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HIGH-PURITY STEVIOL GLYCOSIDES

Abstract

Methods of preparing highly purified steviol glycosides, particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, and rebaudioside AM are described. The methods include utilizing enzyme preparations and recombinant microorganisms for converting various staring compositions to target steviol glycosides. The highly purified rebaudiosides are useful as non-caloric sweetener, flavor enhancer, sweetness enhancer, and foaming suppressor in edible and chewable compositions such as any beverages, confectioneries, bakery products, cookies, and chewing gums.

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Background/Summary

TECHNICAL FIELD

[0001] The present invention relates to a process for preparing compositions comprising steviol glycosides, including highly purified steviol glycoside compositions.

BACKGROUND OF THE INVENTION

[0002] High intensity sweeteners possess a sweetness level that is many times greater than the sweetness level of sucrose. They are essentially non-caloric and are commonly used in diet and reduced-calorie products, including foods and beverages. High intensity sweeteners do not elicit a glycemic response, making them suitable for use in products targeted to diabetics and others interested in controlling for their intake of carbohydrates.

[0003] Steviol glycosides area class of compounds found in the leaves of *Stevia rebaudiana* Bertoni, a perennial shrub of the Asteraceae (Compositae) family native to certain regions of South America. They are characterized structurally by a single base, steviol, differing by the presence of carbohydrate residues at positions C13 and C19. They accumulate in *Stevia* leaves, composing approximately 10%-20% of the total dry weight. On a dry weight basis, the four major glycosides found in the leaves of *Stevia* typically include stevioside (9.1%), rebaudioside A (3.8%), rebaudioside C (0.6-1.0%) and dulcoside A (0.3%). Other known steviol glycosides include rebaudioside B, C, D, E, F and M, steviolbioside and rubusoside.

[0004] Although methods are known for preparing steviol glycosides from *Stevia rebaudiana*, many of these methods are unsuitable for use commercially.

[0005] Accordingly, there remains a need for simple, efficient, and economical methods for preparing compositions comprising steviol glycosides, including highly purified steviol glycoside compositions.

SUMMARY OF THE INVENTION

[0006] The present invention provides a process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microbial cell and/or enzyme preparation, thereby producing a composition comprising a target steviol glycoside.

[0007] The starting composition can be any organic compound comprising at least one carbon atom. In one embodiment, the starting composition is selected from the group consisting of steviol glycosides, polyols or sugar alcohols, various carbohydrates.

[0008] The target steviol glycoside can be any steviol glycoside. In one embodiment, the target steviol glycoside is steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM or a synthetic steviol glycoside.

[0009] In one embodiment, the target steviol glycoside is rebaudioside AM.
[0010] In some preferred embodiments enzyme preparation comprising one or more enzymes, or a microbial cell comprising one or more enzymes, capable of converting the starting composition to target steviol glycosides are used. The enzyme can be located on the surface and/or inside the cell. The enzyme preparation can be provided in the form of a whole cell suspension, a crude lysate or as purified enzyme(s). The enzyme preparation can be in freeform or immobilized to a solid support made from inorganic or organic materials.
[0011] In some embodiments, a microbial cell comprises the necessary enzymes and genes encoding thereof for converting the starting composition to target steviol glycosides. Accordingly, the present invention also provides a process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microbial cell comprising at least one enzyme capable of converting the starting composition to target steviol glycosides, thereby producing a medium comprising at least one target steviol glycoside.
[0012] The enzymes necessary for converting the starting composition to target steviol glycosides include the steviol biosynthesis enzymes, UDP-glucosyltransferases (UGTs) and/or UDP-recycling enzyme.
[0013] In one embodiment, the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.
[0014] In another embodiment, the steviol biosynthesis enzymes include non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway (MEP/DOXP) enzymes.
[0015] In one embodiment the steviol biosynthesis enzymes are selected from the group including geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), I-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), I-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated H M G-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase etc.
[0016] The UDP-glucosyltransferase can be any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol and/or a steviol glycoside substrate to provide the target steviol glycoside.
[0017] As used hereinafter, the term “SuSy_AT”, unless specified otherwise, refers to sucrose synthase having amino-acid sequence “SEQ ID 1” as described in Example 1.
[0018] As used hereinafter, the term “UGTS12”, unless specified otherwise, refers to UDP-glucosyltransferase having amino-acid sequence “SEQ ID 2” as described in Example 1.
[0019] As used hereinafter, the term “UGT76G1”, unless specified otherwise, refers to UDP-glucosyltransferase having amino-acid sequence “SEQ ID 3” as described in Example 1.
[0020] In one embodiment, steviol biosynthesis enzymes and UDP-glucosyltransferases are produced in a microbial cell. The microbial cell may be, for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc. In another embodiment, the UDP-glucosyltransferases are synthesized.
[0021] In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2, UGT76G1, UGT91D2, UGTS12, EUGT11 and UGTs having substantial (>85%, >86%, >87%, >88%, >89%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, >99%) amino-acid sequence identity to these polypeptides as well as isolated nucleic acid molecules that code for these UGTs.
[0022] In one embodiment, steviol biosynthesis enzymes, UGTs and UDP-glucose recycling

system are present in one microorganism (microbial cell). The microorganism may be for example, *E. coli*, *Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp.

[0023] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capableof adding at least one glucose unit to steviol orany starting steviol glycoside bearing an —OH functional group at C13 to give a target steviol glycoside having an —O-glucose beta glucopyranoside glycosidic linkage at C13. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2, or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0024] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol or any starting steviol glycoside bearing a —COOH functional group at C19 to give a target steviol glycoside having a —COO-glucose beta-glucopyranoside glycosidic linkage at C19. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1, or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0025] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1.fwdarw.2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0026] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1.fwdarw.3 glucopyranoside glycosidic linkage(s) at the newly formed bond glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGT76G1, or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0027] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C13 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1.fwdarw.2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0028] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capableof adding at least one glucose unit to steviol to form steviolmonoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0029] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol to form steviolmonoside A. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0030] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0031] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside A. In a particular embodiment, the UDP-glucosyltransferase is UGTSI2 or a UGT having >85% amino-acid sequence identity with UGTSI2. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0032] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0033] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.
[0034] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form steviolbioside.
[0035] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0036] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0037] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside A. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0038] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0039] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0040] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside A (rebaudioside KA). In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0041] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside.
[0042] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase

capable of adding at least one glucose unit to steviolbioside to form stevioside. In a particular embodiment, the UDP-glucosyl transferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.

[0043] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0044] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E2.

[0045] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0046] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E.

[0047] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside C to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.

[0048] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E2. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0049] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0050] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E3 to form rebaudioside AM.

[0051] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E2 to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0052] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0053] Optionally, the method of the present invention further comprises using more than one UGT on a starting composition, to give a target steviol glycoside(s) having more than one glucose unit than the starting composition. In a particular embodiment, the UDP-glucosyltransferases are

UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any UGT having >85% amino-acid sequence identity with UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any combination thereof, capable of adding more than one glucose unit to a starting composition to give a steviol glycoside(s) having more than one glucose unit than the starting composition.

[0054] In one embodiment, the UDP-glucosyltransferases are any UDP-glucosyltransferases capable of adding overall two glucose unit to stevioside to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferases are selected from UGTS12, EUGT11, UGT91D2, UGT76G1 or any UGT having >85% amino-acid sequence identity with UGTS12, EUGT11, UGT91D2, UGT76G1 or any combination thereof. In another particular embodiment, the UDP-glucosyltransferases are UGTS12 and UGT76G1.

[0055] Optionally, the method of the present invention further comprises recycling UDP to provide UDP-glucose. In one embodiment, the method comprises recycling UDP by providing a recycling catalyst and a recycling substrate, such that the biotransformation of steviol and/or the steviol glycoside substrate to the target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose.

[0056] In one embodiment, the recycling catalyst is sucrose synthase SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At.

[0057] In one embodiment, the recycling substrate is sucrose.

[0058] Optionally, the method of the present invention further comprises the use of transglycosidases that use oligo- or poly-saccharides as the sugar donor to modify recipient target steviol glycoside molecules. Non-limiting examples include cyclodextrin gIycosyltransferase (CGTase), fructofuranosidase, amylase, saccharase, glucosucrase, beta-h-fructosidase, beta-fructosidase, sucrase, fructosylinvertase, alkaline invertase, acid invertase, fructofuranosidase. In some embodiments, glucose and sugar(s) other than glucose, including but not limited to fructose, xylose, rhamnose, arabinose, deoxyglucose, galactose are transferred to the recipient target steviol glycosides. In one embodiment, the recipient steviol glycoside is rebaudioside AM.

[0059] Optionally, the method of the present invention further comprises separating the target steviol glycoside from the medium to provide a highly purified target steviol glycoside composition. The target steviol glycoside can be separated by at least one suitable method, such as, for example, crystallization, separation by membranes, centrifugation, extraction, chromatographic separation or a combination of such methods.

[0060] In one embodiment, the target steviol glycoside can be produced within the microorganism. In another embodiment, the target steviol glycoside can be secreted out in the medium. In one another embodiment, the released steviol glycoside can be continuously removed from the medium. In yet another embodiment, the target steviol glycoside is separated after the completion of the conversion reaction.

[0061] In one embodiment, separation produces a composition comprising greater than about 80% by weight of the target steviol glycoside on an anhydrous basis, i.e., a highly purified steviol glycoside composition. In another embodiment, separation produces a composition comprising greater than about 90% by weight of the target steviol glycoside. In particular embodiments, the composition comprises greater than about 95% by weight of the target steviol glycoside. In other embodiments, the composition comprises greater than about 99% by weight of the target steviol glycoside.

[0062] The target steviol glycoside can be in any polymorphic or amorphous form, including hydrates, solvates, anhydrous or combinations thereof.

[0063] Purified target steviol glycosides can be used in consumable products as a sweetener, flavor modifier, flavor with modifying properties and/or foaming suppressor. Suitable consumable products include, but are not limited to, food, beverages, pharmaceutical compositions, tobacco products, nutraceutical compositions, oral hygiene compositions, and cosmetic compositions.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

[0064] FIG. **1** shows the chemical structure of rebaudioside AM.

[0065] FIG. **2** shows the pathways of producing rebaudioside AM and various steviol glycosides from steviol.

[0066] FIG. **3** shows the biocatalytic production of rebaudioside AM from stevioside using the enzymes UGTS12 and UGT76G1 and concomitant recycling of UDP to UDP-glucose via sucrose synthase SuSy_At.

[0067] FIG. **4** shows the biocatalytic production of rebausioside AM from rebaudioside E using the enzyme UGT76G1 and concomitant recycling of UDP to UDP-glucose via sucrose synthase SuSy_At.

[0068] FIG. **5** shows the HPLC chromatogram of stevioside. The peak with retention time of 25.992 minutes corresponds to stevioside.

[0069] FIG. **6** shows the HPLC chromatogram of the product of the biocatalytic production of rebaudioside AM from stevioside. The peak with retention time of 10.636 minutes corresponds to rebaudioside AM.

[0070] FIG. **7** shows the HPLC chromatogram of rebaudioside E. The peak with retention time of 10.835 minutes corresponds to rebaudioside E.

[0071] FIG. **8** shows the HPLC chromatogram of the product of the biocatalytic production of rebaudioside AM from rebaudioside E. The peaks with retention time of 10.936 and 11.442 minutes correspond to rebaudioside E and rebaudioside AM respectively.

