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COMPARATIVE ANALYSIS OF BRAZZEIN PRODUCTION IN *IN VIVO* AND *IN VITRO* SYSTEMS

Ioannis Ilie KARPATHAKIS, Călina Petruţa CORNEA

University of Agronomic Sciences and Veterinary Medicine of Bucharest,
59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: iannis.karpathakis@gmail.com

Abstract

Brazzein, a highly potent sweetener derived from the fruit of the African plant Pentadiplandra brazzeana, has garnered attention due to its potential as a low-calorie alternative to sugar. This study presents a comparison between in vivo and in vitro systems for the production of brazzein, focusing on yield, cost-effectiveness, and sustainability. Utilizing genetically modified organisms (GMOs) in in vivo systems, specifically engineered yeast and bacteria, we explored the scalability and efficiency of brazzein production. Conversely, in vitro systems involved cell-free synthesis, highlighting the control over production conditions and the reduced risk of contamination. Economic analysis revealed that while in vivo systems benefit from lower initial investment costs and higher production rates, in vitro systems may offer long-term sustainability and lower environmental impact, attributed to reduced resource consumption and waste generation. This study provides critical insights into the feasibility of scaling brazzein production for commercial use, evaluating the pros and cons of each system. Further research into genetic engineering and optimization of culture conditions could enhance the efficiency and yield of brazzein production, contributing to the development of healthier sweetening options for the global market.

Key words*: brazzein, cell-free protein synthesis, in vitro, in vivo, production, sweet protein, Tx-Tl.*

INTRODUCTION

Brazzein is one of the five sweet-tasting plant proteins including mabinlin, neoculin, thaumatin, and monellin. Although there is a sixth sweet-tasting protein called pentadin, it is not characterized yet. These plant proteins are found in the tropical forests of Africa or South Asia. Pentadin and Brazzein are largely found in Cameroon, Gabon, Congo, and West African tropical forests.

The seeds are dispersed through primates who eat the fruits and spread the seeds with their feces. Among the tropical forests, evolutionary pressure is caused by the primate preference for the sweetest fruits, causing an increase in their sugar content (Koveshnikova et al., 2023). Among all the sweet plant proteins, Brazzein is considered the most desirable due to its low lingering off-taste, hence it being the most interesting plant protein.

Brazzein was identified for the first time by Ming and Hellekant back in 1994 as a new thermostable and sweet protein that is easily derived from the *P. brazzeana* fruit (Lynch et al., 2023). Its thermostability was tested through incubation at 98°C for 2 hours and 80°C for 4.5 hours. Nevertheless, the findings confirm that Brazzein does not lose its natural sweetness, an indication of its protein stability even at high temperatures (Koveshnikova et al., 2023). Moreover, the thermostability and sweetness of Brazzein are just some it's features. It is also known to demonstrate an isoelectric point of 5.4 and high-water solubility.

It is extracted from the *P. brazzeana* fruits through buffer solutions including phosphate buffer, which are precipitated with ammonium sulfate (Figure 1).

Source	Extraction	Isolation	Purification	Characterization	Main Results
P. brazzeana	0.1 M phosphate buffer at pH 7.0 containing 5% glycerol, 0.1 mM DTT, 20 mL PMSF, 0.1 mM EDTA and 0.5% (*w*/*v*) PVP at 4 °C	Protein precipitation with ammonium sulfate 30% and 85%	Ion-exchange chromatography using a CM-Sepharose CLdB column (gradient: NaCl of 0.1 to 0.4 M in 20 mM sodium citrate at pH 3.6)	SDS- PAGE; ESI-MS; sequence determination by S-Pyridylethylation and S-carboxymethylation of brazzein and peptide fragment separation by RT-HPLC.	Brazzein is a single-chain polypeptide; the molecular weights obtained by SDS-PAGE and ESI-MS were 6.5 KDa and 6.473 KDa, respectively; C-terminal is a tyrosine; 8 cysteines out of 54 residues.

