be judged against the same target. This decision converts the vague endpoint of "Brazzein production" into a testable release claim and establishes the denominator for subsequent mass-balance calculations.

### 3.2. Upstream host selection and comparable titres

The host decision is a trade-off among volumetric production, product quality, development speed, and downstream burden. Bacterial systems are attractive for rapid variant screening and can exploit intracellular aggregation as a capture aid. Yeast and fungal hosts offer secretion and oxidative folding but may introduce heterogeneous processing, extracellular proteolysis, and complex supernatants. Plant seed production separates cultivation from fermentation infrastructure but adds agricultural and extraction constraints.

Reported titres should be normalized before route selection. At minimum, the same mature sequence should be measured with a validated assay at a defined sampling time and classified as total expressed, soluble intracellular, secreted, purified, or specification-compliant active product. Culture volume, wet or dry biomass, cell density, induction regime, and bioreactor scale should accompany the result. A high secreted titre can lose its advantage during concentration and polishing, whereas a lower intracellular titre can remain competitive if capture is selective and cleavage is efficient.

A matched host comparison should therefore begin at bench bioreactor scale with one analytical standard and one quantification workflow. The first screen may compare K. phaffii, T. reesei, and an E. coli recovery construct, but its decision criterion should be specification-compliant activity after primary recovery, not supernatant or lysate titre. Only the best two routes need progress to full polishing and economic modelling, which avoids expensive optimization of a host whose product cannot meet the release specification.

### 3.3. Recovery and cleavage route comparison

The recovery decision begins with the location and physical state of the target. Secreted protein requires clarification, concentration, and separation from host proteins and medium components. Soluble intracellular fusion requires disruption, clarification, capture, cleavage, and tag removal.

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Engineered inclusion bodies replace clarification of a soluble target with centrifugation of a dense particle, followed by cleavage or controlled release. Each route shifts rather than removes the downstream burden.

L6KD-SUMO and gnd_v2-TEV illustrate complementary strategies. The former offers simple primary capture and high local target concentration, but the decisive unknowns are cleavage accessibility, soluble recovery after release, and direct functional activity. The latter provides soluble expression and sensory evidence, but soluble lysate handling and added protease may increase impurity and polishing loads. An intein route could reduce external protease use, although premature or incomplete self-cleavage may trade material cost for yield uncertainty.

The correct comparison is a stepwise mass balance. For each route, target mass should be recorded after expression, primary capture, cleavage, clarification, polishing, and final activity testing. Cleavage conversion must be separated from product recovery: a reaction may convert most fusion protein yet lose released Brazzein on membranes, particles, or chromatography. Enzyme dose, reaction time, buffer volume, and number of unit operations should be reported because they drive scale-up cost and environmental burden.

Table 1 shows why existing platforms cannot yet be ranked by a single reported value. The next decisive experiment is a same-sequence, same-scale comparison of L6KD-SUMO and gnd_v2-TEV in a shared E. coli background, using common assays and a fixed purity target. If an intein construct is included, its cleavage should be triggered under conditions already shown not to reduce Brazzein activity.

Table 1. Evidence-aware comparison of representative Brazzein production routes

| Route | Strongest demonstrated endpoint | Boundary for comparison |
|---|---|---|
| K. phaffii secretion | Host engineering and fed-batch titre; secretion evidence [16, 34, 35] | Different constructs and quantified fractions; purified active yield not uniformly reported. |
| T. reesei secretion | Approx. 1.3 g/L with mass, disulfide, terminal, and folding analytics [17] | Direct receptor or sensory response not demonstrated. |
| K. lactis secretion | Secretory production, defined medium, and quantification development [31-33] | Scale and final recovery differ across studies. |
| E. coli gnd_v2-TEV | Soluble fusion, engineered cleavage, ultrafiltration, and sensory sweetness [20] | Complete culture-to-active-product balance unavailable. |
| E. coli L6KD-SUMO | Brz53 aggregate capture, release, and chromatographic comparison [19] | Direct functional response and scale-up cleavage recovery remain open. |
| Maize seed | High seed accumulation and application-oriented production evidence [18] | Agricultural cycle, extraction, containment, and batch variability alter the comparison. |