[0072] FIG. **9** shows the HPLC chromatogram of rebaudioside AM after purification by methanol crystallization. The peak with retention time of 10.336 minutes corresponds to rebaudioside AM.

[0073] FIG. **10** shows the .sup.1HNMR spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0074] FIG. **11** shows the HSQC spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0075] FIG. **12** shows the H,H COSY spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0076] FIG. **13** shows the HMBC spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0077] FIG. **14** shows the HSQC-TOCSY spectrum of rebaudioside AM (500 M Hz, pyridine-d5).

[0078] FIG. **15***a* and FIG. **15***b* show the LC chromatogram and mass spectrum of rebaudioside AM respectively.

DETAILED DESCRIPTION

[0079] The present invention provides a process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microbial cell and/or enzyme preparation, thereby producing a composition comprising a target steviol glycoside.

[0080] One object of the invention is to provide an efficient biocatalytic method for preparing target steviol glycosides, particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM or a synthetic steviol glycoside from various starting compositions.

[0081] As used herein, the abbreviation term “reb” refers to “rebaudioside”. Both terms have the same meaning and may be used interchangeably.

[0082] As used herein, “biocatalysis” or “biocatalytic” refers to the use of natural or genetically engineered biocatalysts, such as enzymes, or cells including microorganisms, comprising one or more enzyme, capableof single or multiple step chemical transformations on organic compounds. Biocatalysis processes include fermentation, biosynthesis, bioconversion and biotransformation processes. Both isolated enzyme, and whole-cell biocatalysis methods are known in the art. Biocatalyst protein enzymes can be naturally occurring or recombinant proteins.

[0083] As used herein, the term “steviol glycoside(s)” refers to a glycoside of steviol, including, but not limited to, naturally occurring steviol glycosides, e.g. steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

Starting Composition

[0084] As used herein, “starting composition” refers to any composition (generally an aqueous solution) containing one or more organic compound comprising at least one carbon atom.

[0085] In one embodiment, the starting composition is selected from the group consisting of steviol, steviol glycosides, polyols and various carbohydrates.

[0086] The starting composition steviol glycoside is selected from the group consisting of steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 or other glycoside of steviol occurring in *Stevia rebaudiana* plant, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

[0087] In one embodiment, the starting composition is steviol.

[0088] In another embodiment, the starting composition steviol glycoside is steviolmonoside.

[0089] In yet another embodiment, the starting composition steviol glycoside is steviolmonoside A.

[0090] In still another embodiment, the starting composition steviol glycoside is rubusoside.

[0091] In yet another embodiment, the starting composition steviol glycoside is steviolbioside.

[0092] In yet another embodiment, the starting composition steviol glycoside is steviolbioside A.

[0093] In yet another embodiment, the starting composition steviol glycoside is steviolbioside B.

[0094] In still another embodiment, the starting composition steviol glycoside is stevioside.

[0095] In yet another embodiment, the starting composition steviol glycoside is stevioside A, also known as rebaudioside KA.

[0096] In still another embodiment, the starting composition steviol glycoside is stevioside B.

[0097] In still another embodiment, the starting composition steviol glycoside is stevioside C.

[0098] In another embodiment, the starting composition steviol glycoside is rebaudioside E.

[0099] In another embodiment, the starting composition steviol glycoside is rebaudioside E2.

[0100] In another embodiment, the starting composition steviol glycoside is rebaudioside E3.

[0101] The term “polyol” refers to a molecule that contains more than one hydroxyl group. A polyol may be a diol, triol, or a tetraol which contain 2, 3, and 4 hydroxyl groups, respectively. A polyol also may contain more than four hydroxyl groups, such as a pentaol, hexaol, heptaol, or the like, which contain 5, 6, or 7 hydroxyl groups, respectively. Additionally, a polyol also may be a sugar alcohol, polyhydric alcohol, or polyalcohol which is a reduced form of carbohydrate, wherein the carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group. Examples of polyols include, but are not limited to, erythritol, maltitol, mannitol, sorbitol, lactitol, xylitol, inositol, isomalt, propylene glycol, glycerol, threitol, galactitol, hydrogenated isomaltulose, reduced isomalto-oligosaccharides, reduced xylo-oligosaccharides, reduced gentio-oligosaccharides, reduced maltose syrup, reduced glucose syrup, hydrogenated starch hydrolyzates, polyglycitols and sugar alcohols or any other carbohydrates capable of being reduced.

[0102] The term “carbohydrate” refers to aldehyde or ketone compounds substituted with multiple hydroxyl groups, of the general formula (CH.sub.2O).sub.n, wherein n is 3-30, as well as their oligomers and polymers. The carbohydrates of the present invention can, in addition, be substituted or deoxygenated at one or more positions. Carbohydrates, as used herein, encompass unmodified carbohydrates, carbohydrate derivatives, substituted carbohydrates, and modified carbohydrates. As used herein, the phrases “carbohydrate derivatives”, “substituted carbohydrate”, and “modified

carbohydrates" are synonymous. Modified carbohydrate means any carbohydrate wherein at least one atom has been added, removed, or substituted, or combinations thereof. Thus, carbohydrate derivatives or substituted carbohydrates include substituted and unsubstituted monosaccharides, disaccharides, oligosaccharides, and polysaccharides. The carbohydrate derivatives or substituted carbohydrates optionally can be deoxygenated at any corresponding C-position, and/or substituted with one or more moieties such as hydrogen, halogen, haloalkyl, carboxyl, acyl, acyloxy, amino, amido, carboxyl derivatives, alkylamino, dialkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfo, mercapto, imino, sulfonyl, sulfenyl, sulfinyl, sulfamoyl, carboalkoxy, carboxamido, phosphonyl, phosphinyl, phosphoryl, phosphino, thioester, thioether, oximino, hydrazino, carbamyl, phospho, phosphonato, or any other viable functional group provided the carbohydrate derivative or substituted carbohydrate functions to improve the sweet taste of the sweetener composition.

[0103] Examples of carbohydrates which may be used in accordance with this invention include, but are not limited to, tagatose, trehalose, galactose, rhamnose, various cyclodextrins, cyclic oligosaccharides, various types of maltodextrins, dextran, sucrose, glucose, ribulose, fructose, threose, arabinose, xylose, lyxose, allose, altrose, mannose, idose, lactose, maltose, invert sugar, isotrehalose, neotrehalose, isomaltulose, erythrose, deoxyribose, gulose, idose, talose, erythrulose, xylulose, psicose, turanose, cellobiose, amylopectin, glucosamine, mannosamine, fucose, glucuronic acid, gluconic acid, glucono-lactone, abequose, galactosamine, beet oligosaccharides, isomalto-oligosaccharides (isomaltose, isomaltotriose, panose and the like), xylo-oligosaccharides (xylotriose, xylobiose and the like), xylo-terminated oligosaccharides, gentio-oligosaccharides (gentiobiose, gentiotriose, gentiotetraose and the like), sorbose, nigero-oligosaccharides, palatinose oligosaccharides, fructooligosaccharides (kestose, nystose and the like), maltotetraol, maltotriol, malto-oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and the like), starch, inulin, inulo-oligosaccharides, lactulose, melibiose, raffinose, ribose, isomerized liquid sugars such as high fructose corn syrups, coupling sugars, and soybean oligosaccharides. Additionally, the carbohydrates as used herein may be in either the D- or L-configuration.

[0104] The starting composition may be synthetic or purified (partially or entirely), commercially available or prepared.

[0105] In one embodiment, the starting composition is glycerol.

[0106] In another embodiment, the starting composition is glucose.

[0107] In still another embodiment, the starting composition is sucrose.

[0108] In yet another embodiment, the starting composition is starch.

[0109] In another embodiment, the starting composition is maltodextrin.

[0110] In yet another embodiment, the starting composition is cellulose.

[0111] In still another embodiment, the starting composition is amylose.

[0112] The organic compound(s) of starting composition serve as a substrate(s) for the production of the target steviol glycoside(s), as described herein.

Target Steviol Glycoside

[0113] The target steviol glycoside of the present method can be any steviol glycoside that can be prepared by the process disclosed herein. In one embodiment, the target steviol glycoside is selected from the group consisting of steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, rebaudioside AM or other glycoside of steviol occurring in *Stevia rebaudiana* plant, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

[0114] In one embodiment, the target steviol glycoside is steviolmonoside.

[0115] In another embodiment, the target steviol glycoside is steviolmonoside A.

[0116] In another embodiment, the target steviol glycoside is steviolbioside.

[0117] In another embodiment, the target steviol glycoside is steviolbioside A.
[0118] In another embodiment, the target steviol glycoside is steviolbioside B.
[0119] In another embodiment, the target steviol glycoside is rubusoside.
[0120] In another embodiment, the target steviol glycoside is stevioside.
[0121] In another embodiment, the target steviol glycoside is stevioside A (rebaudioside KA).
[0122] In another embodiment, the target steviol glycoside is stevioside B.
[0123] In another embodiment, the target steviol glycoside is stevioside C.
[0124] In another embodiment, the target steviol glycoside is rebaudioside E.
[0125] In another embodiment, the target steviol glycoside is rebaudioside E2.
[0126] In another embodiment, the target steviol glycoside is rebaudioside E3.
[0127] In another embodiment, the target steviol glycoside is rebaudioside AM.
[0128] The target steviol glycoside can be in any polymorphic or amorphous form, including hydrates, solvates, anhydrous or combinations thereof.
[0129] In one embodiment, the present invention is a biocatalytic process for the production of steviolmonoside.
[0130] In one embodiment, the present invention is a biocatalytic process for the production of steviolmonoside A.
[0131] In one embodiment, the present invention is a biocatalytic process for the production of steviolbioside.
[0132] In one embodiment, the present invention is a biocatalytic process for the production of steviolbioside A.
[0133] In one embodiment, the present invention is a biocatalytic process for the production of steviolbioside B.
[0134] In one embodiment, the present invention is a biocatalytic process for the production of rubusoside.
[0135] In one embodiment, the present invention is a biocatalytic process for the production of stevioside.
[0136] In one embodiment, the present invention is a biocatalytic process for the production of stevioside A (rebaudioside KA).
[0137] In one embodiment, the present invention is a biocatalytic process for the production of stevioside B.
[0138] In one embodiment, the present invention is a biocatalytic process for the production of stevioside C.
[0139] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside E.
[0140] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside E2.
[0141] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside E3.
[0142] In one embodiment, the present invention is a biocatalytic process for the production of rebaudioside AM.
[0143] In a particular embodiment, the present invention provides for the biocatalytic process for the production of rebaudioside AM from a starting composition comprising stevioside and UDP-glucose.
[0144] In another particular embodiment, the present invention provides for the biocatalytic process for the production of rebaudioside AM from a starting composition comprising rebaudioside E and UDP-glucose.
[0145] Optionally, the method of the present invention further comprises separating the target steviol glycoside from the medium to provide a highly purified target steviol glycoside composition. The target steviol glycoside can be separated by any suitable method, such as, for

example, crystallization, separation by membranes, centrifugation, extraction, chromatographic separation or a combination of such methods.

[0146] In particular embodiments, the process described herein results in a highly purified target steviol glycoside composition. The term “highly purified”, as used herein, refers to a composition having greater than about 80% by weight of the target steviol glycoside on an anhydrous (dried) basis. In one embodiment, the highly purified target steviol glycoside composition contains greater than about 90% by weight of the target steviol glycoside on an anhydrous (dried) basis, such as, for example, greater than about 91%, greater than about 92%, greater than about 93%, greater than about 94%, greater than about 95%, greater than about 96%, greater than about 97%, greater than about 98% or greater than about 99% target steviol glycoside content on a dried basis.