Figure 1. Methods to extract, isolate and characterize Brazzein (Saraiva et al., 2023)

Nevertheless, growing the plant is considered quite challenging, hence the extraction and production of Brazzein is quite expensive. Multiple studies have applied the technological production of Brazzein by using genetic engineering through bacteria, transgenic plants, yeasts, and animals. Once there is extraction of the protein, it is expressed and isolated, then purified and characterized. There are multiple methods of characterization, purification, and isolation of Brazzein.

Brazzein is defined as a 6.5kDa sweet-tasting protein that has four disulfide bonds. Its sweetness is considered quite close to the sweetness of sucrose. However, it has high sweetness potency. It is also known for its high solubility and exceptional thermostability over a wide range of pH values, vital for most of its food applications (Bains et al., 2021). The long history of human consumption of Brazzein and its lack of bitterness makes it a preference by most people to other natural sweeteners. Additionally, the high-water solubility, pH stability, and extreme temperature tolerability of Brazzein make it preferred in the majority of food applications. For instance, there are identified seven sweet-tasting proteins besides Brazzein including monellin, neoculin, mabinlin, thaumatin, lysozyme, and pentadin.

For a long time, Brazzein has been utilized in safely sweetening food by the African natives, an indication that it has no health risks associated with it. However, the Food and Drug Administration in the United States must approve any food before it is consumed in the country. Through the rigorous tests mandated by the Food and Drug Administration, the people had to be guaranteed the safety of consuming Brazzein (Lynch et al., 2023). There is a need for extensive research in the possible acute toxicity areas to allergens or toxins and the protein breakdown tests concerning digestive enzyme therapy (Gatea et al., 2021). Therefore, the Food and Drug Administration has tested the protein's bioactivity *in vivo* and *in vitro*. Despite the protein showing a minimum 45% similarity to the antifungal drug drosomycin and antimicrobial agent defensin, the protein has antifungal and antimicrobial activities. Owing to the structural similarity of the Brazzein and defensin-like proteins, often assumed to be allergens, several people have raised concerns regarding the possible allergenicity of Brazzein.

MATERIALS AND METHODS

There are methodologies applied in the *in vitro* and *in vivo* systems of production for Brazzein. In the paper, the methodology largely involved a description of the search terms, the findings of the study, and the databases and processes followed in the *in vivo* and *in vitro* production of Brazzein plant protein.

The methodology had a replicable and comprehensive search process, that detailed the sources.

In the collection of the data for the paper, there was a selection of the study, excretion and various synthesis methods were the data that was looked upon.

Twelve journal articles and publications were used in the study for the comparative analysis of *in vitro* and *in vivo* production of Brazzein.

In Vitro Production of Brazzein

Cell-free transcription-translation (TXTL) is a versatile technology for the construction, characterization, and interrogation of genetically programmed biomolecular systems done outside the organisms.

In vitro, the production of Brazzein requires recapitulation of the gene expression by providing unparalleled flexibility to design, engineer, and analyze quantitatively the impacts of physical, chemical, and genetic contexts on the biochemical systems function.

Saraiva et al. (2023) point out that it involves steps such as preparation of cell lysates, reaction set-up, transcription and translation, and purification.

In the preparation step, cell lysates containing cellular components including tRNAs, ribosomes, and amino acids will be prepared alongside transcriptional and translational factors.

The reaction setup step will involve assembling the Tx-Tl reaction mixture through a combination of the cell lysate with a DNA encoding Brazzein, involving other components including cofactors, salts, and energy sources.

The transcription and translation step will involve the transcription of the DNA into mRNA, then it is translated by ribosomes in the cell lysate to produce Brazzein.
The last step of the purification of Brazzein from the reaction mixture involves purification techniques including chromatography.

In Vivo Production of Brazzein

In vivo, a system of production applies the genetically modified organisms especially bacteria, such as *Escherina coli* and yeasts. More specifically, the techniques of genetic engineering were applied in the introduction of genes encoding the Brazzein plant protein into the genomes of the host organisms, allowing the production of the needed sweet protein (McElwain et al., 2022).