### 3.4. Stability engineering and functional validation

The stability decision should optimize retained sweetness, not structural persistence in isolation. Candidate residues can be prioritized where heat-sensitive regions, computational predictions, and

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receptor evidence overlap. These regions are scientifically attractive but also risky because a substitution may stabilize the fold while weakening receptor binding. A staged screen reduces that risk: expression and identity first, thermal or pH challenge second, receptor activation third, and sensory comparison only for the best candidates [26-30].

Treatments must reproduce the intended application. A beverage candidate should be tested across realistic pH, ionic strength, storage temperature, and preservative conditions; a heated food candidate requires time-temperature history and matrix controls. Pre-treatment and post-treatment concentration alone are insufficient. Retained activity should be calculated relative to an untreated matched sample, while degradation, aggregation, oxidation, and terminal changes are measured in parallel.

The proposed decision rule is to advance a variant only if it improves post-treatment functional yield without increasing purification burden or producing an unacceptable sensory profile. This prevents a nominally stable but weakly sweet variant from displacing a less stable sequence that performs better at the required use concentration. Combination variants should be rebuilt and retested rather than predicted by adding single-mutation effects.

### 3.5. Circular feedstock and sustainability boundary

The circular-input decision should begin only after a baseline production route is reproducible. Introducing spent-yeast hydrolysate before the host and recovery process are stable makes it difficult to assign changes in titre, folding, or purification to the feedstock. The near-term route should therefore use a validated host and substitute a characterized hydrolysate fraction for part of the commercial nutrient input [21, 22, 39 ,40].

The experimental control is equal available nitrogen, not equal wet biomass. Commercial yeast extract and a defined nitrogen mixture should serve as comparators. Growth, productivity, product quality, proteolysis, colour, foam, membrane fouling, and chromatographic load should be measured, because a medium that raises upstream titre may still increase downstream cost. Feedstock lots should be profiled to identify a compositional acceptance window.

Economic and environmental claims should use one gram of specification-compliant active Brazzein as the functional unit. Collection, cold storage or drying, debittering, lysis, sterilization, hydrolysis, chemicals, water, energy, solids disposal, and wastewater must enter the boundary. Avoided yeast-extract production may create a credit, but only when the replaced product and allocation method are explicit. The recovered-cell host route remains a separate, longer-term study because it requires strain-level control and stable genetic performance.

### 3.6. Integrated experimental pathway and decision criteria

A staged programme can close the evidence gaps efficiently. Phase 1 establishes the reference sequence, analytical standard, release specification, and receptor assay. Phase 2 compares recovery architectures in one bacterial background and compares the winning intracellular route with one secretion host. Phase 3 tests a small set of stability variants in application-relevant matrices. Phase 4 introduces standardized spent-yeast hydrolysate into the established process. Phase 5 integrates techno-economic and life-cycle models with experimentally measured yields and burdens.

Go or no-go criteria should be defined before each phase. A recovery route advances only if it meets identity, purity, and functional thresholds at an acceptable overall recovery. A variant advances only if retained post-treatment activity improves relative to the parent. A circular medium advances only if lot variability is controllable and total cost or environmental burden per gram of

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active product decreases. These criteria make negative results informative and prevent headline titre from dominating the decision.

Table 2 links each unresolved question to a measurement and decision node. Together with the mass balance, this matrix creates a reproducible path from construct design to a food-relevant manufacturing claim. It also separates evidence needed for scientific comparison from evidence needed for commercialization, where scale, impurity clearance, safety, sensory quality, and regulatory consistency all become binding constraints.