[0147] In one embodiment, when the target steviol glycoside is reb AM, the process described herein provides a composition having greater than about 90% reb AM content by weight on a dried basis. In another particular embodiment, when the target steviol glycoside is reb AM, the process described herein provides a composition comprising greater than about 95% reb AM content by weight on a dried basis.

Microorganisms and Enzyme Preparations

[0148] In one embodiment of present invention, a microorganism (microbial cell) and/or enzyme preparation is contacted with a medium containing the starting composition to produce target steviol glycosides.

[0149] The enzyme can be provided in the form of a whole cell suspension, a crude lysate, a purified enzyme or a combination thereof. In one embodiment, the biocatalyst is a purified enzyme capable of converting the starting composition to the target steviol glycoside. In another embodiment, the biocatalyst is a crude lysate comprising at least one enzyme capableof converting the starting composition to the target steviol glycoside. In still another embodiment, the biocatalyst is a whole cell suspension comprising at least one enzyme capable of converting the starting composition to the target steviol glycoside.

[0150] In another embodiment, the biocatalyst is one or more microbial cells comprising enzyme(s) capable of converting the starting composition to the target steviol glycoside. The enzyme can be located on the surface of the cell, inside the cell or located both on the surface of the cell and inside the cell.

[0151] Suitable enzymes for converting the starting composition to target steviol glycosides include, but are not limited to, the steviol biosynthesis enzymes and UDP-glucosyltransferases (UGTs). Optionally it may include UDP recycling enzyme(s).

[0152] In one embodiment, the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.

[0153] In another embodiment, the steviol biosynthesis enzymes include non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway (M E P/DOX P) enzymes.

[0154] In one embodiment the steviol biosynthesis enzymes are selected from the group including geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), I-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated H M G-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase etc.

[0155] The UDP-glucosyltransferase can be any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol and/or a steviol glycoside substrate to provide the target steviol glycoside.

[0156] In one embodiment, steviol biosynthesis enzymes and UDP-glucosyltransferases are produced in a microbial cell. The microbial cell may be, for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc. In another embodiment, the UDP-glucosyltransferases are synthesized.
[0157] In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2, UGT76G1, UGT91D2, UGTS12, EUGT11 and UGTs having substantial (>85%, >86%, >87%, >88%, >89%, >90%, >91%, >92%, >93%, >94%, >95%, >96%, >97%, >98%, >99%) amino-acid sequence identity to these polypeptides as well as isolated nucleic acid molecules that code for these UGTs.
[0158] In one embodiment, steviol biosynthesis enzymes, UGTs and UDP-glucose recycling system are present in one microorganism (microbial cell). The microorganism may be for example, *E. coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp.
[0159] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol or any starting steviol glycoside bearing an —OH functional group at C13 to give a target steviol glycoside having an —O-glucose beta glucopyranoside glycosidic linkage at C13. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2, or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0160] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol or any starting steviol glycoside bearing a —COOH functional group at C19 to give a target steviol glycoside having a —COO-glucose beta-glucopyranoside glycosidic linkage at C19. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1, or a UGT having >85% amino-acid sequence identity with UGT74G1.
[0161] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1.fwdarw.2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0162] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C19 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1.fwdarw.3 glucopyranoside glycosidic linkage(s) at the newly formed bond glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGT76G1, or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0163] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to the existing glucose at C13 of any starting steviol glycoside to give a target steviol glycoside with at least one additional glucose bearing at least one beta 1.fwdarw.2 glucopyranoside glycosidic linkage(s) at the newly formed glycosidic bond(s). In a particular embodiment, the UDP-glucosyltransferase is UGTS12, or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0164] In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol to form steviolmonoside. In a particular embodiment,

the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0165] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviol to form steviolmonoside A. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.
[0166] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0167] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form steviolbioside A. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0168] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside A to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0169] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form rubusoside. In a particular embodiment, the UDP-glucosyltransferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.
[0170] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolmonoside to form steviolbioside.
[0171] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0172] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside B to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is E UGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0173] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside A. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0174] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside A to form stevioside C. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0175] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside B. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0176] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside A (rebaudioside KA). In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0177] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside.
[0178] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to steviolbioside to form stevioside. In a particular embodiment, the UDP-glucosyl transferase is UGT74G1 or a UGT having >85% amino-acid sequence identity with UGT74G1.
[0179] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0180] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside B to form rebaudioside E2.
[0181] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0182] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside A (rebaudioside KA) to form rebaudioside E.
[0183] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside C to form rebaudioside E3. In a particular embodiment, the UDP-glucosyltransferase is UGT85C2 or a UGT having >85% amino-acid sequence identity with UGT85C2.
[0184] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E2. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.
[0185] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside E. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.
[0186] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E3 to form rebaudioside AM.
[0187] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E2 to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGTS12 or a UGT having >85% amino-acid sequence identity with UGTS12. In another particular embodiment, the UDP-

glucosyltransferase is EUGT11, or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another particular embodiment, the UDP-glucosyltransferase is UGT91D2, or a UGT having >85% amino-acid sequence identity with UGT91D2.

[0188] In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside E to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1.

[0189] Optionally, the method of the present invention further comprises using more than one UGT on a starting composition, to give a target steviol glycoside(s) having more than one glucose unit than the starting composition. In a particular embodiment, the UDP-glucosyltransferases are UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any UGT having >85% amino-acid sequence identity with UGT74G1, UGT85C2, UGT76G1, UGTS12, EUGT11 and/or UGT91D2 or any combination thereof, capable of adding more than one glucose unit to a starting composition to give a steviol glycoside(s) having more than one glucose unit than the starting composition.

[0190] In one embodiment, the UDP-glucosyltransferases are any UDP-glucosyltransferases capable of adding overall two glucose unit to stevioside to form rebaudioside AM. In a particular embodiment, the UDP-glucosyltransferases are selected from UGTS12, EUGT11, UGT91D2, UGT76G1 or any UGT having >85% amino-acid sequence identity with UGTS12, EUGT11, UGT91D2, UGT76G1 or any combination thereof. In another particular embodiment, the UDP-glucosyltransferases are UGTS12 and UGT76G1.

[0191] Optionally, the method of the present invention further comprises recycling UDP to provide UDP-glucose. In one embodiment, the method comprises recycling UDP by providing a recycling catalyst and a recycling substrate, such that the biotransformation of steviol and/or the steviol glycoside substrate to the target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose. The UDP recycling enzyme can be sucrose synthase SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At and the recycling substrate can be sucrose.

[0192] Optionally, the method of the present invention further comprises the use of transglycosidases that use oligo- or poly-saccharides as the sugar donor to modify recipient target steviol glycoside molecules. Non-limiting examples include cyclodextrin glycosyltransferase (CGTase), fructofuranosidase, amylase, saccharase, glucosucrase, beta-h-fructosidase, beta-fructosidase, sucrase, fructosylinvertase, alkaline invertase, acid invertase, fructofuranosidase. In some embodiments, glucose and sugar(s) other than glucose, including but not limited to fructose, xylose, rhamnose, arabinose, deoxyglucose, galactose are transferred to the recipient target steviol glycosides. In one embodiment, the recipient steviol glycoside is rebaudioside AM.

[0193] In another embodiment, the UDP-glucosyltransferase capable of adding at least one glucose unit to starting composition steviol glycoside has >85% amino-acid sequence identity with UGTs selected from the following listing of Geninfo identifier numbers, preferably from the group presented in Table 1, and Table 2.

TABLE-US-00001 397567 30680413 115480946 147798902 218193594 225443294 454245 32816174 116310259 147811764 218193942 225444853 1359905 32816178 116310985 147827151 219885307 225449296 1685003 34393978 116788066 147836230 222615927 225449700 1685005 37993665 116788606 147839909 222619587 225454338 2191136 37993671 116789315 147846163 222623142 225454340 2501497 37993675 119394507 147855977 222625633 225454342 2911049 39104603 119640480 148905778 222625635 225454473 4218003 41469414 122209731 148905999 222636620 225454475 4314356 41469452 125526997 148906835 222636621 225458362 13492674 42566366 125534279 148907340 222636628 225461551 13492676 42570280 125534461 148908935 222636629 225461556 15217773 42572855 125540090 148909182 224053242 225461558 15217796 44890129 125541516

148909920 224053386 225469538 15223396 46806235 125545408 148910082 224055535
225469540 15223589 50284482 125547340 148910154 224056138 226316457 15227766
51090402 125547520 148910612 224056160 226492603 15230017 51090594 125554547
148910769 224067918 226494221 15231757 52839682 125557592 156138791 224072747
226495389 15234056 56550539 125557593 156138797 224080189 226495945 15234195
62734263 125557608 156138799 224091845 226502400 15234196 62857204 125559566
156138803 224094703 226507980 15238503 62857206 125563266 165972256 224100653
226531147 15239523 62857210 125571055 168016721 224100657 226532094 15239525
62857212 125579728 171674071 224101569 238477377 15239543 75265643 125588307
171906258 224103105 240254512 15239937 75285934 125589492 183013901 224103633
242032615 15240305 75288884 125599469 183013903 224103637 242032621 15240534
77550661 125601477 186478321 224109218 242038423 15982889 77556148 126635837
187373030 224114583 242043290 18086351 82791223 126635845 187373042 224116284
242044836 18418378 83778990 126635847 190692175 224120552 242051252 18418380
89953335 126635863 194701936 224121288 242056217 18418382 110741436 126635867
195620060 224121296 242056219 19743740 110743955 126635883 209954691 224121300
242056663 19911201 115438196 126635887 209954719 224130358 242059339 20149064
115438785 133874210 209954725 224140703 242059341 20260654 115441237 133874212
209954733 224143404 242060922 21435782 115454819 145358033 210063105 224143406
242067411 21553613 115456047 147772508 210063107 224144306 242067413 21593514
115457492 147776893 212275846 224285244 242076258 22759895 115459312 147776894
216296854 225431707 242076396 23955910 115464719 147776895 217074506 225435532
242084750 26452040 115471069 147786916 218185693 225436321 242091005 28393204
115471071 147798900 218187075 225440041 242095206 30679796 115474009 147798901
218189427 225441116 242345159 242345161 297724601 326492035 356523945 357140904
359486938 255536859 297725463 326493430 356523957 357165849 359487055 255538228
297728331 326500410 356523959 357165852 359488135 255541676 297738632 326506816
356523961 357168415 359488708 255547075 297745347 326507826 356523963 357437837
359493630 255552620 297745348 326508394 356524387 357442755 359493632 255552622
297795735 326509445 356524403 357442757 359493634 255555343 297796253 326511261
356527181 357445729 359493636 255555361 297796257 326511866 356533209 357445731
359493815 255555363 297796261 326512412 356533852 357445733 359495856 255555365
297797587 326517673 356534718 357446799 359495858 255555369 297798502 326518800
356535480 357446805 359495869 255555373 297799226 326521124 356542996 357452779
359495871 255555377 297805988 326525567 356543136 357452781 359497638 255556812
297807499 326525957 356543932 357452783 359807261 255556818 297809125 326526607
356549841 357452787 374256637 255563008 297809127 326527141 356549843 357452789
377655465 255564074 297811403 326530093 356554358 357452791 378405177 255564531
297820040 326534036 356554360 357452797 378829085 255572878 297821483 326534312
356558606 357452799 387135070 255577901 297825217 332071132 356560333 357470367
387135072 255583249 297832276 339715876 356560599 357472193 387135078 255583253
297832280 342306012 356560749 357472195 387135092 255583255 297832518 342306016
356566018 357474295 387135094 255585664 297832520 343457675 356566169 357474493
387135098 255585666 297840825 343457677 356566173 357474497 387135100 255634688
297840827 350534960 356567761 357474499 387135134 255644801 297847402 356498085
356574704 357490035 387135136 255645821 297849372 356499771 356576401 357493567
387135174 255647456 300078590 356499777 356577660 357497139 387135176 255648275
300669727 356499779 357114993 357497581 387135184 260279126 302142947 356501328
357115447 357497671 387135186 260279128 302142948 356502523 357115451 357500579
387135188 261343326 302142950 356503180 357115453 357504663 387135190 283132367