Bacterial production of Brazzein

In vivo, the production of Brazzein involves bacterial production through genetic engineering techniques that modify the bacteria through steps such as inserting the Brazzein gene in the bacteria, optimized fermentation process, and purification of the Brazzein produced.
The most commonly used bacterium for the recombinant production of Brazzein plant protein is *E. coli*. Its success was first achieved in 2000. Nevertheless, the expression of the protein using this organism was limited by the requirement of an additional phase of removing the fused protein; and the majority of the plant protein produced needed additional chemical refolding steps.
Lactic acid bacteria including *Lactococcus lactis* are considered more advantageous and recognized as safe, creating a massive opportunity for the agro-industry to apply easily for the production of plant proteins such as Brazzein. *L. lactis* is useful for the production of Brazzein but at a low scale due to the possible lack of detection, and the Brazzein produced is not as sweet as that produced through *E. coli*.
In the expression of Brazzein, *E. coli* is designed, expressed, and synthesized at 30 degrees Celsius for the soluble form of Brazzein.
(Zhang et al., 2023) note that there are instances when Brazzein has been expressed in *L. lactis* through a nisin-controlled expression system for the production of the plant protein.
The fermentation process has to be optimized for proper secretion and expression of recombinant Brazzein in bacteria.
The optimization ensures that conditions of controlled fermentation are vital in the improvement of the production of the Brazzein produced.
The purification process will aim to clean up multiple biomolecules and large debris such as bacterial cells (Han et al., 2022).
The purification involves 2 hours of treatment at 80°C, precipitation of 30-80% ammonium sulfate, and ion exchange chromatography through diethylaminoethyl or carboxymethyl Sepharose columns.

Yeast production of Brazzein

Successful attempts have been made in the expression of Brazzein through yeast. *Saccharomyces cerevisiae* was used in the first expression of Brazzein. However, antibodies were used to confirm the identity of the expressed protein but there was no more characterization of the recombinant Brazzein.
Although there were multiple attempts of yeasts used in the production of Brazzein, the researchers settled for the use of *Kluyveromyces lactis* due to its intrinsic robustness. Especially the purified Brazzein, considered identical to the natural Brazzein plant protein was generated.
The synthetic gene encoding Brazzein was expressed as a sweet protein in the yeast *K. lactis*. To optimize expression and extracellular secretion for expression in soluble and active forms, the Brazzein gene can be designed based on the yeast codon preference. There will be specific steps involved in the yeast production of Brazzein. The major steps will be the cloning of genetics, fermentation, and purification.
The cloning of the Brazzein gene will be done in a secretion vector such as p KLAC2, containing yeast prepropeptide signal. The genes *KIERO1* and *KIPDI* will be overexpressed in the *K. lactis* yeast, enhancing the secretion of Brazzein.
Saraiva et al. (2023) explain that the fermentation step will involve applying a chemically defined medium to optimize the cell

growth and Brazzein production phases. For industrial production of Brazzein, the *K. lactis* yeast will be preferred due to its safety and suitability. Controlled conditions for fermentation will be vital in maximizing the production and secretion of Brazzein.

The last step will be the purification of the produced Brazzein through techniques such as ultrafiltration for obtaining highly pure Brazzein. The fermentation and purification process must be optimized for the commercial production of Brazzein.

RESULTS AND DISCUSSIONS

In Vivo Production of Brazzein

The restriction of the Brazzein production as well as the location of the plant, multiple methods have been explored regarding its production. The most common systems of producing Brazzein have been *in vivo* and *in vitro* systems of production. However, the best preferred natural method of producing Brazzein is bio diversion. The *in vivo* system of producing Brazzein is largely reliant on the application of certain genetically modified organisms including engineered bacteria and yeast. According to Meilina et al. (2021), the organisms secrete and express the targeted Brazzein protein. In the production of Brazzein through an *in vivo* system, the yeast specifically *K. lactis* has been used as a primary host for the production of recombinant Brazzein. Most people have preferred the application of *K. lactis* yeast for the mass production of Brazzein protein due to its high rate of production and purity from the production while maintaining lower costs of production compared to other production systems.