Table 2. Proposed validation matrix and decision gates

| Node | Question | Core measurement | Decision gate |
|---|---|---|---|
| Product identity | Which molecular form is released? | Intact mass; peptide and terminal mapping; disulfide evidence | Reject material outside the fixed sequence and processing specification. |
| Recovery | Where is target mass lost? | Stepwise mass balance; cleavage conversion; membrane and chromatography recovery | Advance the route with the highest specification-compliant functional recovery. |
| Function | Does analytical quality predict sweetness? | T1R2-T1R3 dose-response plus blinded sensory confirmation | Require activity within a predefined reference interval. |
| Processing | Is activity retained in the intended food? | Matched untreated and treated matrix tests; degradation and aggregation analytics | Advance only when post-treatment functional yield improves. |
| Circular input | Does spent-yeast hydrolysate reduce burden? | Equal-nitrogen controls; lot profiling; downstream load; TEA and LCA inventory | Require controllable variability and lower burden per gram of active product. |

## 4. Conclusion and future research

Brazzein manufacturing is best evaluated as a linked product-process system. Structural studies establish that termini, disulfides, and distributed receptor-facing residues are essential quality attributes. Production studies show that yeast and fungal secretion, soluble bacterial fusion, engineered active inclusion bodies, and plant expression are all credible routes, but their headline titres represent different materials and stages. The appropriate common endpoint is sequence-verified, tag-free, functionally active Brazzein recovered per litre after the intended treatment.

The first research priority is a matched, end-to-end comparison of recovery routes. One mature sequence should be expressed in a common E. coli background using L6KD-SUMO and gnd_v2-TEV, with intein self-cleavage included only after junction feasibility is shown. The study should report target mass at every unit operation, terminal identity, disulfide or fold evidence, purity, receptor activity, and sensory performance. The better intracellular route should then be compared with a leading secretion host under the same release specification.

The second priority is to connect sequence engineering to food performance. Candidate mutations should be selected from the intersection of heat-sensitive regions, receptor-contact evidence, and manufacturability predictions. Their value must be judged after realistic beverage or food

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treatments, using post-treatment receptor response and blinded sensory analysis rather than soluble protein alone. Parallel digestion studies should follow standardized INFOGEST conditions and quantify intact protein, peptides, and residual activity without conflating digestive persistence with processing stability.

The third priority is responsible scale-up and circular integration. Spent brewer's yeast should first be developed as a standardized nutrient input, supported by compositional acceptance limits, equal-nitrogen controls, impurity monitoring, and downstream-load measurements. Pilot-scale data should then feed techno-economic and life-cycle assessments whose functional unit is one gram of specification-compliant active Brazzein. Future safety and regulatory work must remain linked to the selected sequence, host, cleavage route, impurity profile, exposure, and final food application. This research sequence would convert a collection of promising platform results into an evidence base suitable for robust, scalable, and sustainable manufacture.

## 5. References


[1] Ming, D., & Hellekant, G. (1994). Brazzein, a new high-potency thermostable sweet protein from Pentadiplandra brazzeana B. *FEBS Letters, 355*(1), 106–108. doi:10.1016/0014-5793(94)01184-2.

[2] Caldwell, J. E., Abildgaard, F., Dzakula, Z., Ming, D., Hellekant, G., & Markley, J. L. (1998). Solution structure of the thermostable sweet-tasting protein brazzein. *Nature Structural Biology, 5*(6), 427–431. doi:10.1038/nsb0698-427.

[3] Izawa, H., Ota, M., Kohmura, M., & Ariyoshi, Y. (1996). Synthesis and characterization of the sweet protein brazzein. *Biopolymers, 39*(1), 95–101. doi:10.1002/(SICI)1097-0282(199607)39:1<95::AID-BIP10>3.0.CO;2-B.

[4] Kohmura, M., Ota, M., Izawa, H., Ming, D., Hellekant, G., & Ariyoshi, Y. (1996). Assignment of the disulfide bonds in the sweet protein brazzein. *Biopolymers, 38*(4), 553–556. doi:10.1002/(SICI)1097-0282(199604)38:4<553::AID-BIP10>3.0.CO;2-B.