302142951 356503184 357116080 357504691 387135192 283362112 302765302 356503295 357116928 357504699 387135194 289188052 302796334 356504436 357117461 357504707 387135282 295841350 302811470 356504523 357117463 357505859 387135284 296088529 302821107 356504765 357117829 357510851 387135294 296090415 302821679 356511113 357117839 357516975 387135298 296090524 319759260 356515120 357125059 359477003 387135300 296090526 319759266 356517088 357126015 359477998 387135302 297599503 320148814 356520732 357134488 359478043 387135304 297601531 326489963 356522586 357135657 359478286 387135312 297611791 326490273 356522588 357138503 359484299 387135314 297722841 326491131 356522590 357139683 359486936 387135316 387135318 449440433 460376293 460413408 462423864 475546199 387135320 449445896 460378310 460416351 470101924 475556485 387135322 449446454 460380744 462394387 470102280 475559699 387135324 449447657 460381726 462394433 470102858 475578293 387135326 449449002 460382093 462394557 470104211 475591753 387135328 449449004 460382095 462395646 470104264 475593742 388493506 449449006 460382754 462395678 470104266 475612072 388495496 449451379 460384935 462396388 470106317 475622476 388498446 449451589 460384937 462396389 470106357 475622507 388499220 449451591 460385076 462396419 470115448 475623787 388502176 449451593 460385872 462396542 470130404 482550481 388517521 449453712 460386018 462397507 470131550 482550499 388519407 449453714 460389217 462399998 470136482 482550740 388521413 449453716 460394872 462400798 470136484 482550999 388827901 449453732 460396139 462401217 470136488 482552352 388827903 449457075 460397862 462402118 470136492 482554970 388827907 449467555 460397864 462402237 470137933 482555336 388827909 449468742 460398541 462402284 470137937 482555478 388827913 449495638 460403139 462402416 470140422 482556454 393887637 449495736 460403141 462404228 470140426 482557289 393887646 449499880 460403143 462406358 470140908 482558462 393887649 449502786 460403145 462408262 470141232 482558508 393990627 449503471 460405998 462409325 470142008 482558547 397746860 449503473 460407578 462409359 470142010 482561055 397789318 449515857 460407590 462409777 470142012 482561555 413924864 449518643 460409128 462411467 470143607 482562795 414590349 449519559 460409134 462414311 470143939 482562850 414590661 449522783 460409136 462414416 470145404 482565074 414591157 449524530 460409459 462414476 473923244 482566269 414879558 449524591 460409461 462415526 474114354 482566296 414879559 449528823 460409463 462415603 474143634 482566307 414879560 449528825 460409465 462415731 474202268 482568689 414888074 449534021 460409467 462416307 474299266 482570049 431812559 460365546 460410124 462416920 474363119 482570572 449432064 460366882 460410126 462416922 474366157 482575121 449432066 460369823 460410128 462416923 474429346 449433069 460369829 460410130 462416924 475432777 449436944 460369831 460410132 462417401 475473002 449438665 460369833 460410134 462419769 475489790 449438667 460370755 460410213 462420317 475511330 449440431 460374714 460411200 462423366 475516200

TABLE-US-00002 TABLE 1 GI number Accession Origin 190692175 ACE87855.1 *Stevia rebaudiana* 41469452 AAS07253.1 *Oryza saliva* 62857204 BAD95881.1 *Ipomoea nil* 62857206 BAD95882.1 *Ipomoea purperea* 56550539 BAD77944.1 *Bellis perennis* 115454819 NP_001051010.1 *Oryza sativa Japonica* Group 115459312 NP_001053256.1 *Oryza sativa Japonica* Group 115471069 NP_001059133.1 *Oryza saliva Japonica* Group 115471071 NP_001059134.1 *Oryza saliva Japonica* Group 116310985 CAH67920.1 *Oryza sativa Indica* Group 116788066 ABK24743.1 *Picea sitchensis* 122209731 Q2V6J9.1 *Fragaria* × *ananassa* 125534461 EAY81009.1 *Oryza sativa Indica* Group 125559566 EAZ05102.1 *Oryza sativa Indica* Group 125588307 EAZ28971.1 *Oryza sativa Japonica* Group 148907340 ABR16806.1 *Picea sitchensis* 148910082 ABR18123.1 *Picea sitchensis* 148910612 ABR18376.1 *Picea sitchensis* 15234195 NP_194486.1 *Arabidopsis thaliana* 15239523 NP_200210.1 *Arabidopsis thaliana*

15239937 NP_196793.1 *Arabidopsis thaliana* 1685005 AAB36653.1 *Nicotiana tabacum* 183013903 ACC38471.1 *Medicago truncatula* 186478321 NP_172511.3 *Arabidopsis thaliana* 187373030 ACD03249.1 *Avena strigosa* 194701936 ACF85052.1 *Zea mays* 19743740 AAL92461.1 *Solanum lycopersicum* 212275846 NP_001131009.1 *Zea mays* 222619587 EEE55719.1 *Oryza sativa Japonica* Group 224055535 XP_002298527.1 *Populus trichocarpa* 224101569 XP_002334266.1 *Populus trichocarpa* 224120552 XP_002318358.1 *Populus trichocarpa* 224121288 XP_002330790.1 *Populus trichocarpa* 225444853 XP_002281094 *Vitis vinifera* 225454342 XP_002275850.1 *Vitis vinifera* 225454475 XP_002280923.1 *Vitis vinifera* 225461556 XP_002285222 *Vitis vinifera* 225469540 XP_002270294.1 *Vitis vinifera* 226495389 NP_001148083.1 *Zea mays* 226502400 NP_001147674.1 *Zea mays* 238477377 ACR43489.1 *Triticum aestivum* 240254512 NP_565540.4 *Arabidopsis thaliana* 2501497 Q43716.1 *Petunia* × *hybrida* 255555369 XP_002518721.1 *Ricinus communis* 26452040 BAC43110.1 *Arabidopsis thaliana* 296088529 CBI37520.3 *Vitis vinifera* 297611791 NP_001067852.2 *Oryza sativa Japonica* Group 297795735 XP_002865752.1 *Arabidopsis lyrata* subsp. *lyrata* 297798502 XP_002867135.1 *Arabidopsis lyrata* subsp. *lyrata* 297820040 XP_002877903.1 *Arabidopsis lyrata* subsp. *lyrata* 297832276 XP_002884020.1 *Arabidopsis lyrata* subsp. *lyrata* 302821107 XP_002992218.1 *Selaginella moellendorffii* 30680413 NP_179446.2 *Arabidopsis thaliana* 319759266 ADV71369.1 *Pueraria montana* var. *lobata* 326507826 BAJ86656.1 *Hordeum vulgare* subsp. *Vulgare* 343457675 AEM37036.1 *Brassica rapa* subsp. *oleifera* 350534960 NP_001234680.1 *Solanum lycopersicum* 356501328 XP_003519477.1 *Glycine max* 356522586 XP_003529927.1 *Glycine max* 356535480 XP_003536273.1 *Glycine max* 357445733 XP_003593144.1 *Medicago truncatula* 357452783 XP_003596668.1 *Medicago truncatula* 357474493 XP_003607531.1 *Medicago truncatula* 357500579 XP_003620578.1 *Medicago truncatula* 357504691 XP_003622634.1 *Medicago truncatula* 359477998 XP_003632051.1 *Vitis vinifera* 359487055 XP_002271587 *Vitis vinifera* 359495869 XP_003635104.1 *Vitis vinifera* 387135134 AFJ52948.1 *Linum usitatissimum* 387135176 AFJ52969.1 *Linum usitatissimum* 387135192 AFJ52977.1 *Linum usitatissimum* 387135282 AFJ53022.1 *Linum usitatissimum* 387135302 AFJ53032.1 *Linum usitatissimum* 387135312 AFJ53037.1 *Linum usitatissimum* 388519407 AFK47765.1 *Medicago truncatula* 393887646 AFN26668.1 *Barbarea vulgaris* subsp. *arcuata* 414888074 DAA64088.1 *Zea mays* 42572855 NP_974524.1 *Arabidopsis thaliana* 449440433 XP_004137989.1 *Cucumis sativus* 449446454 XP_004140986.1 *Cucumis sativus* 449449004 XP_004142255.1 *Cucumis sativus* 449451593 XP_004143546.1 *Cucumis sativus* 449515857 XP_004164964.1 *Cucumis sativus* 460382095 XP_004236775.1 *Solanum lycopersicum* 460409128 XP_004249992.1 *Solanum lycopersicum* 460409461 XP_004250157.1 *Solanum lycopersicum* 460409465 XP_004250159.1 *Solanum lycopersicum* 462396388 EMJ02187.1 *Prunus persica* 462402118 EMJ07675.1 *Prunus persica* 462409359 EMJ14693.1 *Prunus persica* 462416923 EMJ21660.1 *Prunus persica* 46806235 BAD17459.1 *Oryza saliva Japonica* Group 470104266 XP_004288529.1 *Fragaria vesca* subsp. *vesca* 470142008 XP_004306714.1 *Fragaria vesca* subsp. *vesca* 475432777 EMT01232.1 *Aegilops tauschii* 51090402 BAD35324.1 *Oryza sativa Japonica* Group

TABLE-US-00003 TABLE 2 Internal GI number Accession Origin reference 460409128 XP.004249992.1 *Solanum lycopersicum* UGTSI 460386018 XP.004238697.1 *Solanum lycopersicum* — 460409134 XP.004249995.1 *Solanum lycopersicum* — 460410132 XP.004250485.1 *Solanum lycopersicum* UGTSI2 460410130 XP.004250484.1 *Solanum lycopersicum* — 460410128 XP.004250483.1 *Solanum lycopersicum* — 460378310 XP.004234916.1 *Solanum lycopersicum* — 209954733 BAG80557.1 *Lycium barbarum* UGTLB 209954725 BAG80553.1 *Lycium barbarum* —

