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RECENT ADVANCES IN THE EXPRESSION OF SWEET-TASTING PROTEINS IN MICROBIAL SYSTEMS

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Annotation: In response to growing health concerns about excessive sugar consumption, the field of food biotechnology has turned increasing attention to sweet-tasting proteins as promising non-nutritive sugar substitutes. These proteins—such as brazzein, monellin, thaumatin and others—exhibit potent sweetness, low caloric content, and are of natural origin. However, their commercial application is hindered by difficulties in sourcing sufficient quantities from native plant sources and by challenges in cost-effective production. This review examines recent advances in heterologous expression of sweet-tasting proteins in microbial systems (bacteria, yeast and fungal hosts). Key themes include: optimisation of gene design (e.g., codon usage, signal peptides), host engineering (secretion pathways, protein folding/chaperones, reduction of proteolysis), and fermentation process improvements (promoter selection, gene copy number, feed-batch strategies). Overall, the paper underscores that microbial expression of sweet-tasting proteins is advancing rapidly and holds genuine promise, though further work is needed to translate lab-scale successes into commercially viable processes.

Keywords: sweet-tasting proteins; heterologous expression; microbial production; monellin; thaumatin; yeast expression systems; gene engineering; fermentation; metabolic engineering; non-nutritive sweeteners; food biotechnology; recombinant protein production.

1. Introduction: The Biological and Economic Imperative for Recombinant Sweet Proteins

The global effort to curb rising rates of obesity, diabetes, and hyperlipemia has intensified the search for sustainable, non-caloric sugar alternatives [1]. Traditional approaches, relying on both caloric sweeteners and artificial non-nutritive sweeteners, are increasingly scrutinized. Artificial sweeteners, such as aspartame, have been associated with adverse health outcomes including gastrointestinal issues, headaches, and dizziness, driving consumer preference toward naturally derived alternatives [2]. Sweet-Tasting Proteins (STPs) represent a highly promising class of biomacromolecules that exhibit extraordinary sweetening power, often exceeding that of sucrose by thousands of times [3].

1.1. Physicochemical Properties of Sweet-Tasting Proteins (STPs)

To date, eight primary STPs have been characterized, predominantly originating from tropical plants: thaumatin, monellin, brazzein, mabinlin, pentadin, curculin, miraculin, and neoculin [1]. The mechanism by which STPs elicit sweetness involves binding to and activating the human sweet taste receptor, the heterodimer T1R2/T1R3 [1]. Beyond simple sweetness, some STPs

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possess unique functional properties; for example, miraculin acts as a taste modifier, functioning as an antagonist at neutral pH but converting sour stimuli into sweetness under acidic conditions, thus becoming an agonist [1]. However, these desirable properties are constrained by intrinsic molecular shortcomings, such as low thermostability or variable sweetness profiles, necessitating detailed molecular engineering to realize their full commercial potential [1].

1.2. Challenges of Natural Extraction and the Transition to Microbial Expression

STPs are naturally isolated from exotic tropical plants, leading to high extraction costs, challenging purification protocols, and fundamental issues of resource sustainability, including the ecological impact on rainforest ecosystems [4]. These constraints establish a strong commercial justification for migrating production to engineered microbial host systems. Recombinant DNA technology, leveraging established success in the biopharmaceutical industry (which already features over 400 approved protein drugs), offers a sustainable, scalable, and cost-effective means for the mass biosynthesis of STPs [4]. The economic viability of STPs hinges directly upon the successful development of high-yield fermentation processes capable of delivering a steady and affordable supply, thereby offsetting the prohibitive costs and logistical instability associated with plant extraction [3].

2. Molecular Design and Engineering of Enhanced Sweet Proteins

The structural complexity of STPs necessitates intensive molecular engineering before high-level expression can be achieved in heterologous hosts. Many STPs, such as thaumatin, require precise tertiary structures stabilized by numerous disulfide bonds (thaumatin possesses eight) [7].

2.1. Structural Complexity and Functional Requirements

The precise configuration required for sweetness is non-trivial. For instance, thaumatin's sweet taste is predominantly ascribed to the positive electrical charge distribution on its surface, with specific residues like Arg76, Lys49, and Lys163 reported as critical for preserving its function [5]. Monellin, naturally a non-covalent heterodimer composed of A and B chains, suffers from inherent instability. This has been successfully mitigated by rational design, resulting in the creation of a thermostable, single-chain variant known as Monellin E-I (MNEI), which fuses the two chains via a Gly-Phe dipeptide junction [9]. The effectiveness of a recombinant sweet protein, therefore, relies on its ability to mimic the exact conformational structure of the native protein [11].

