

**“FORMULATION FOR IMPROVING SWEETNESS OF THE
RARE SUGARS WITHOUT ALTERING THE TEXTURE AND
FLAVOUR AND TO THE METHOD THEREOF”**

5 TECHNICAL FIELD OF THE INVENTION:

The present invention relates to a formulation comprising sweet protein with carbohydrate and/ or non-carbohydrates selected from rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners for improving the sweetness without changing the texture and flavours and to the method for improving the sweetness
10 of the rare sugars. More particularly, the present invention relates to method for improving the sweetness of the rare sugars without changing the texture and flavours of the primary sugars.

The present invention further relates to addition of Brazzein with Liquid Rare
15 sugars before crystallization or at pre caramalization stage or post caramalization stage of rare sugars at different temperatures to increase the sweetness. Brazzein increases the sweetness of Rare sugars not limited to D-Allulose, D-tagatose, D-Allose, Isomaltulose, Trehalulose etc.

20 The present invention also relates to evaluation of sugar substitutes and/ or non-nutritive sweeteners such as aspartame, acesulfame-K, Cyclamate, Neotame, Saccharin, Sucralose Stevia etc., as each one has limitations for taste quality if used with Rare sugars. However, the use of Brazzein did not change the flavour and texture of the rare sugars and the taste profile matches the sucrose.

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BACKGROUND AND PRIOR ART OF THE INVENTION:

Rare sugars and their derivatives rarely exist in nature. These sugars possess the properties like sweeteners but lack calorific value. Therefore, these could be used as an alternative to other sweeteners. Rare sugars are not metabolized as natural
30 sugars by the body.

There is currently increased demand for sugar substitutes because many harmful effects of sugar have been reported. Some of these detrimental effects include the increased risk of coronary heart disease, contribution to obesity and diabetes, and suppression of the immune system. Alternative sugars can be divided into two
5 classes of compounds, natural and artificial.

The synthetic sugar substitutes include acesulfame potassium, aspartame, neotame, saccharin, and sucralose. The Food and Drug Administration from various countries has strictly regulated artificial sweeteners as food additives due to the controversial
10 health risks of synthetic sugar substitutes.

In addition to synthetic sugar substitutes, consumers are exposed to many natural sugars as sugar alternatives including isomaltulose, tagatose, allulose, trehalulose, turanose, allose which are referred as rare sugars.

15 Isomaltulose, also known by the trade name Palatinose and chemical name 6-O- α -D-glucopyranosyl-D-fructose, is a disaccharide carbohydrate formed from glucose and fructose. It can be found naturally in honey and sugarcane extracts, and it is also manufactured industrially from sucrose, serving as a substitute for sugar.

20 Tagatose, a hexose monosaccharide, can be found in small amounts in various foods and has garnered interest as a potential alternative sweetener. It is generated during the heating process of milk, particularly in dairy products. It closely resembles sucrose (table sugar) in texture and appearance, being 92% as sweet, but
25 containing only 38% of the calories.

D-allulose or simply allulose is a low-calorie isomer of the monosaccharide fructose. It is utilized by several prominent food and beverage companies as a low-calorie sweetener. Initially discovered in wheat during the 1940s, allulose occurs
30 naturally in small amounts in specific food items.

Trehalulose is a disaccharide consisting of one fructose molecule linked to one glucose molecule. It serves as a structural isomer of sucrose and is found in minor amounts in honey. This compound constitutes 50% of the sugars found in the honeydew produced by silverleaf whiteflies and is synthesized from sucrose by
5 certain bacteria, including *Protaminobacter rubrum*.

Allose is classified as an aldohexose sugar and found in the form of a 6-O-cinnamyl glycoside within the leaves of the African shrub *Protea rubropilosa*. Additionally, this sugar is present in extracts from the freshwater alga *Ochromas malhamensis*,
10 although its absolute configuration remains unidentified. Furthermore, it is identified as a C-3 epimer of glucose.