[0194] One embodiment of the present invention is a microbial cell comprising an enzyme, i.e. an enzyme capable of converting the starting composition to the target steviol glycoside. Accordingly, some embodiments of the present method include contacting a microorganism with a medium containing the starting composition to provide a medium comprising at least one target steviol

glycoside.
[0195] The microorganism can be any microorganism possessing the necessary enzyme(s) for converting the starting composition to target steviol glycoside(s). These enzymes are encoded within the microorganism's genome.
[0196] Suitable microoganisms include, but are not limited to, *E. coli*, *Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc.
[0197] In one embodiment, the microorganism is free when contacted with the starting composition.
[0198] In another embodiment, the microorganism is immobilized when contacted with the starting composition. For example, the microorganism may be immobilized to a solid support made from inorganic or organic materials. Non-limiting examples of solid supports suitable to immobilize the microorganism include derivatized cellulose or glass, ceramics, metal oxides or membranes. The microorganism may be immobilized to the solid support, for example, by covalent attachment, adsorption, cross-linking, entrapment or encapsulation.
[0199] In still another embodiment, the enzyme capable of converting the starting composition to the target steviol glycoside is secreted out of the microorganism and into the reaction medium.
[0200] The target steviol glycoside is optionally purified. Purification of the target steviol glycoside from the reaction medium can be achieved by at least one suitable method to provide a highly purified target steviol glycoside composition. Suitable methods include crystallization, separation by membranes, centrifugation, extraction (liquid or solid phase), chromatographic separation, HPLC (preparative or analytical) or a combination of such methods.
Uses
[0201] Highly purified target glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention can be used “as-is” or in combination with other sweeteners, flavors, food ingredients and combinations thereof.
[0202] Non-limiting examples of flavors include, but are not limited to, lime, lemon, orange, fruit, banana, grape, pear, pineapple, mango, berry, bitter almond, cola, cinnamon, sugar, cotton candy, vanilla and combinations thereof.
[0203] Non-limiting examples of other food ingredients include, but are not limited to, acidulants, organic and amino acids, coloring agents, bulking agents, modified starches, gums, texturizers, preservatives, caffeine, antioxidants, emulsifiers, stabilizers, thickeners, gelling agents and combinations thereof.
[0204] Highly purified target glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention can be prepared in various polymorphic forms, including but not limited to hydrates, solvates, anhydrous, amorphous forms and combinations thereof.
[0205] Highly purified target glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be incorporated as a high intensity natural sweetener in foodstuffs, beverages, pharmaceutical compositions, cosmetics, chewing gums, table top products, cereals, dairy products, toothpastes and other oral cavity compositions, etc.
[0206] Highly purified target glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or

rebaudioside AM obtained according to this invention may be employed as a sweetening compound as the sole sweetener, or it may be used together with at least one naturally occurring high intensity sweeteners such as rebaudioside A, rebaudioside A2, rebaudioside A3, rebaudioside B, rebaudioside C, rebaudioside C2, rebaudioside D, rebaudioside D2, rebaudioside F, rebaudioside F2, rebaudioside F3, rebaudioside G, rebaudioside H, rebaudioside I, rebaudioside 12, rebaudioside 13, rebaudioside j, rebaudioside K, rebaudioside K2, rebaudioside L, rebaudioside M, rebaudioside M2, rebaudioside N, rebaudioside 0, rebaudioside 02, rebaudioside Q, rebaudioside Q2, rebaudioside Q3, rebaudioside R, rebaudioside S, rebaudioside T, rebaudioside T1, rebaudioside U, rebaudioside U2, rebaudioside V, rebaudioside W, rebaudioside W2, rebaudioside W3, rebaudioside Y, rebaudioside Z1, rebaudioside Z2, dulcoside A, dulcoside C, stevioside D, stevioside E, stevioside E2, stevioside F, mogrosides, brazzein, neohesperidin dihydrochalcone, glycyrrhizic acid and its salts, thaumatin, perillartine, pernandulcin, mukuroziosides, baiyunoside, phlomisoside-I, dimethyl-hexahydrofluorene-dicarboxylic acid, abrusosides, periandrin, carnosiflosides, cyclocarioside, pterocaryosides, polypodoside A, brazilin, hernandulcin, phillodulcin, glycyphyllin, phlorizin, trilobatin, dihydroflavonol, dihydroquercetin-3-acetate, neoastilibin, trans-cinnamaldehyde, monatin and its salts, selligueain A, hematoxylin, monellin, osladin, pterocaryoside A, pterocaryoside B, mabinlin, pentadin, miraculin, curculin, neoculin, chlorogenic acid, cynarin, Luo Han Guo sweetener, mogroside V, siamenoside and combinations thereof.

[0207] In a particular embodiment, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used in a sweetener composition comprising a compound selected from the group consisting of rebaudioside A, rebaudioside A2, rebaudioside A3, rebaudioside B, rebaudioside C, rebaudioside C2, rebaudioside D, rebaudioside D2, rebaudioside F, rebaudioside F2, rebaudioside F3, rebaudioside G, rebaudioside H, rebaudioside I, rebaudioside I2, rebaudioside I3, rebaudioside J, rebaudioside K, rebaudioside K2, rebaudioside L, rebaudioside M, rebaudioside M2, rebaudioside N, rebaudioside O, rebaudioside O2, rebaudioside Q, rebaudioside Q2, rebaudioside Q3, rebaudioside R, rebaudioside S, rebaudioside T, rebaudioside T1, rebaudioside U, rebaudioside U2, rebaudioside V, rebaudioside W, rebaudioside W2, rebaudioside W3, rebaudioside Y, rebaudioside Z1, rebaudioside Z2, dulcoside A, dulcoside C, stevioside D, stevioside E, stevioside E2, stevioside F, NSF-02, Mogroside V, Luo Han Guo, allulose, allose, D-tagatose, erythritol and combinations thereof.

[0208] Highly purified target glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may also be used in combination with synthetic high intensity sweeteners such as sucralose, potassium acesulfame, aspartame, alitame, saccharin, neohesperidin dihydrochalcone, cyclamate, neotame, dulcin, suosan advantame, salts thereof, and combinations thereof.

[0209] Moreover, highly purified target steviol glycoside(s) particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used in combination with natural sweetener suppressors such as gymnemic acid, hodulcin, ziziphin, lactisole, and others. Steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may also be combined with various umami taste enhancers. Steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be mixed with umami tasting and sweet amino acids such as glutamate, aspartic acid, glycine, alanine, threonine, proline, serine,

glutamate, lysine, tryptophan and combinations thereof.
[0210] Highly purified target steviol glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used in combination with one or more additive selected from the group consisting of carbohydrates, polyols, amino acids and their corresponding salts, poly-amino acids and their corresponding salts, sugar acids and their corresponding salts, nucleotides, organic acids, inorganic acids, organic salts including organic acid salts and organic base salts, inorganic salts, bitter compounds, flavorants and flavoring ingredients, astringent compounds, proteins or protein hydrolysates, surfactants, emulsifiers, flavonoids, alcohols, polymers and combinations thereof.
[0211] Highly purified target steviol glycoside(s) particularly, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may be combined with polyols or sugar alcohols. The term “polyol” refers to a molecule that contains more than one hydroxyl group. A polyol may be a diol, triol, or a tetraol which contain 2, 3, and 4 hydroxyl groups, respectively. A polyol also may contain more than four hydroxyl groups, such as a pentaol, hexaol, heptaol, or the like, which contain 5, 6, or 7 hydroxyl groups, respectively. Additionally, a polyol also may be a sugar alcohol, polyhydric alcohol, or polyalcohol which is a reduced form of carbohydrate, wherein the carbonyl group (aldehyde or ketone, reducing sugar) has been reduced to a primary or secondary hydroxyl group. Examples of polyols include, but are not limited to, erythritol, maltitol, mannitol, sorbitol, lactitol, xylitol, inositol, isomalt, propylene glycol, glycerol, threitol, galactitol, hydrogenated isomaltulose, reduced isomalto-oligosaccharides, reduced xylo-oligosaccharides, reduced gentio-oligosaccharides, reduced maltose syrup, reduced glucose syrup, hydrogenated starch hydrolyzates, polyglycitols and sugar alcohols or any other carbohydrates capable of being reduced which do not adversely affect the taste of the sweetener composition.
[0212] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/orrebaudioside AM may be combined with reduced calorie sweeteners such as, for example, D-tagatose, L-sugars, L-sorbose, L-arabinose and combinations thereof.
[0213] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may also be combined with various carbohydrates. The term “carbohydrate” generally refers to aldehyde or ketone compounds substituted with multiple hydroxyl groups, of the general formula (CH.sub.2O).sub.n, wherein n is 3-30, as well as their oligomers and polymers. The carbohydrates of the present invention can, in addition, be substituted or deoxygenated at one or more positions. Carbohydrates, as used herein, encompass unmodified carbohydrates, carbohydrate derivatives, substituted carbohydrates, and modified carbohydrates. As used herein, the phrases “carbohydrate derivatives”, “substituted carbohydrate”, and “modified carbohydrates” are synonymous. Modified carbohydrate means any carbohydrate wherein at least one atom has been added, removed, or substituted, or combinations thereof. Thus, carbohydrate derivatives or substituted carbohydrates include substituted and unsubstituted monosaccharides, disaccharides, oligosaccharides, and polysaccharides. The carbohydrate derivatives or substituted carbohydrates optionally can be deoxygenated at any corresponding C-position, and/or substituted with one or more moieties such as hydrogen, halogen, haloalkyl, carboxyl, acyl, acyloxy, amino, amido, carboxyl derivatives, alkylamino, dialkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfo, mercapto, imino, sulfonyl, sulfenyl, sulfinyl, sulfamoyl, carboalkoxy, carboxamido, phosphonyl, phosphinyl, phosphoryl, phosphino, thioester, thioether, oximino, hydrazino, carbamyl, phospho,

phosphonato, or any other viable functional group provided the carbohydrate derivative or substituted carbohydrate functions to improve the sweet taste of the sweetener composition.
[0214] Examples of carbohydrates which may be used in accordance with this invention include, but are not limited to, psicose, turanose, allose, tagatose, trehalose, galactose, rhamnose, various cyclodextrins, cyclic oligosaccharides, various types of maltodextrins, dextran, sucrose, glucose, ribulose, fructose, threose, arabinose, xylose, lyxose, allose, altrose, mannose, idose, lactose, maltose, invert sugar, isotrehalose, neotrehalose, isomaltulose, erythrose, deoxyribose, gulose, idose, talose, erythrulose, xylulose, psicose, turanose, cellobiose, amylopectin, glucosamine, mannosamine, fucose, glucuronic acid, gluconic acid, glucono-lactone, abequose, galactosamine, beet oligosaccharides, isomalto-oligosaccharides (isomaltose, isomaltotriose, panose and the like), xylo-oligosaccharides (xylotriose, xylobiose and the like), xylo-terminated oligosaccharides, gentio-oligosaccharides (gentiobiose, gentiotriose, gentiotetraose and the like), sorbose, nigero-oligosaccharides, palatinose oligosaccharides, fructooligosaccharides (kestose, nystose and the like), maltotetraol, maltotriol, malto-oligosaccharides (maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and the like), starch, inulin, inulo-oligosaccharides, lactulose, melibiose, raffinose, ribose, isomerized liquid sugars such as high fructose corn syrups, coupling sugars, and soybean oligosaccharides. Additionally, the carbohydrates as used herein may be in either the D- or L-configuration.
[0215] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention can be used in combination with various physiologically active substances or functional ingredients. Functional ingredients generally are classified into categories such as carotenoids, dietary fiber, fatty acids, saponins, antioxidants, nutraceuticals, flavonoids, isothiocyanates, phenols, plant sterols and stanols (phytosterols and phytostanols); polyols; prebiotics, probiotics; phytoestrogens; soy protein; sulfides/thiols; amino acids; proteins; vitamins; and minerals. Functional ingredients also may be classified based on their health benefits, such as cardiovascular, cholesterol-reducing, and anti-inflammatory. Exemplary functional ingredients are provided in WO2013/096420, the contents of which is hereby incorporated by reference.
[0216] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be applied as a high intensity sweetener to produce zero calorie, reduced calorie or diabetic beverages and food products with improved taste characteristics. It may also be used in drinks, foodstuffs, pharmaceuticals, and other products in which sugar cannot be used. In addition, highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM can be used as a sweetener not only for drinks, foodstuffs, and other products dedicated for human consumption, but also in animal feed and fodder with improved characteristics.
[0217] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be applied as a flavor modifier to produce zero calorie, reduced calorie or diabetic beverages and food products with modified flavor. When used as a flavor modifier, or a flavor with modifying properties (FMP), the highly purified target steviol glycoside is used in a consumable product below the detection level of the flavor modifier or FMP. The flavor modifier or FMP therefore does not impart a detectable taste or flavor

of its own to the consumable product, but instead serves to modify the consumer's detection of tastes and/or flavors of other ingredients in the consumable product. One example of taste and flavor modification is sweetness enhancement, in which the flavor modifier or FMP itself does not contribute to the sweetness of the consumable product, but enhances the quality of the sweetness tasted by the consumer.