2.2. Rational Protein Engineering Guided by Structural Insights

Recent advancements have shifted the focus from random site-directed mutagenesis to rational design guided by structural biology. A conceptual understanding known as the "protein sector" suggests that specific biological properties arise from the cooperative action of a small subset of physically connected residues within the tertiary structure [1]. Targeting these "protein sectors" provides meaningful guidelines for engineering, allowing researchers to optimize the sweet

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properties of STPs systematically [1].

This approach confirms that modifications within these sectors must consider combinatorial effects rather than single-site changes. For example, the brazzein mutant H31R/E36D/E41A displayed significantly improved sweetness compared to any of the corresponding single-site or double-site mutants [1]. This phenomenon underscores the necessity of cooperative intramolecular interactions for desired function. Furthermore, proprietary computational methods, such as Agile Integrative Computational Protein Design (AI-CPD), are being applied to design "Designer Protein" sweeteners and optimize sequences for mass food market application, particularly where resolved crystal structures are limited [12]. Since correct folding and stability are primary bottlenecks in microbial production, this strategic upfront molecular design, aimed at enhancing inherent protein stability and potency (e.g., MNEI design), significantly simplifies the subsequent heterologous expression and purification stages.

3. Advanced Strategies in Microbial Host System Selection and Optimization

The choice of microbial host is the single most critical factor influencing yield and purity, primarily dictated by the protein's requirement for complex post-translational modifications, specifically disulfide bond formation.

3.1. Bacterial Systems: The Escherichia coli Trade-Off

Escherichia coli provides an attractive platform due to its low cost, rapid growth, and simplicity [13]. However, its inherent limitations become stark when producing complex STPs. The reducing environment of the E. coli cytoplasm actively inhibits the formation of disulfide bonds, leading to the accumulation of inactive and insoluble inclusion bodies (IBs), a challenge noted even for highly studied proteins like thaumatin [5]. Overcoming IBs necessitates energy-intensive and often low-efficiency in vitro refolding processes [14].

3.1.1. Overcoming Misfolding: Periplasmic Targeting and Folding Machinery

To mitigate IBs, several advanced engineering strategies are employed in bacteria. One key method is expressing the STP in the oxidizing periplasmic space via an appended signal peptide, which facilitates correct folding [15]. Alternatively, the cytoplasmic environment can be engineered to be more oxidizing through the use of specific host strains, such as TrxB- and gor-mutants, or by incorporating specialized enzymatic systems like CyDisCo (Cysteine Disulfide Bond formation catalyzed by the sulfhydryl oxidase Erv1p [15]. Furthermore, optimizing the gene sequence via codon optimization to align with the host's preferred tRNA supply is essential for reducing translational bottlenecks that lead to IBs and improving overall protein solubility [7]. Beyond E. coli, novel platforms are emerging, such as Lactococcus lactis NZ9000, which has demonstrated success in producing MNEI using cost-effective, sustainable dairy by-products like cheese whey, highlighting the potential for circular-economy bioproduction [17].

3.2. Yeast Systems: The Workhorses of STP Production

Eukaryotic hosts, particularly yeast species, offer a critical advantage: the presence of an

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endoplasmic reticulum (ER) and Golgi apparatus, which provide an oxidizing environment and the necessary enzymatic machinery (foldases and chaperones) for the formation of correct disulfide bonds and secretion [13]. The methylotrophic yeast Pichia pastoris (now referred to as Komagataella phaffii) is currently considered the most promising host, favored for its high-level secretion capabilities, ease of scale-up, and utilization of tightly regulated, strong promoters [2].

3.2.1. Promoter and Secretion Pathway Optimization

Achieving high secretion yields requires rigorous optimization of the expression cassette. Commonly, strong promoters like the methanol-inducible AOX1 promoter in P. pastoris or the PglaA promoter in Aspergillus niger are selected [2]. To guide the recombinant protein out of the cell, the Saccharomyces cerevisiae prepro α-mating factor secretion signal is frequently fused to the STP gene [2]. However, this is not always seamless; improper N-terminal processing can sometimes occur, indicating that precise optimization of the STP's inherent pre- and pro-sequences is often required to yield the native, active product [7].

3.2.2. Chaperone and Folding Machinery Modulation

Recent research demonstrates that simply utilizing the secretion pathway is insufficient; modulating the host's endogenous folding environment is essential. Targeted overexpression of molecular chaperones, such as Protein Disulfide Isomerase PDI and BipA, significantly assists in the correct folding kinetics and improves secretion efficiency [5]. Notably, optimizing the PDI level in Aspergillus awamori to between two and four times the native level resulted in a five-fold increase in thaumatin secretion [7]. Similarly, optimizing monellin expression in A. niger involved not only chaperone overexpression but also attenuating the Endoplasmic Reticulum-Associated Degradation (ERAD) pathway to prevent premature degradation of nascent proteins [9].