Rare sugars are monosaccharides with a limited availability in the nature. Therefore, the research was focussed on industrial synthesis of the rare sugars using
15 various chemical, enzymatic and microbial processes and thus reduces their production costs and availability of these molecules thereby making research on these molecules more accessible. Thereafter, a number of studies on the medical/clinical applications of rare sugars carried out by various studies and consequently, rare sugars are emerged as potential candidates to replace
20 conventional sugars in human nutrition due to their beneficial health effects. Further, the use of rare sugars in agriculture was also reported recently. Above mentioned rare sugars do not have equivalent sweetness like sucrose. For example, D-tagatose is 8% less sweet than sucrose but contains only 1.5 kcal/g [Torrico, D.D.; Tam, J.; Fuentes, S.; Viejo, C.G.; Dunshea, F.R. D-tagatose as a sucrose
25 substitute and its effect on the physicochemical properties and acceptability of strawberry-flavored yogurt. *Foods* 2019, 8, 256].

US20080182752A1 discloses an agricultural composition comprising a rare sugar selected from the group consisting of D-psicose, a mixture of D-psicose and D-fructose, D-allose, D-altrose and L-galactose as an active ingredient, which has an
30 effect of inducing systemic acquired resistance in a plant.

US9717267B2 discloses a low-calorie carbonated soft drink beverage having sugar-sweetened characteristics comprising D-psicose and Rebaudioside A, as sweetness enhancer. Sweet proteins have the potential to replace these artificial
5 sweeteners, by acting as natural, good, low calorie sweeteners, as we know that proteins do not trigger a demand for insulin in these patients whereas sucrose does. So far there are seven known sweet and taste-modifying proteins, namely Brazzein, Thaumatin, Monelin, Curculin, Mabinlin, Miraculin and Pentadin. All of these sweet proteins have been isolated from plants that grow in tropical rainforests.

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Brazzein is first disclosed in WO1994019467A1 as a protein sweetener that has been isolated from *Pentadiplandra brazzeana* Baillon. This publication further discloses that the Brazzein is thermostable, lysine rich and has a relative long-lasting taste. WO2008112475A2 and PCT/KR2009/004855 discloses variants of Brazzein,
15 and nucleotide sequences capable of expressing them.

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WO2010030999A1 discloses codon-optimized brazzein coding sequences and the expression of brazzein and variants thereof using yeast expression systems. This publication further discloses methods of expression of proteins to enhance the
sweetness taste profile of foods and/or beverages.

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WO2018056746A1 discloses a brazzein multiplex variant having antiallergic and antioxidative activities and, more specifically, to a brazzein multiplex variant capable of simultaneously exhibiting both an antioxidative effect through GST and
tyrosinase activities and an antiallergic effect through an inhibitory activity against the release of β -hexosaminidase, which is a degranulation-indicating substance, released with histamine. Accordingly, the brazzein multiplex variant according to
the present invention can be usefully employed in a pharmaceutical composition for allergic disease prevention or treatment, an antiallergic cosmetic composition,
an antioxidative composition, an antioxidative cosmetic composition, and a health
30 functional food.

The above literature shows that there is a continued commercial interest in rare sugars and sweet proteins such as brazzein. However, since the early days of brazzein discovery and rare sugars in the late 1980s, there appears no industrial
5 solution for the provision of successful compositions of rare sugars with brazzein or any technical solution published with the potential to meet the market needs.

In the light of the foregoing, there remains a need in the art to improve the sweetness index of the naturally available rare sugars by employing sweet proteins such as
10 Brazzein to meet the growing health care market needs.

Summary of the invention:

In line with the above, the present invention relates to a formulation comprising sweet protein with carbohydrate and/ or non-carbohydrates selected from rare
15 sugars and/or sugar substitutes and/ or non-nutritive sweeteners for improving the sweetness without changing the texture and flavours.

The sweet protein is selected from the group consisting of brazzein, curculin/neoculin, egg white lysozyme, mabinlin, miraculin, monellin, pentadin,
20 and thaumatin, and the like.