[5] Nagata, K., Hongo, N., Kameda, Y., Yamamura, A., Sasaki, H., Lee, W. C., et al. (2013). The structure of brazzein, a sweet-tasting protein from the wild African plant Pentadiplandra brazzeana. *Acta Crystallographica Section D: Biological Crystallography, 69*(Pt 4), 642–647. doi:10.1107/S0907444913001005.

[6] Li, X., Staszewski, L., Xu, H., Durick, K., Zoller, M., & Adler, E. (2002). Human receptors for sweet and umami taste. *Proceedings of the National Academy of Sciences of the United States of America, 99*(7), 4692–4696. doi:10.1073/pnas.072090199.

[7] Assadi-Porter, F. M., Aceti, D. J., & Markley, J. L. (2000). Sweetness determinant sites of brazzein, a small, heat-stable, sweet-tasting protein. *Arch Biochem Biophys, 376*(2), 259-265. doi:10.1006/abbi.2000.1726.

[8] Jin, Z., Danilova, V., Assadi-Porter, F. M., Aceti, D. J., Markley, J. L., & Hellekant, G. (2003). Critical regions for the sweetness of brazzein. *FEBS Lett, 544*(1-3), 33-37. doi:10.1016/S0014-5793(03)00383-1.

[9] Assadi-Porter, F. M., Maillet, E. L., Radek, J. T., Quijada, J., Markley, J. L., & Max, M. (2010). Key amino acid residues involved in multi-point binding interactions between brazzein and the T1R2-T1R3 human sweet receptor. *J Mol Biol, 398*(4), 584-599. doi:10.1016/j.jmb.2010.03.017.

[10] Dittli, S. M., Rao, H., Tonelli, M., Quijada, J., Markley, J. L., Max, M., et al. (2011). Structural role of the terminal disulfide bond in the sweetness of brazzein. *Chem Senses, 36*(9), 821-830. doi:10.1093/chemse/bjr057.

[11] Liu, B., Jiang, H., Wang, H., & Yang, L. (2021). Removal of the N-terminal methionine improves the sweetness of the recombinant expressed sweet-tasting protein brazzein and its mutants in Escherichia coli. *J Food Biochem, 45*(3), e13354. doi:10.1111/jfbc.13354.

[12] Assadi-Porter, F. M., Aceti, D. J., Cheng, H., & Markley, J. L. (2000). Efficient production of recombinant brazzein, a small, heat-stable, sweet-tasting protein of plant origin. *Arch Biochem Biophys, 376*(2), 252-258. doi:10.1006/abbi.2000.1725.

[13] Berlec, A., Jevnikar, Z., Majhenic, A. C., Rogelj, I., & Strukelj, B. (2006). Expression of the sweet-tasting plant protein brazzein in Escherichia coli and Lactococcus lactis: A path toward sweet lactic acid bacteria. *Appl Microbiol Biotechnol, 73*(1), 158-165. doi:10.1007/s00253-006-0438-y.

[14] Kazemi-Nasab, A., & Shahpiri, A. (2020). Expression of brazzein, a small sweet-tasting protein in Saccharomyces cerevisiae: An introduction for production of sweet yeasts. *Protein Pept Lett, 27*(10), 945-952. doi:10.2174/0929866527666200331134431.