The rationale for preferring eukaryotic secretion systems is rooted in efficiency: while E. coli offers faster, cheaper initial biomass production, the cost and complexity of refolding inactive IBs often negate this advantage. The ability of yeast to secrete the product in its correctly folded, active form inherently streamlines downstream processing, ultimately lowering the total cost of goods sold (COGS) for complex STPs [13].

4. Genetic and Bioprocess Breakthroughs for High-Yield Production

High-titer production relies on coupling sophisticated molecular engineering with optimized bioprocess control, moving beyond simple lab-scale flasks to large-scale fermenters.

4.1. Strategies for Increasing Gene Expression and Yield

Advances in genetic manipulation include increasing the gene dosage of the STP within the host genome, which directly correlates with enhanced protein synthesis capacity [9]. Furthermore, protein fusion techniques are powerful for enhancing yield. Fusion tags, such as C-terminal FLAG-tags, serve multiple purposes: they facilitate affinity purification and can also enhance protein solubility and folding stability, without compromising the protein's secondary or tertiary

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structure [5]. For research purposes, especially when dealing with ultra-low expression levels, the use of highly sensitive detection methods is crucial. For example, the HiBiT-Tag, a small 1.3 kDa peptide, has been fused with codon-optimized monellin to allow highly sensitive quantitative bioluminescence detection, enabling researchers to accurately measure expression in systems where conventional antibody-based methods fail [9].

4.2. Bioprocess Control: The Key to Industrial Titer

The ceiling for microbial production is not just the genetic capacity of the strain but the ability of the bioprocess to sustain high cell density and protein synthesis flux. The use of optimized fed-batch fermentation in industrial bioreactors is mandatory for achieving commercial titers [18]. During fermentation, non-genetic parameters are essential for maintaining protein quality and yield. These include tactical adjustments such as lowering the culture temperature during induction, decreasing the concentration of the inducer (e.g., IPTG concentration reduction from 0.5–1.0 mM to 0.01–0.05 mM), or implementing a brief heat shock prior to expression. These measures are designed to reduce the synthesis rate, minimize aggregation, and induce the host's endogenous chaperone machinery [15].

4.3. Quantitative Comparison of High-Titer Microbial Expression

The efficacy of these combined strategies is best measured quantitatively. The most significant recent achievement is the high-titer production of Brazzein. Through systematic engineering of Komagataella phaffii (involving promoter and signal peptide evaluation, gene copy number optimization, and folding modulation), researchers achieved a final titer of 639.11 mg/L via fed-batch fermentation in a 5 L bioreactor [18]. This represents the highest level reported for microbially produced brazzein.

This breakthrough signifies that the primary technical bottleneck has successfully moved from the inability to fold the protein correctly (misfolding/IBs) to the challenge of maximizing total, active cellular output. However, achieving this high titer requires sustained cellular activity, confirming the crucial role of advanced bioprocess control (fed-batch) in capitalizing on engineered strains.

Other key achievements provide critical benchmarks:

- An engineered Pichia pastoris strain achieved a yield of approximately 400 mg/L of recombinant lysozyme (a sweet-tasting protein) [20].
- Early thaumatin II production in P. pastoris reached approximately 25 mg/L [2].
- Codon-optimized Monellin expression in E. coli yielded 43 mg/g dry cell weight [7].

A critical analysis of these titers reveals a substantial difference between the yield achieved for Brazzein (639.11 mg/L and Thaumatin (around 25 mg/L) in comparable yeast systems. Since Thaumatin requires eight disulfide bonds and Brazzein requires four, this discrepancy strongly suggests that structural complexity remains the most challenging factor for achieving

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commercial titers, demanding more intensive systems engineering efforts for the most complex STPs.

Table 1: High-Titer Production of Key Sweet Proteins in Recent Microbial Systems

Sweet Protein	Microbial Host	Key Engineering Strategy	Reported Maximum Titer/Yield	Reference
Brazzein	Komagataella phaffii (Yeast)	Optimized expression cassette, gene copy number, fed-batch fermentation	639.11 mg/L	[18]
Lysozyme (STP)	Pichia pastoris (Yeast)	Blasticidin S selection system	~400 mg/L	[20]
Monellin	Escherichia coli (Bacteria)	Codon-optimized synthetic gene, soluble expression	43 mg/g dry cell wt	[7]
Thaumatin II	Pichia pastoris (Yeast)	S. cerevisiae prepro α-mating factor signal	~25 mg/L	[2]

5. Downstream Processing and Quality Control

The economic viability of STP production hinges not only on high yield but also on efficient and cost-effective downstream processing (DSP).

5.1. Purification Challenges and Mitigation Strategies

For proteins expressed as IBs in E. coli, the most significant challenge is the required refolding step, which often results in significant protein loss and adds complexity to the process [8]. Conversely, proteins secreted by yeast or fungi require less rigorous initial steps but must contend with protease degradation, particularly in the extracellular environment. The inclusion of protease inhibitors and engineering protease-deficient host strains are critical mitigating steps [9].