In an aspect, the sweet protein used to improve the sweetness of the rare sugars is brazzein.

25 In another aspect, the sweet protein Brazzein can also be used in formulation containing rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners such as and not limited to aspartame, acesulfame-K, Cyclamate, Neotame, Saccharin, Sucralose, Stevia etc.,

In a preferred aspect, the present invention relates to a formulation comprising Brazzein with rare sugars for improving the sweetness of the rare sugars with low glycemic index without changing the texture and flavours of said rare sugars.

- 5 In an aspect, Brazzein is used at a concentration ranging 1 to 100 mg per 100g of the rare sugar.

In an aspect, appropriate amounts of Brazzein can be used as the sole sweetener or added to rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners
10 depending on the practical application of the formulation, wherein the purpose of Brazzein is for enhancement of sweetness and/ or compensation for bitterness.

The rare sugars are selected from the group including but not limited to D-Allulose, D-Tagatose, D-Allose, Isomaltulose, Trehalulose, Leucrose, Turanose and the like.
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In another aspect, the present invention provides a method for improving the sweetness of the rare sugars without changing the texture and flavours of the rare sugars comprising adding brazzein to the rare sugar at pre caramalization stage or post caramalization stage to increase the sweetness.
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More particularly, the present invention discloses a method for improving the sweetness of the rare sugars without changing the sweetness profile, texture and flavours of the primary sugars by adding Brazzein at pre caramalization stage or post caramalization stage of rare sugars.
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Detailed description of the invention:

The invention will now be described in detail in connection with certain preferred and optional embodiments, so that various aspects thereof may be more fully understood and appreciated.
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The term "rare sugar" or "primary sugar" used herein interchangeably relates and means the rare sugars D-Allulose, D-tagatose, D-Allose, Isomaltulose, Trehalulose, Turanose, Leucrose, Sorbose used in the present invention.

5 In an embodiment, the present invention provides a formulation comprising sweet protein with carbohydrate and/ or non-carbohydrates selected from rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners for improving the sweetness without changing the texture and flavours.

10 In another embodiment, the sweet protein is selected from the group consisting of brazzein, curculin/neoculin, egg white lysozyme, mabinlin, miraculin, monellin, pentadin, thaumatin, and the like..

In one embodiment, the sweet protein used to improve the sweetness of the rare
15 sugars is brazzein.

In a preferred embodiment, the present invention relates to a formulation comprising Brazzein with rare sugars for improving the sweetness of the rare sugars with low glycemic index without changing the texture and flavours of said rare
20 sugars.

In an embodiment, Brazzein is used at a concentration ranging between 1 to 100 mg per 100g of the rare sugar.

25 In an embodiment, appropriate amounts of Brazzein can be used as the sole sweetener or added to rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners depending on the practical application of the formulation, wherein the purpose of Brazzein is for enhancement of sweetness and/ or compensation for bitterness.

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The rare sugars are selected from the group including but not limited to D-Allulose, D-Tagatose, D-Allose, D-Sorbose, Isomaltulose, Trehalulose, Turanose, Leucrose and the like.

- 5 In another embodiment, the sweet protein Brazzein can also be used in formulation containing rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners such as and not limited to aspartame, acesulfame-K, Cyclamate, Neotame, Saccharin, Sucralose, Stevia etc., In another embodiment, the present invention provides a method for improving the sweetness of the rare sugars without changing
10 the texture and flavours of the rare sugars comprising adding brazzein to the rare sugar at pre caramalization stage or post caramalization stage.

- In another embodiment, the sweet protein used in the method is selected from the group consisting of brazzein, curculin/neoculin, egg white lysozyme, mabinlin,
15 miraculin, monellin, pentadin, thaumatin, and the like.

- In another embodiment, the said primary sugars are the rare sugars which are also alternate diet sugars such as and not limited to D-Allulose, D-Tagatose, D-Allose, Isomaltulose, Trehalulose, Sorbose, Turanose, Leucrose and the like.