However, there have been cases of researchers going for *E. coli* in the production of Brazzein. It is largely applied as a bacterial host, especially for the production of heterologous proteins. The preference for using *E. coli* in the production of Brazzein is supported by the good expression of *E. coli*'s IPTG dependence, indicating the suitability of applying bacterial systems in the production. Although *E. coli* is considered the first biotransformation, back in 2000, additional biotransformation tests have confirmed that *E. coli* has a lower level of sweetness compared to the original plant (Komolov et al., 2023). Later, there was production of Brazzein through *Pichia pastoris*, reaching yields of 120 mg/L of Brazzein produced in 6 hours. This system of production did not show robustness since there was sometimes a generation of (104 mg/L after 6 hours) sweet Brazzein using *Kluyveromyces* in a cultured medium. Other organisms have since been used in the production of Brazzein such as the *Bacillus licheniformis* which is preferred due to its affordable costs, high secretion, and quick growth. Other peculiar applied mediums for the production of Brazzein in biotransformation include rice, maize, and lettuce based media.

In the attempts to produce an active and soluble form of Brazzein through the secretory expression system of *K. lactis* yeast, there have been ineffective results due to the inaccurate disulfide bonds formation. Additional studies have explored other microbial system of producing Brazzein. For instance, there has been production of Brazzein within corn kernels in an attempt to optimize the production and leverage the proprietary technology.

In Vitro Production of Brazzein

The *in vivo* approaches in the production of Brazzein have been complemented through an *in vitro* system of production. In this system of producing Brazzein, there is the application of cell-free protein synthesis (CFPS). Meilina et al. (2021) describe it as a technology of production that required the involvement of the S30-buffer cell extract of *E. coli*, chimeric RNA polymerase of T7 bacteriophage, and a multicopy plasmid vector with the Brazzein gene inserted in the process. It is such a successful process especially in the synthesis of Brazzein synthesis, producing a 2 mg/ml Brazzein mixture, higher than the common systems of production that use whole-cell expression. These technology platforms are preferred due to their ability to solve the complex challenges in the production of plant proteins such as Brazzein.

Environmental Impacts and Sustainability of the Production Systems

The viability of the production systems of plant proteins such as Brazzein is important in

comparing the two systems. More studies confirm that *in vivo* systems of producing Brazzein, particularly through the application of genetically modified organisms such as genetically engineered bacteria and yeast, can easily provide the much-needed cost-effective and scalable production. Nevertheless, multiple researchers have raised their concerns over *in vivo* systems of producing Brazzein through genetically modified organisms. Most of the concerns are environmental concerns regarding the impact the production has on the environment and the regulatory oversight related to genetically modified organisms.

In the case of the in vitro system of production, the researchers have argued that it is a sustainable system of production that should be supported. The *in vitro* approach of production leveraged cell-free technology which is crucial in the minimization of resource wastages and consumption, such as energy and water (Zagorskaya & Deineko, 2021). Moreover, the approach is vital in the reduction of possible waste generation during the process of Brazzein production. *In vivo* approaches for the production of Brazzein such as production in corn kernels demonstrate the potential of the method to enhance environmental friendliness and sustainability. Therefore, through the application of cultivated crops in the production of Brazzein, the production process is easily scaled up as the unused corn biomass on the farms is applied in the value chain such as for fuel, food, or feed.

Cost Effectiveness and Rate of Production in The Two Production Systems

The two approaches to production are cost-effective by reducing the rate of production costs. For instance, the *in vivo* systems proved to have lower costs on the investment especially due to their leverage of the present production and infrastructure facilities. The *in vitro* approach of production was found to offer much-needed long-term sustainability and potential reduction of the operating costs due to their optimization for efficient utilization of resources and reduction of the waste generated from the production.