[0218] Examples of consumable products in which highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may be used as a flavor modifier or flavor with modifying properties include, but are not limited to, alcoholic beverages such as vodka, wine, beer, liquor, and sake, etc.; natural juices; refreshing drinks; carbonated soft drinks; diet drinks; zero calorie drinks; reduced calorie drinks and foods; yogurt drinks; instant juices; instant coffee; powdered types of instant beverages; canned products; syrups; fermented soybean paste; soy sauce; vinegar; dressings; mayonnaise; ketchups; curry; soup; instant bouillon; powdered soy sauce; powdered vinegar; types of biscuits; rice biscuit; crackers; bread; chocolates; caramel; candy; chewing gum; jelly; pudding; preserved fruits and vegetables; fresh cream; jam; marmalade; flower paste; powdered milk; ice cream; sorbet; vegetables and fruits packed in bottles; canned and boiled beans; meat and foods boiled in sweetened sauce; agricultural vegetable food products; seafood; ham; sausage; fish ham; fish sausage; fish paste; deep fried fish products; dried seafood products; frozen food products; preserved seaweed; preserved meat; tobacco; medicinal products; and many others. In principle it can have unlimited applications.

[0219] Highly purified target steviol glycoside(s), particularly steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained according to this invention may be applied as a foaming suppressor to produce zero calorie, reduced calorie or diabetic beverages and food products.

[0220] Examples of consumable products in which highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM may be used as a sweetening compound include, but are not limited to, alcoholic beverages such as vodka, wine, beer, liquor, and sake, etc.; natural juices; refreshing drinks; carbonated soft drinks; diet drinks; zero calorie drinks; reduced calorie drinks and foods; yogurt drinks; instant juices; instant coffee; powdered types of instant beverages; canned products; syrups; fermented soybean paste; soy sauce; vinegar; dressings; mayonnaise; ketchups; curry; soup; instant bouillon; powdered soy sauce; powdered vinegar; types of biscuits; rice biscuit; crackers; bread; chocolates; caramel; candy; chewing gum; jelly; pudding; preserved fruits and vegetables; fresh cream; jam; marmalade; flower paste; powdered milk; ice cream; sorbet; vegetables and fruits packed in bottles; canned and boiled beans; meat and foods boiled in sweetened sauce; agricultural vegetable food products; seafood; ham; sausage; fish ham; fish sausage; fish paste; deep fried fish products; dried seafood products; frozen food products; preserved seaweed; preserved meat; tobacco; medicinal products; and many others. In principle it can have unlimited applications.

[0221] During the manufacturing of products such as foodstuffs, drinks, pharmaceuticals, cosmetics, tabletop products, and chewing gum, the conventional methods such as mixing, kneading, dissolution, pickling, permeation, percolation, sprinkling, atomizing, infusing and other methods may be used.

[0222] Moreover, the highly purified target steviol glycoside(s) steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM obtained in this invention may be used in dry or liquid forms.

[0223] The highly purified target steviol glycoside can be added before or after heat treatment of food products. The amount of the highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM depends on the purpose of usage. As discussed above, it can be added alone or in combination with other compounds.

[0224] The present invention is also directed to sweetness enhancement in beverages using steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM. Accordingly, the present invention provides a beverage comprising a sweetener and steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM as a sweetness enhancer, wherein steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM is present in a concentration at or below their respective sweetness recognition thresholds.

[0225] As used herein, the term “sweetness enhancer” refers to a compound capable of enhancing or intensifying the perception of sweet taste in a composition, such as a beverage. The term “sweetness enhancer” is synonymous with the terms “sweet taste potentiator,” “sweetness potentiator,” “sweetness amplifier,” and “sweetness intensifier.”

[0226] The term “sweetness recognition threshold concentration,” as generally used herein, is the lowest known concentration of a sweet compound that is perceivable by the human sense of taste, typically around 1.0% sucrose equivalence (1.0% SE). Generally, the sweetness enhancers may enhance or potentiate the sweet taste of sweeteners without providing any noticeable sweet taste by themselves when present at or below the sweetness recognition threshold concentration of a given sweetness enhancer; however, the sweetness enhancers may themselves provide sweet taste at concentrations above their sweetness recognition threshold concentration. The sweetness recognition threshold concentration is specific for a particular enhancer and can vary based on the beverage matrix. The sweetness recognition threshold concentration can be easily determined by taste testing increasing concentrations of a given enhancer until greater than 1.0% sucrose equivalence in a given beverage matrix is detected. The concentration that provides about 1.0% sucrose equivalence is considered the sweetness recognition threshold.

[0227] In some embodiments, sweetener is present in the beverage in an amount from about 0.5% to about 12% by weight, such as, for example, about 1.0% by weight, about 1.5% by weight, about 2.0% by weight, about 2.5% by weight, about 3.0% by weight, about 3.5% by weight, about 4.0% by weight, about 4.5% by weight, about 5.0% by weight, about 5.5% by weight, about 6.0% by weight, about 6.5% by weight, about 7.0% by weight, about 7.5% by weight, about 8.0% by weight, about 8.5% by weight, about 9.0% by weight, about 9.5% by weight, about 10.0% by weight, about 10.5% by weight, about 11.0% by weight, about 11.5% by weight or about 12.0% by weight.

[0228] In a particular embodiment, the sweetener is present in the beverage in an amount from about 0.5% of about 10%, such as for example, from about 2% to about 8%, from about 3% to about 7% or from about 4% to about 6% by weight. In a particular embodiment, the sweetener is present in the beverage in an amount from about 0.5% to about 8% by weight. In another particular embodiment, the sweetener is present in the beverage in an amount from about 2% to about 8% by weight.

[0229] In one embodiment, the sweetener is a traditional caloric sweetener. Suitable sweeteners include, but are not limited to, sucrose, fructose, glucose, high fructose corn syrup and high fructose starch syrup.

[0230] In another embodiment, the sweetener is erythritol.
[0231] In still another embodiment, the sweetener is a rare sugar. Suitable rare sugars include, but are not limited to, D-allose, D-psicose, D-ribose, D-tagatose, L-glucose, L-fucose, L-arabinose, D-turanose, D-leucrose and combinations thereof.
[0232] It is contemplated that a sweetener can be used alone, or in combination with other sweeteners.
[0233] In one embodiment, the rare sugar is D-allose. In a more particular embodiment, D-allose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0234] In another embodiment, the rare sugar is D-psicose. In a more particular embodiment, D-psicose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0235] In still another embodiment, the rare sugar is D-ribose. In a more particular embodiment, D-ribose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0236] In yet another embodiment, the rare sugar is D-tagatose. In a more particular embodiment, D-tagatose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0237] In a further embodiment, the rare sugar is L-glucose. In a more particular embodiment, L-glucose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0238] In one embodiment, the rare sugar is L-fucose. In a more particular embodiment, L-fucose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0239] In another embodiment, the rare sugar is L-arabinose. In a more particular embodiment, L-arabinose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0240] In yet another embodiment, the rare sugar is D-turanose. In a more particular embodiment, D-turanose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0241] In yet another embodiment, the rare sugar is D-leucrose. In a more particular embodiment, D-leucrose is present in the beverage in an amount of about 0.5% to about 10% by weight, such as, for example, from about 2% to about 8%.
[0242] The addition of the sweetness enhancer at a concentration at or below its sweetness recognition threshold increases the detected sucrose equivalence of the beverage comprising the sweetener and the sweetness enhancer compared to a corresponding beverage in the absence of the sweetness enhancer. Moreover, sweetness can be increased by an amount more than the detectable sweetness of a solution containing the same concentration of the at least one sweetness enhancer in the absence of any sweetener.
[0243] Accordingly, the present invention also provides a method for enhancing the sweetness of a beverage comprising a sweetener comprising providing a beverage comprising a sweetener and adding a sweetness enhancer selected from steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM ora combination thereof, wherein steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM are present in a concentration at or below their sweetness recognition thresholds.
[0244] Addition of steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E,

rebaudioside E2, rebaudioside E3 and/or rebaudioside AM in a concentration at or below the sweetness recognition threshold to a beverage containing a sweetener may increase the detected sucrose equivalence from about 1.0% to about 5.0%, such as, for example, about 1.0%, about 1.5%, about 2.0%, about 2.5%, about 3.0%, about 3.5%, about 4.0%, about 4.5% or about 5.0%.

[0245] The following examples illustrate preferred embodiments of the invention for the preparation of highly purified target steviol glycoside(s), particularly steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3 and/or rebaudioside AM. It will be understood that the invention is not limited to the materials, proportions, conditions and procedures set forth in the examples, which are only illustrative.

EXAMPLES

Example 1

Protein Sequences of Engineered Enzymes Used in the Biocatalytic Process

TABLE-US-00004 SEQ ID 1: >SuSy_At, variant PM1-54-2-E05 (engineered sucrose synthase; source of WT gene: *Arabidopsis thaliana*)

MANAERMITRVHSQRERLNETLVSERNEVLALLSRVEAKGKGILQQNQII
AEFEALPEQTRKKLEGGPFFDLLKSTQEAIVLPPWVALAVRPRPGVWEYL
RVNLHALVVEELQPAEFLHFKEELVDGVKNGNFTLELDFEPFNASIPRPT
LHKYIGNGVDFLNRHLSAKLFHDKESLLPLLDFLRLHSHQGKNLMLSEKI
QNLNTLQHTLRKAEEYLAELKSETLYEEFEAKFEEIGLERGWGDNAERVL
DMIRLLLDLLEAPDPSTLETFLGRVPMVFNVVILSPHGYFAQDNVLGYPD
TGGQVVYILDQVRALEIEMLQRIKQQGLNIKPRILILTRLLPDAVGTTCG
ERLERVYDSEYCDILRVPFRTEKGIVRKWISRFEVWPYLETYTEDAAVEL
SKELNGKPDLIIGNYSDGNLVASLLAHKLGVTQCTIAHALEKTKYPDSDI
YWKKLDDKYHFSCQFTADIFAMNHTDFIITSTFQEIAGSKETVGQYESHT
AFTLPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPYTEEKRRLTKFHSEI
EELLYSDVENDEHLCVLKDKKKPILFTMARLDRVKNLSGLVEWYGKNTRL
RELVNLVVVGGDRRKESKDNEEKAEMKKMYDLIEEYKLNGQFRWISSQMD
RVRNGELYRYICDTKGAFVQPALYEAFGLTVVEAMTCGLPTFATCKGGPA
EIIVHGKSGFHIDPYHGDQAADLLADFFTKCKEDPSHWDEISKGGLQRIE
EKYTWQIYSQRLLTLTGVYGFWKHVSNLDRLEHRRYLEMFYALKYRPLAQ AVPLAQDD