- 20 In another embodiment, the process includes processes of sweet protein addition to the primary sugars such as and not limited to addition at pre caramalization stage or post caramalization stage of the synthesis of the rare sugars at different temperatures to increase the sweetness.

- 25 More particularly, the present invention discloses a method for improving the sweetness of the rare sugars without changing the flavours of the primary sugars by adding Brazzein at pre caramalization stage or post caramalization stage of the synthesis of the rare sugars at different temperatures to increase the sweetness.

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According to the present invention, addition of Brazzein at pre caramelization stage or post caramelization stage of the synthesis of the rare sugars increases the sweetness of Rare sugars but not limited to D-Allulose, D-tagatose, D-Allose, Isomaltulose, Trehalulose etc.

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According to one embodiment, to increase the sweetness of the rare sugars, alternative naturally available sweetener, having more sweetness index than sucrose, like sweet protein selected from the group consisting of brazzein, curculin/neoculin, egg white lysozyme, mabinlin, miraculin, monellin, pentadin, and thaumatin is formulated with rare sugar by in house developed method.

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Compared to other sweet proteins, brazzein is attractive as a candidate sweetener as the same has other health benefits also such as controlling obesity, oral health, and diabetic management, in addition to its potential sweetness, sugar-like taste.

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Brazzein was isolated in two forms from the fruit of the West African *Pentadiplandra brazzeana Baillon* plant. The major form contains a pyroglutamate at its N-terminus, while the minor form lacks this N-terminal pyroglutamate. The major form has an intrinsic sweetness 500 times sweeter than sucrose in weight-weight comparisons (9500 times higher on a per-molecule basis).

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The minor form Brazzein is about 5,890 Daltons in size and is about 2,000 times sweeter in 2% solution than a 2% sucrose solution. Thermostability tests according to the invention indicate that this protein has desired heat stability characteristics (thus making it much more suitable for use in connection with formulating or baking). In this regard, the protein still tasted very sweet after two hours of incubation at 98°C.

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Thus, Brazzein is especially suitable to being mixed with other known rare sugars like, D-Allulose, Isomaltulose, Turanose, Trehalulose etc. which has low Glycemic index (GI) and low caloric value and thus introduction of Brazzein with these rare sugars will enhance the sweetness index of rare sugar more than sucrose.

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In another embodiment, the formulation of the present invention can be applied to food applications such as and not limited to addition in beverages, in chocolates, in cookies, and the like.

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The processing of the liquid to obtain solid form of sugar and protein together can be achieved by the methods selected from methods such as and not limited to co-crystallization, lyophilization, spray drying, etc.

10 The present invention has the following advantages:

1. The present process improves the sweetness of the rare sugars with low glycemic index without changing the texture and flavours of said rare sugars.
2. Brazzein can be mixed with rare sugars like, D-Allulose, Isomaltulose,
15 Turanose, Trehalulose etc. which have low Glycemic index (GI) and low caloric value.
3. Addition of Brazzein to the rare sugars enhances the sweetness index of rare sugar more than sucrose.

20 The invention can be better understood by the following non-limiting examples. The examples given are mere an illustration of the instant invention and should not be construed as limiting the scope of the present invention in any manner.

EXAMPLES

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Example 1: Assessment of sweetness of Allulose sugar solution by addition of Brazzein

In the natural form Allulose has 70% sweetness as compared to sucrose.

30 The sweetness lacking in Allulose solution was compensated by adding Brazzein to the the Allulose.

Brazzein preparations of different concentrations (5 to 25 mg) were added to 100g Allulose solution. Sweetness was assessed by tasting analysis and was recorded on a sweetness scale of 0 – 10.

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Note:

On a sweetness scale of 0-10: where 0 represents no sweetness and 10 represent the highest level of sweetness.