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The primary purification strategy for STPs, which are generally positively charged (Monellin has a net positive charge of 3 at pH 5.5) [21], involves ion exchange chromatography. This method, often utilizing carboxymethyl Sepharose or DEAE columns, is typically preceded by bulk concentration methods such as salt precipitation (e.g., ammonium sulfate) [21]. The use of fusion tags, as discussed previously, streamlines purification significantly by allowing for affinity chromatography, providing an integrated solution that reduces overall DSP complexity [5].

5.2. Functional Characterization and Quality Assurance

The final product must be rigorously characterized to ensure it is structurally identical and functionally equivalent to the native plant protein. Recombinant thaumatin II produced in P. pastoris, for example, demonstrated secondary and tertiary structures almost identical to the plant protein and elicited the same sweetness threshold in humans (around 50 nM).11 Functional testing via mass spectrometry, HPLC, and sensory analysis in humans is mandatory to confirm both purity and bioactivity, assuring the integrity required for food applications [11].

6. Commercialization, Safety, and Future Directions: Systems Metabolic Engineering

6.1. Safety, Regulatory Approval, and Consumer Acceptance

The regulatory pathway for STPs is well-established by Thaumatin, which was the first sweet plant protein to be approved and commercialized (E957 in the European Union since 1984) [24]. Recent safety studies for recombinant monellin and brazzein confirm they are non-toxic, non-allergenic, and safe for consumption across tested mammalian species [10].

Crucially, comparative studies tracking the influence on gut microbiota composition provide a significant advantage over conventional sweeteners. Prolonged consumption of recombinant brazzein and monellin did not cause significant changes in rat gut microbiota or lead to the appearance of opportunistic bacteria, in contrast to a sucrose-fed group, which saw an increase in Faecalibaculum rodentium—a species potentially contributing to obesity [3]. This robust safety profile, particularly regarding the microbiome, addresses a major consumer and regulatory concern associated with artificial sweeteners and strengthens the commercial case for large-scale STP adoption.

6.2. Strategic Outlook and Patent Landscape

Despite significant academic breakthroughs, establishing industrial-scale biotechnological production facilities for STPs remains an area of active development [6]. The field is highly competitive, evidenced by an active patent landscape. Brazzein, noted for its high sweetness and stability, is the subject of multiple patents focusing on expression [26]. Companies like Amai Proteins are strategically employing computational protein design methods (AI-CPD) protected by numerous patents to optimize their Designer Protein ingredients for the mass market [12].

6.3. The Future: Systems Metabolic Engineering for Predictive Optimization

Moving forward, achieving multi-gram per liter titers necessary for full cost competitiveness requires a transition from sequential optimization to comprehensive Systems Metabolic Engineering and Synthetic Biology approaches [5]. The success achieved in Brazzein production

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(639.11 mg/L) highlights that marginal gains through isolated genetic modifications are diminishing, requiring a deeper understanding of the host cell's holistic physiology.
Systems biology provides crucial tools, such as omics technologies and flux balance analysis, to map the complex metabolism and cellular stress responses of the host [2]. This knowledge allows for the predictive redesign of biological systems using standardized engineering principles [27]. Future strategies will therefore focus on systematically manipulating multiple cellular functions simultaneously, including balancing the supply of precursor building blocks, optimizing energy expenditure for synthesis, and coordinating protein biogenesis stages (from transcription and codon choice to co-translational folding, secretion, and post-translational modification) [9]. By fully integrating the rational design of the STP molecule with the comprehensive metabolic capacity of the host cell, researchers aim to overcome the inherent limitations that currently prevent the highest-complexity STPs, like Thaumatin, from reaching commercial-scale yields.

Conclusions

Recent advances in the microbial expression of sweet-tasting proteins confirm their potential as viable and superior alternatives to sugar and artificial sweeteners. The technological frontier has moved from struggling with protein misfolding to maximizing and sustaining active protein titer, as evidenced by the breakthrough Brazzein yield of 639.11 mg/L achieved in Komagataella phaffii via systematic strain engineering and advanced fed-batch fermentation [18]. The success of these systems relies on a critical understanding of protein structure (using "protein sector" analysis and AI-CPD) and the strategic selection of eukaryotic hosts that can execute complex folding requirements, thereby avoiding the costly refolding steps associated with bacterial inclusion bodies. Furthermore, documented safety and a favorable impact on the gut microbiome provide a compelling commercial advantage for these recombinant sweeteners over synthetic counterparts [3]. The continued commercialization of these proteins will depend on the effective implementation of Systems Metabolic Engineering and Synthetic Biology to unlock the necessary multi-gram per liter production capacity, ensuring cost competitiveness and global market penetration.

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