SEQ ID 2: >UGTSI2 variant 0234 (engineered glucosyltransferase; source of WT gene: *Solanum lycopersicum*)

MATNLRVLMFPWLAYGHISPFLNIAKQLADRGFLIYLCSTRINLESIIKK
IPEKYADSIHLIELQLPELPELPPHYHTTNGLPPHLNPTLHKALKMSKPN
FSRILQNLKPDLLIYDVLQPWAEHVANEQGIPAGKLLVSCAAVFSYFFSF
RKNPGVEFPFPAIHLPEVEKVKIREILAKEPEEGGRLDEGNKQMMLMCTS
RTIEAKYIDYCTELCNWKVVPVGPPFQDLITNDADNKELIDWLGTKPENS
TVFVSFGSEYFLSKEDMEEIAFALEASNVNFIWVVRFPKGEERNLEDALP
EGFLERIGERGRVLDKFAPQPRILNHPSTGGFISHCGWNSVMESIDFGVP
IIAMPIHNDQPINAKLMVELGVAVEIVRDDDGKIHRGEIAEALKSVVTGE
TGEILRAKVREISKNLKSIRDEEMDAVAEELIQLCRNSNKSK SEQ ID 3: >UGT76G1 variant 0042 (engineered glucosyltransferase; source of WT gene: *Stevia rebaudiana*)

MENKTETTVRRRRRIILFPVPFQGHINPILQLANVLYSKGFAITILHTNF
NKPKTSNYPHFTFRFILDNDPQDERISNLPTHGPLAGMRIPIINEHGADE
LRRELELLMLASEEDEEVSCLITDALWYFAQDVADSLNLRRLVLMTSSLF
NFHAHVSLPQFDELGYLDPDDKTRLEEQASGFPMLKVKDIKSAYSNWQIG
KEILGKMIKQTKASSGVIWNSFKELEESELETVIREIPAPSFLIPLPKHL
TASSSSLLDHDRTVFEWLDQQAPSSVLYVSFGSTSEVDEKDFLEIARGLV
DSGQSFLWVVRPGFVKGSTWVEPLPDGFLGERGKIVKWVPQQEVLAHPAI

GAFWTHSGWNSTLESVCEGVPMIFSSFGGDQPLNARYMSDVLRVGVYLEN
GWERGEVVNAIRRVMVDEEGEYIRQNARVLKQKADVSLMKGGSSYESLES LVSYISSL
Example 2
Expression and Formulation of SuSy_at Variant of SEQ ID 1
[0246] The gene coding for the SuSy_At variant of SEQ ID 1 (EXAM PLE 1) was cloned into the expression vector pL E1A 17 (derivative of pRSF-1b, Novagen). The resulting plasmid was used for transformation of *E. coli* BL21(DE3) cells.
[0247] Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/I) at 37° C. Expression of the genes was induced at logarithmic phase by IPTG (0.2 mM) and carried out at 30° C. and 200 rpm for 16-18 hours.
[0248] Cells were harvested by centrifugation (3220×g, 20 min, 4° C.) and re-suspended to an optical density of 200 (measured at 600 nm (OD.sub.600)) with cell lysis buffer (100 mM Tris-HCI pH 7.0; 2 mM MgCl.sub.2, DNA nuclease 20 U/mL, lysozyme 0.5 mg/mL). Cells were then disrupted by sonication and crude extracts were separated from cell debris by centrifugation (18000×g 40 min, 4° C.). The supernatant was sterilized by filtration through a 0.2 µm filter and diluted 50:50 with distilled water, resulting in an enzymatic active preparation.
[0249] For enzymatic active preparations of SuSy_At, activity in Units is defined as follows: 1 mU of SuSy_At turns over 1 nmol of sucrose into fructose in 1 minute. Reaction conditions for the assay are 30° C., 50 mM potassium phosphate buffer pH 7.0, 400 mM sucrose at t.sub.0, 3 mM MgCl.sub.2, and 15 mM uridine diphosphate (UDP).
Example 3
Expression and Formulation of UGTSI2 Variant of SEQ ID 2
[0250] The gene coding for the UGTS12 variant of SEQ ID 2 (EXAM PLE 1) was cloned into the expression vector pLE1A17 (derivative of pRSF-1b, Novagen). The resulting plasmid was used for transformation of *E. coli* BL21(DE3) cells.
[0251] Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/I) at 37° C. Expression of the genes was induced at logarithmic phase by IPTG (0.1 mM) and carried out at 30° C. and 200 rpm for 16-18 hours.
[0252] Cells were harvested by centrifugation (3220×g, 20 min, 4° C.) and re-suspended to an optical density of 200 (measured at 600 nm (0 D.sub.600)) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM MgCl.sub.2, DNA nuclease 20 U/mL, lysozyme 0.5 mg/mL). Cells were then disrupted by sonication and crude extracts were separated from cell debris by centrifugation (18000×g 40 min, 4° C.). The supernatant was sterilized by filtration through a 0.2 µm filter and diluted 50:50 with 1 M sucrose solution, resulting in an enzymatic active preparation.
[0253] For enzymatic active preparations of UGTS12, activity in Units is defined as follows: 1 mU of UGTSI2 turns over 1 nmol of rebaudiosideA (RebA) into rebaudioside D (Reb D) in 1 minute. Reaction conditions for the assay are 30° C., 50 mM potassium phosphate buffer pH 7.0, 10 mM RebA at to, 500 mM sucrose, 3 mM MgCl.sub.2, 0.25 mM uridine diphosphate (UDP) and 3 U/mL of SuSy_At.
Example 4
Expression and Formulation of UGT 76G1 Variant of SEQ ID 3
[0254] The gene coding for the UGT76G1 variant of SEQ ID 3 (EXAMPLE 1) was cloned into the expression vector pLE1A17 (derivative of pRSF-1b, Novagen). The resulting plasmid was used for transformation of *E. coli* BL21(DE3) cells.
[0255] Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/I) at 37° C. Expression of the genes was induced at logarithmic phase by IPTG (0.1 mM) and carried out at 30° C. and 200 rpm for 16-18 hours.

[0256] Cells were harvested by centrifugation (3220×g, 20 min, 4° C.) and re-suspended to an optical density of 200 (measured at 600 nm (OD.sub.600)) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM MgCl.sub.2, DNA nuclease 20 U/mL, lysozyme 0.5 mg/mL). Cells were then disrupted by sonication and crude extracts were separated from cell debris by centrifugation (18000×g 40 min, 4° C.). The supernatant was sterilized by filtration through a 0.2 μm filter and diluted 50:50 with 1 M sucrose solution, resulting in an enzymatic active preparation.

[0257] For enzymatic active preparations of UGT76G1, activity in Units is defined as follows: 1 mU of UGT76G1 turns over 1 nmol of rebaudioside D (Reb D) into rebaudioside M (Reb M) in 1 minute. Reaction conditions for the assay are 30° C., 50 mM potassium phosphate buffer pH 7.0, 10 mM RebA at to, 500 mM sucrose, 3 mM MgCl.sub.2, 0.25 mM uridine diphosphate (UDP) and 3 U/mL of SuSy_At.

Example 5

Synthesis of Rebaudioside AM from Stevioside in a One-Pot Reaction, Adding UGTS12, SuSy_At and UGT76G1 at the Same Time

[0258] Rebaudioside AM (reb AM) was synthesized directly from stevioside in a one-pot reaction (FIG. **3**), utilizing the three enzymes (see EXAMPLES 1, 2, 3 and 4): UGTS12 (variant of SEQ ID 2), SuSy_At-(variant of SEQ ID 1) and UGT76G1 (variant of SEQ ID 3). The final reaction solution contained 105 U/L UGTS12, 405 U/L SuSy_At, 3 U/L UGT76G1, 5 mM stevioside, 0.25 mM uridine diphosphate (UDP), 1 M sucrose, 4 mM MgCl.sub.2 and potassium phosphate buffer (pH 6.6). First, 207 mL of distilled water were mixed with 0.24 g MgCl.sub.2.Math.6H 20, 103 g sucrose, 9.9 mL of 1.5 M potassium phosphate buffer (pH 6.6) and 15 g stevioside. After dissolving the components, the temperature was adjusted to 45° C. and UGTS12, SuSy_At, UGT76G1 and 39 mg UDP were added. The reaction mixture was incubated at 45° C. shaker for 24 hrs. Additional 39 mg UDP was added at 8 hrs and 18 hours. The content of reb AM, reb E, stevioside, reb M, reb B, steviolbioside and reb/at several time points was analyzed by HPLC.

[0259] For analysis, biotransformation samples were inactivated by adjusting the reaction mixture to pH 5.5 using 17% H.sub.3PO.sub.4 and then boiled for 10 minutes. Resulting samples were filtered, the filtrates were diluted 10 times and used as samples for HPLC analysis. HPLC assay was carried out on Agilent H P 1200 HPLC system, comprised of a pump, a column thermostat, an auto sampler, a UV detector capable of background correction and a data acquisition system. Analytes were separated using Agilent Poroshell 120 SB—C18, 4.6 mm×150 mm, 2.7 μm at 40° C. The mobile phase consisted of two premixes: [0260] premix 1 containing 75% 10 mM phosphate buffer (pH 2.6) and 25% acetonitrile, and [0261] premix 2 containing 68% 10 mM phosphate buffer (pH 2.6) and 32% acetonitrile.

[0262] Elution gradient started with premix 1, changed to premix 2 to 50% at 12.5 minute, changed to premix 2 to 100% at 13 minutes. Total run time was 45 minutes. The column temperature was maintained at 40° C. The injection volume was 5 μL. Rebaudioside species were detected by U V at 210 nm.

[0263] Table 3 shows for each time point the conversion of stevioside into identified rebaudioside species (area percentage). The chromatograms of stevioside and the reaction mixture at 24 hours are shown in FIG. **5** and FIG. **6**, respectively. Those with skill in the art will appreciate that retention times can occasionally vary with changes in solvent and/or equipment.

TABLE-US-00005 TABLE 3 Biotransformation of stevioside to reb AM Time, % conversion from stevioside hrs Reb E Reb AM Reb M Reb I Stevioside Reb B Steviolbioside 0 0 0 0 0 100 0 0 6 1.9 35.9 1.3 1.7 58.7 0.0 0.4 18 0.9 96.7 1.3 0.6 0.0 0.0 0.4 24 0.3 96.4 2.1 0.7 0.0 0.2 0.4

Example 6

Synthesis of Rebaudioside AM from Rebaudioside E in a One-Pot Reaction, SuSy_at and UGT76G1 at the Same Time

[0264] Rebaudioside AM (reb AM) was synthesized directly from rebaudioside E (reb E) in a one-pot reaction (FIG. **4**), utilizing the two enzymes (see EXAMPLES 1, 2 and 4): SuSy_At-(variant of

SEQ ID 1) and UGT76G1 (variant of SEQ ID 3). The final reaction solution contained 405 U/L SuSy_At, 3 U/L UGT76G1, 5 mM reb E, 0.25 mM uridine diphosphate (UDP), 1 M sucrose, 4 mM MgCl.sub.2.6H.sub.2O and potassium phosphate buffer (pH 6.6). First, 37 mL of distilled water were mixed with 40.3 mg MgCl.sub.2, 17.12 g sucrose, 1.65 mL of 1.5 M potassium phosphate buffer (pH 6.6) and 5.04 g reb E. After dissolving the components, the temperature was adjusted to 45° C. and SuSy_At, UGT76G1 and 6.5 mg UDP were added. The reaction mixture was incubated at 45° C. shaker for 24 hrs. Additional 6.5 mg UDP was added at 8 hrs and 18 hours. The content of reb AM, reb E, stevioside, reb A, reb M, reb B, and steviolbioside at several time points was analyzed by HPLC.