S.No.	Allulose	Brazzein	Sweetness
1	100 g	0 mg	+++
2	100 g	5 mg	+++
3	100 g	10 mg	++++
4	100 g	20 mg	+++++
5	100 g	25 mg	+++++

- 10 Results: Addition of 5 to 25 mg Brazzein to 100g Allulose compensated for the lacking sweetness, and enhanced the sweetness.

Conclusion:

- 15 It can be concluded that the addition of brazzein to Allulose resulted in significant increase in sweetness.

Example 2: Assessment of Sweetness of Tagatose sugar solution by addition of Brazzein

- 20 In the natural form Tagatose has 90% sweetness as compared to sucrose.
The sweetness lacking in Tagatose solution was compensated by adding brazzein to the Tagatose solution before caramalisation.

Brazzein preparations of different concentrations (2 to 20 mg) were added to 100g Tagatose solution. Sweetness was assessed by tasting analysis and was recorded on a sweetness scale of 0 – 10.

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- Note:
On a sweetness scale of 0-10: where 0 represents no sweetness and 10 represent the highest level of sweetness.

S.No.	Tagatose	Brazzein	Sweetness
1	100 g	0 mg	+++
2	100 g	2 mg	++++
3	100 g	10 mg	+++++
4	100 g	20 mg	+++++
5	100 g	20 mg	+++++

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- Addition of 2 to 20 mg Brazzein to 100g Tagatose compensated for the lacking sweetness, and enhanced the sweetness.

Conclusion:
It can be concluded that the addition of brazzein to Tagatose resulted in significant increase in sweetness.

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- Although the invention has been described in detail in the foregoing for the purpose of illustration, it is to be understood that such detail is solely for that purpose and that variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention except as it may be limited by the claims.

WE CLAIM,

1. A formulation comprising sweet protein with carbohydrate and/ or non-carbohydrates selected from rare sugars and/or sugar substitutes and/ or
5 non-nutritive sweeteners for improving the sweetness without changing the texture and flavours.
2. The formulation as claimed in Claim 1, wherein the sweet protein is selected from the group consisting of brazzein, curculin/neoculin, egg white lysozyme, mabinlin, miraculin, monellin, pentadin, thaumatin, and the like.
- 10 3. The formulation as claimed in Claim 1 and 2, wherein the sweet protein used to improve the sweetness of the rare sugar is brazzein.
4. The formulation as claimed in Claim 1, wherein the formulation comprises Brazzein with rare sugars for improving the sweetness of the rare sugars without changing the texture and flavours.
- 15 5. The formulation as claimed in Claim 4, wherein the Brazzein is used at a concentration ranging between 1 to 100 mg per 100g of the rare sugar.
6. The formulation as claimed in claim 1 and 2, wherein appropriate amounts of Brazzein can be added to rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners depending on the practical application of the
20 formulation, wherein the purpose of Brazzein is for enhancement of sweetness and/ or compensation for bitterness.
7. The formulation as claimed in Claim 1, wherein the rare sugars are selected from D-Allulose, D-tagatose, D-Allose, Isomaltulose, Trehalulose, Sorbose, Turanose, Leucrose and the like.
- 25 8. The formulation as claimed in claim 1 and 2, wherein sweet protein Brazzein can also be used in formulation containing rare sugars and/or sugar substitutes and/ or non-nutritive sweeteners such as and not limited to aspartame, acesulfame-K, Cyclamate, Neotame, Saccharin, Sucralose, Stevia and the like.

9. A method for improving the sweetness of the rare sugars without changing the texture and flavours of the rare sugars comprising adding brazzein to the rare sugar at pre caramalization stage or post caramalization stage.
10. The method as claimed in claim 9, wherein the said primary sugars are the rare sugars which are also alternate diet sugars such as and not limited to D-
5 Allulose, D-Tagatose, D-Allose, Isomaltulose, Trehalulose, Sorbose, Turanose, Leucrose and the like.
11. Use of sweet protein with rare sugars as claimed in any one of the preceding claims for improving sweetness in beverages, in chocolates, in cookies, and
10 the like.