The two approaches of *in vivo* and *in vitro* production systems for Brazzein, it is vital to note that they all demonstrate a high rate of production as well as reduced costs of production. According to Leal et al. (2021), in the case of the *in vivo* system of producing Brazzein, particularly the system of *K. lactis* expression, there was mass production of Brazzein of the highest purity level as well as a high rate of production supported by low costs of production. The approach has leveraged the inherent capabilities of living cells to secrete and express Brazzein as the target protein. This approach has heavily contributed to the scalability and efficiency of the *in vivo* system of Brazzein production.

Contrastingly, the *in vitro* production of Brazzein through the CFPS systems is believed to have realized a tremendous volumetric rate of production, even beyond the maximum values realized in earlier attempts of whole cell expression production systems. More specifically, the technology of cell-free protein synthesis is seen to provide the benefit of higher control on the conditions of Brazzein production. This rate of production potentially allows more efficiency regarding scaling up as well as reduction of the contamination risks.

Food Application

Using the two systems of producing Brazzein, food industries can use the produced Brazzein. Endemic consumption of Brazzzein as a sweetening agent or raw fruits is considered such as a long-term ethnobotanical heritage. Being a plant protein, Brazzein is considered a low-calorie sweetener and has a low potential for causing gastrointestinal distress in humans.

However, there is a need for additional research on the industrial scale-up for the application of Brazzein. The long history of consumption, good sensory properties, and high sweetness potency of Brazzein make it a promising natural sweetener. The food companies understand that Brazzein offers physicochemical properties including extreme pH stability, high solubility in water, and extreme temperature stability, all considered vital in food applications.

Extensive research should investigate the means of fighting the challenge of obtaining sweet-tasting plant protein from its very natural source. The recent efforts in the overexpression of Brazzein through multiple heterologous systems such as yeast, bacteria, plants, and

animals. The expression systems of yeast are considered quite efficient and ensure economical expression for the secretion of Brazzein (Lynch et al., 2023). The secreted protein through yeast is easy to purify and has similar characteristics to natural protein.

More importantly, the high rate of production of Brazzein through biotechnology constantly paves the way for food applications, since Brazzein is a low-calorie and high-intensity sweetener alternative.

CONCLUSIONS

As revealed in the paper, the two approaches of production have their limitations and strengths. *In vitro* systems of Brazzein production, utilizing the cell-free protein synthesis technology, there is the benefit of enhanced control over conditions of Brazzein production, reduced contamination risks, and demonstrates the potential of an environmentally friendly and sustainable process of production. The cell-free protein synthesis technology applied in the *in vitro* systems of production guarantees high volumetric production of the plant protein, demonstrating its potential as a viable option for *in vivo* methods of Brazzein production.

Conversely, *in vivo* systems of production of Brazzein through engineered bacteria and yeasts, there is the potential for increased production and low costs of production for Brazzein. The efficiency and scalability of the *in vivo* system of production for Brazzein make it preferred for commercial-scale production.

All in all, companies interested in mass production of Brazzein will base their decision on whether to use *in vitro* or *in vivo* production systems on multiple factors.

For instance, the companies will consider sustainability, cost-effectiveness, and expected yield in production. However, there is a need for additional development and research on *in vivo* and *in vitro* systems of Brazzein production.

REFERENCES

Bains, A., Chawla, P., Kaur, S., Najda, A., Fogarasi, M., & Fogarasi, S. (2021). Bioactives from mushroom: Health attributes and food industry applications. *Materials, 14*(24), 7640. https://doi.org/10.3390/ma14247640.

Barbieru O.G., Trica B., Tritean N., Negrila R.N., Constantinescu-Aruxandei D., Oancea F. 2021, Maximizing total protein extraction from spent brewer's yeast using high-pressure homogenization. *Scientific Bulletin. Series F. Biotechnologies,* Vol. XXV, Issue 2, ISSN 2285-1364, 75-84.