DOI: 10.54254/2753-8818/2027.39416

[15] Poirier, N., Roudnitzky, N., Brockhoff, A., Belloir, C., Maison, M., Thomas-Danguin, T., et al. (2012). Efficient production and characterization of the sweet-tasting brazzein secreted by the yeast Pichia pastoris. *J Agric Food Chem, 60*(39), 9807-9814. doi:10.1021/jf301600m.
[16] Rong, W., Wang, X., Shi, Z., Liu, L., Zhang, Z., Fang, Y., et al. (2025). Systematic engineering of Komagataella phaffii for efficient production of sweet-tasting protein brazzein. *Biotechnol J, 20*(6), e70046. doi:10.1002/biot.70046.
[17] Mojzita, D., Aro, N., Kontturi, J., Kogler, M., Nordlund, E., Hosia, W., et al. (2025). Label-free analytical characterization of brazzein produced with the filamentous fungus Trichoderma reesei. *Front Bioeng Biotechnol, 13*, 1688495. doi:10.3389/fbioe.2025.1688495.
[18] Lamphear, B. J., Barker, D. K., Brooks, C. A., Delaney, D. E., Lane, J. R., Beifuss, K., et al. (2005). Expression of the sweet protein brazzein in maize for production of a new commercial sweetener. *Plant Biotechnol J, 3*(1), 103-114. doi:10.1111/j.1467-7652.2004.00105.x.
[19] Komolov, A. S., Sannikova, E. P., Gorbunov, A. A., Gubaidullin, I. I., Plokhikh, K. S., Konstantinova, G. E., et al. (2024). Synthesis of biologically active proteins as L6KD-SUMO fusions forming inclusion bodies in Escherichia coli. *Biotechnol Bioeng, 121*(2), 535-550. doi:10.1002/bit.28587.
[20] Wang, Y., Zheng, J., Wen, F., Tu, B., & Cui, L. (2024). Novel gnd_v2 fusion tag and engineered TEV protease enable efficient production of brazzein. *J Microbiol Biotechnol, 34*(11), 2310-2320. doi:10.4014/jmb.2407.07047.
[21] Jacob, F. F., Striegel, L., Rychlik, M., Hutzler, M., & Methner, F. J. (2019). Spent yeast from brewing processes: A biodiverse starting material for yeast extract production. *Fermentation, 5*(2), 51. doi:10.3390/fermentation5020051.
[22] Ciobanu, L. T., Constantinescu-Aruxandei, D., Farcasanu, I. C., & Oancea, F. (2024). Spent brewer's yeast lysis enables a best out of waste approach in the beer industry. *Int J Mol Sci, 25*(23), 12655. doi:10.3390/ijms252312655.
[23] Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assuncao, R., Ballance, S., et al. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat Protoc, 14*(4), 991-1014. doi:10.1038/s41596-018-0119-1.
[24] Meetro, J., Nahian, L., Phipps, K. R., Vo, T. D., Dahms, I., Lalpuria, M., et al. (2025). Toxicological evaluation of the sweet protein brazzein derived from Komagataella phaffii for use as a sweetener in foods and beverages. *J Appl Toxicol, 45*(9), 1867-1886. doi:10.1002/jat.4811.
[25] Saraiva, A., Carrascosa, C., Ramos, F., Raheem, D., Pedreiro, S., Vega, A., et al. (2023). Brazzein and monellin: Chemical analysis, food industry applications, safety and quality control, nutritional profile and health impacts. *Foods, 12*(10), 1943. doi:10.3390/foods12101943.
[26] Zuo, J., Zheng, W., Shi, N., Song, R., Han, F., Yang, C., et al. (2024). Study on the thermal stability of the sweet-tasting protein brazzein based on its structure-sweetness relationship. *J Agric Food Chem, 72*(13), 7374-7382. doi:10.1021/acs.jafc.3c09616.
[27] Tang, N., Liu, J., & Cheng, Y. (2021). Potential improvement of the thermal stability of sweet-tasting proteins by structural calculations. *Food Chem, 345*, 128750. doi:10.1016/j.foodchem.2020.128750.
[28] Lee, J. W., Cha, J. E., Jo, H. J., & Kong, K. H. (2013). Multiple mutations of the critical amino acid residues for the sweetness of the sweet-tasting protein brazzein. *Food Chem, 138*(2-3), 1370-1373. doi:10.1016/j.foodchem.2012.10.140.
[29] Kim, T. Y., Woo, E. J., & Yoon, T. S. (2022). Binding mode of brazzein to the taste receptor based on crystal structure and docking simulation. *Biochem Biophys Res Commun, 592*, 119-124. doi:10.1016/j.bbrc.2022.01.004.
[30] Chua, B. N., Guo, W. M., Wong, H. T., Ow, D. S. W., Ho, P. L., Koh, W., et al. (2023). A sweeter future: Using protein language models for exploring sweeter brazzein homologs. *Food Chem, 426*, 136580. doi:10.1016/j.foodchem.2023.136580.
[31] Jo, H. J., Noh, J. S., & Kong, K. H. (2013). Efficient secretory expression of the sweet-tasting protein brazzein in the yeast Kluyveromyces lactis. *Protein Expression and Purification, 90*(2), 84-89. doi:10.1016/j.pep.2013.05.001.
[32] Park, S. W., Kang, B. H., Lee, H. M., Lee, S. J., Kim, H. S., Choi, H. W., et al. (2021). Efficient brazzein production in yeast Kluyveromyces lactis using a chemically defined medium. *Bioprocess and Biosystems Engineering, 44*(4), 913-925. doi:10.1007/s00449-020-02499-y.
[33] Lee, H. M., Park, S. W., Lee, S. J., & Kong, K. H. (2019). Optimized production and quantification of the tryptophan-deficient sweet-tasting protein brazzein in Kluyveromyces lactis. *Preparative Biochemistry and Biotechnology, 49*(8), 790-799. doi:10.1080/10826068.2019.1621892.
[34] Neiers, F., Belloir, C., Poirier, N., Naumer, C., Krohn, M., & Briand, L. (2021). Comparison of different signal peptides for the efficient secretion of the sweet-tasting plant protein brazzein in Pichia pastoris. *Life, 11*(1), 46. doi:10.3390/life11010046.
[35] Ravn, J., Raghavendran, V., & Geijer, C. (2026). Engineering sweetness: Zeocin-based strain screening to identify high-titer Komagataella phaffii clones for brazzein precision fermentation. *Food Science and Biotechnology, 35*,