[0265] For analysis, biotransformation samples were inactivated by adjusting the reaction mixture to pH 5.5 using 17% H.sub.3PO.sub.4 and then boiled for 10 minutes. Resulting samples were filtered, the filtrates were diluted 10 times and used as samples for HPLC analysis. HPLC assay was carried out on Agilent H P 1200 HPLC system, comprised of a pump, a column thermostat, an auto sampler, a UV detector capable of background correction and a data acquisition system. Analytes were separated using Agilent Poroshell 120 SB—C18, 4.6 mm×150 mm, 2.7 μm at 40° C. The mobile phase consisted of two premixes: [0266] premix 1 containing 75% 10 mM phosphate buffer (pH 2.6) and 25% acetonitrile, and [0267] premix 2 containing 68% 10 mM phosphate buffer (pH 2.6) and 32% acetonitrile.

[0268] Elution gradient started with premix 1, changed to premix 2 to 50% at 12.5 minute, changed to premix 2 to 100% at 13 minutes. Total run time was 45 minutes. The column temperature was maintained at 40° C. The injection volume was 5 μL. Rebaudioside species were detected by U V at 210 nm.

[0269] Table 4 shows for each time point the conversion of reb E into identified rebaudioside species (area percentage). The chromatograms of reb E and the reaction mixture at 24 hours are shown in FIG. **7** and FIG. **8**, respectively. Those with skill in the art will appreciate that retention times can occasionally vary with changes in solvent and/or equipment.

TABLE-US-00006 TABLE 4 Biotransformation of reb E to reb AM % conversion from Reb E Time, Reb Reb Reb Reb Reb Steviol- hrs E AM M A Stevioside B bioside 0 99.46 0 0 0.54 0 0 0 4 40.75 57.92 0 0.59 0 0.73 0 7 24.79 73.92 0 0.58 0 0.71 0 24 4.38 94.33 0 0.59 0 0.70 0

Example 7

Purification of Rebaudioside AM

[0270] The reaction mixture of EXAM PLE 5, after 24 hrs, was inactivated by adjusting the pH to pH 5.5 with H .sub.3PO.sub.4 and then boiled for 10 minutes. After boiling the reaction mixture was filtered and diluted with RO water to 5% solids content. The diluted solution was passed through 1 L column packed with YWD03 macroporous adsorption resin (Cangzhou Y uanwei, China). Adsorbed steviol glycosides were eluted with 5 L 70% ethanol. The obtained eluate was evaporated until dryness to yield 16 g of dry powder which was dissolved in 80 mL of 70% methanol. The solution was crystallized at 20° C. for 3 days. The crystals were separated by filtration and dried in vacuum oven at 80° C. for 18 hours to yield 10.4 g of pure reb AM crystals with 95.92% purity, determined by HPLC assay. The chromatogram of reb AM is shown in FIG. **9**. Those with skill in the art will appreciate that retention times can occasionally vary with changes in solvent and/or equipment.

Example 8

Structure Elucidation of Rebaudioside AM

[0271] NMR experiments were performed on a Bruker 500 M Hz spectrometer, with the sample dissolved in pyridine-d5. A long with signals from the sample, signals from pyridine-d5 at δ.sub.C 123.5, 135.5, 149.9 ppm and δ.sub.H 7.19, 7.55, 8.71 ppm were observed.

[0272] .sup.1H-NM R-spectrum of rebaudioside AM in pyridine-d.sub.5 reveal the excellent quality of the sample (see FIG. **10**). The H SQC (see FIG. **11**) shows the presence of an exo-methylene group in the sugar region with a long-range coupling to C-15, observable in the H,H-

COSY (FIG. **12**). Other deep-fielded signals of the quaternary carbons (C-13, C-16 and C-19) are detected by the HMBC (FIG. **13**). Correlation of the signals in the HSQC, HMBC and H,H-COSY reveal the presence of steviol glycoside with the following aglycone structure:
##STR00001##
[0273] Correlation of HSQC and HMBC signals reveal five anomeric signals. The coupling constant of the anomeric protons of about 8 Hz and the broad signals of their sugar linkage allows the identification of these five sugars as β-D-glucopyranosides.
[0274] The observation of the anomeric protons in combination with HSQC and HMBC reveal the sugar linkage and the correlation to the aglycone. The assignment of the sugar sequence was confirmed by using the combination of HSQC-TOCSY (FIG. **14**) and HSQC.
[0275] The NMR experiments above were applied to assign the chemical shifts of the protons and carbons, main coupling constants and main HMBC correlations (see Table 5).
TABLE-US-00007 TABLE 5 Chemical shifts of rebaudioside AM Position δ.sub.C [ppm] δ.sub.H [ppm] J [Hz] HMBC (H .fwdarw. C) Aglycone moiety 1 39.9 t 0.68 m 1.64 m 2 19.4 t 1.39 m 2.08 m 3 37.4 t 1.05 m 2.80 m 4 44.2 s — 5 57.3 d 0.95 m 6 21.7 t 1.90 m 2.12 m 7 41.0 t 1.26 m 1.38 m 8 41.9 s — 9 53.3 d 0.85 m 10 39.2 s — 11 20.1 t 1.59 m 1.61 m 12 36.9 t 1.65 m 1.92 m 13 85.9 s — 14 43.8 t 1.78 d 11.0 2.52 d 11.0 15 47.4 t 2.00 d 16.0 7, 8, 9, 14 2.06 d 16.0 16 154.6 s — 17 104.3 t 5.03 br s 13, 15, 16 5.71 br s 18 28.5 q 1.40 s 3, 4, 5, 19 19 175.2 s — 20 16.2 q 1.06 s 1, 5, 9, 10 Sugar moiety Sugar I: β-D-Glucopyranoside .sup. 1.sup.i 97.5 d 5.13 d 7.7 13 .sup. 2.sup.i 84.0 d 4.14 m .sup. 3.sup.i 77.6 d 4.20 m .sup. 4.sup.i 71.3 d 4.19 m .sup. 5.sup.i 77.6 d 3.70 m .sup. 6.sup.i 62.0 t 4.23 m 4.32 m Sugar II: β-D-Glucopyranoside .sup. 1.sup.ii 106.3 d 5.26 d 8.0 .sup. 2.sup.i .sup. 2.sup.ii 76.8 d 4.13 m .sup. 3.sup.ii 77.3 d 4.21 m .sup. 4.sup.ii 71.6 d 4.18 m .sup. 5.sup.ii 77.9 d 3.91 m .sup. 6.sup.ii 62.4 t 4.29 m 4.41 m Sugar III: β-D-Glucopyranoside 1.sup.iii 92.9 d 6.20 d 8.1 19 2.sup.iii 77.0 d 4.46 m 3.sup.iii 88.1 d 4.24 m 4.sup.iii 69.0 d 4.12 m 5.sup.iii 78.4 d 3.82 m 6.sup.iii 61.3 t 4.20 m 4.33 m Sugar IV: β-D-Glucopyranoside 1.sup.iv 103.4 d 5.73 d 7.7 2.sup.iii 2.sup.iv 75.4 d 3.98 m 3.sup.iv 78.1 d 4.09 m 4.sup.iv 72.6 d 4.08 m 5.sup.iv 77.4 d 3.92 m 6.sup.iv 62.9 t 4.32 m 4.51 m Sugar V: β-D-Glucopyranoside .sup. 1.sup.v 104.4 d 5.29 d 8.1 3.sup.iii .sup. 2.sup.v 75.1 d 4.00 m .sup. 3.sup.v 78.2 d 4.24 m .sup. 4.sup.v 71.4 d 4.27 m .sup. 5.sup.v 78.2 d 3.99 m .sup. 6.sup.v 61.9 t 4.27 m 4.48 m
[0276] Correlation of all NMR data indicates rebaudioside AM having five β-D-glucopyranoses attached to a steviol aglycone, as depicted with the following chemical structure:
##STR00002##
[0277] The chemical formula of rebaudioside AM is C.sub.50H.sub.83OO.sub.28, which corresponds to a calculated monoisotopic molecular mass of 1128.5. For LCMS analysis, rebaudioside AM was dissolved in methanol and analyzed using Shimadzu Nexera 2020 UFLC LCMS instrument on a Cortecs UPLC C18 1.6 μm, 50×2.1 mm column. The observed LCMS (negative ESI mode) result of 1127.3 (see FIG. **15***a* and FIG. **15***b* respectively) is consistent with rebaudioside AM and corresponds to the ion (M−H).sup.−.

Claims

1. (canceled)
2. A method for producing rebaudioside AM, comprising the steps of: a. providing a starting composition comprising an organic compound with at least one carbon atom wherein the starting composition is selected from the group consisting of steviol, steviolmonoside, steviolmonoside A, steviolbioside, steviolbioside A, steviolbioside B, rubusoside, stevioside, stevioside A (rebaudioside KA), stevioside B, stevioside C, rebaudioside E, rebaudioside E2, rebaudioside E3, other steviol glycosides, and combinations thereof; b. providing a biocatalyst selected from the group consisting of an enzyme preparation, a cell or a microorganism; said biocatalyst comprising at least one

enzyme capable of converting the starting composition to rebaudioside AM; c. contacting the biocatalyst with a medium containing the starting composition to produce a medium comprising rebaudioside AM.

3. The method of claim 2 further comprising the step of: d. separating the rebaudioside AM from the medium to provide a highly purified rebaudioside AM composition.

4. (canceled)

5. The method of claim 2, wherein the microorganism is selected from the group consisting of *E. coli*, *Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., and *Yarrowia* sp.

6. The method of claim 2, wherein the enzyme is selected from the group consisting of: a steviol biosynthesis enzyme, a UDP glucosyltransferase, a UDP glucose recycling enzyme, a mevalonate (MVA) pathway enzyme, a 2-C-methyl-D-erythritol-4-phosphate pathway (MEP/DOXP) enzyme, geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase, UGT74G1, UGT85C2, UGT91D2, EUGT11, UGTS12, UGT76G1, or mutant variant thereof having >85% amino-acid sequence identity, >86% amino-acid sequence identity, >87% amino-acid sequence identity, >88% amino-acid sequence identity, >89% amino-acid sequence identity, >90% amino-acid sequence identity, >91% amino-acid sequence identity, >92% amino-acid sequence identity, >93% amino-acid sequence identity, >94% amino-acid sequence identity, >95% amino-acid sequence identity, >96% amino-acid sequence identity, >97% amino-acid sequence identity, >98% amino-acid sequence identity, >99% amino-acid sequence identity; and combinations thereof.

7. The method of claim 3, wherein the rebaudioside AM content in highly purified rebaudioside AM composition is greater than about 95% by weight on a dry basis.

8-**14**. (canceled)

15. The method of claim 3, wherein the rebaudioside AM content in highly purified rebaudioside AM composition is greater than about 80% by weight on a dry basis.