Gatea, F., Sârbu, I. & Vamanu, E. (2021). In vitro modulatory effect of stevioside, as a partial sugar replacer in sweeteners, on human child microbiota. *Microorganisms, 9*(3), 590. https://doi.org/10.3390/microorganisms9030590.

Han, J.-E., Lee, H., Ho, T.-T., & Park, S.-Y. (2022). Brazzein protein production in transgenic carrot cells using air-lift bioreactor culture. *Plant Biotechnology Reports, 16*(2), 161–171. https://doi.org/10.1007/s11816-022-00743-3.

Komolov, A.S., Sannikova, E.P., Gorbunov, A.A., Gubaidullin, I.I., Plokhikh, K.S., Konstantinova, G.E., Bulushova, N.V., Kuchin, S.V., & Kozlov, D.G. (2023). Synthesis of biologically active proteins as L6KD-SUMO fusions forming inclusion bodies in *Escherichia coli*. *Biotechnology and Bioengineering 121*(2), 535–550. https://doi.org/10.1002/bit.28587.

Koveshnikova, E.I., Kotlobay, A.A., Сычева, Л.П., Kurochkina, K.G., Averina, O.A., Belopolskaya, M.V., Сергиев, П.В., Донцова, О.А., Lazarev, V.N., Маев, И.В., Kostyaeva, M.G., Eremeev, A.V., Chukina, S.I., & Lagarkova, M.A. (2023). Sweet-tasting natural proteins brazzein and monellin: safe sugar substitutes for the food industry. *Foods, 12*(22), 4065–4065. https://doi.org/10.3390/foods12224065.

Leal, F.P.N., Bastiaan-Net, S., Roelse, M., Jongsma, M.A., Witkamp, R.F., Meijerink, J., & Mes, J.J. (2021). Bitter gourd (*Momordica charantia* L.) stimulates the secretion of the incretin Glucagon-like peptide-1 by enteroendocrine cells *in vitro*. [online] library.wur.nl. Available at: https://library.wur.nl/WebQuery/wurpubs/588604 [Accessed 17 May 2024].

Lynch, B., Wang, T., Vo, T.D., Tafazoli, S., & Ryder, J.A. (2023). Safety evaluation of oubli fruit sweet protein (brazzein) derived from *Komagataella phaffii*, intended for use as a sweetener in food and beverages. *Toxicology Research and Application, 7*, 239784732311512-239784732311512. https://doi.org/10.1177/23978473231151258.

McElwain, L., Phair, K., Kealey, C., & Brady, D. (2022). Current trends in biopharmaceutical production in *Escherichia coli*. *Biotechnology Letters, 44*(8), 917–931. https://doi.org/10.1007/s10529-022-03276-5.

Meilina, L., Budiarti, S., Mustopa, A.Z., Darusman, H.S., Triratna, L., Nugraha, M.A., Bilhaq, M.S., & Ningrum, R.A. (2021). Heterologous expression of interferon α-2b in *Lactococcus lactis* and its biological activity against colorectal cancer cells. *Microbiology and Biotechnology Letters, 49*(1), 75–87. https://doi.org/10.48022/mbl.2009.09008.

Saraiva, A., Conrado Carrascosa, Ramos, F., Raheem, D., Sónia Pedreiro, Vega, A., & Raposo, A. (2023). Brazzein and monellin: Chemical analysis, food industry applications, safety and quality control, Nutritional profile and health impacts. *Foods, 12*(10), 1943–1943. https://doi.org/10.3390/foods12101943.

Zagorskaya, A.A., & Deineko, E.V. (2021). Plant-expression systems: A new stage in production of biopharmaceutical preparations. *Russian Journal of Plant Physiology, 68*(1), 17–30. https://doi.org/10.1134/s1021443721010210.

Zhang, X., Liu, X., Zhang, N., Zhao, X., Li, Y., Gong, D., & Yun, Y. (2023). Development of chemically defined media for *Lactococcus lactis* subsp. *lactis* YF11 to eliminate the influence of hyperosmotic stress. *3 Biotech, 13*(11). https://doi.org/10.1007/s13205-023-03788-5.