DOI: 10.54254/2753-8818/2027.39416

1885-1894. doi:10.1007/s10068-026-02150-8.
[36] Garcia-Fruitos, E., Gonzalez-Montalban, N., Morell, M., Vera, A., Ferraz, R. M., Aris, A., et al. (2005). Aggregation as bacterial inclusion bodies does not imply inactivation of enzymes and fluorescent proteins. *Microbial Cell Factories, 4*, 27. doi:10.1186/1475-2859-4-27.
[37] Butt, T. R., Edavettal, S. C., Hall, J. P., & Mattern, M. R. (2005). SUMO fusion technology for difficult-to-express proteins. *Protein Expression and Purification, 43*(1), 1-9. doi:10.1016/j.pep.2005.03.016.
[38] Wood, D. W., Wu, W., Belfort, G., Derbyshire, V., & Belfort, M. (1999). A genetic system yields self-cleaving inteins for bioseparations. *Nature Biotechnology, 17*(9), 889-892. doi:10.1038/12879.
[39] Lamoolphak, W., Goto, M., Sasaki, M., Suphantharika, M., Muangnapoh, C., Prommuag, C., et al. (2006). Hydrothermal decomposition of yeast cells for production of proteins and amino acids. *Journal of Hazardous Materials, 137*(3), 1643-1648. doi:10.1016/j.jhazmat.2006.05.029.
[40] Jiang, M., Chen, K., Liu, Z., Wei, P., Ying, H., & Chang, H. (2010). Succinic acid production by Actinobacillus succinogenes using spent brewer's yeast hydrolysate as a nitrogen source. *Applied Biochemistry and Biotechnology, 160*(1), 244-254. doi:10.1007/s12010-009-8649-1.