HIGH-PURITY STEVIOL GLYCOSIDES

TECHNICAL FIELD

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

SEQUENCE LISTING

The text file entitled "PC_71PROV_Seq_Listing_ST25.txt," created on May 15, 2017, having 15 kilobytes of data, and filed concurrently herewith, is hereby incorporated by reference in its entirety in this application.

BACKGROUND OF THE INVENTION

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.

Steviol glycosides are a 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.

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

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

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.

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.

The target steviol glycoside can be any steviol glycoside. In one embodiment, the target steviol glycoside is steviolmonoside, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N*, rebaudioside *O* or a synthetic steviol glycoside.

In one embodiment, the target steviol glycoside is rebaudioside *A*.

In another embodiment, the target steviol glycoside is rebaudioside *E*.

In still another embodiment, the target steviol glycoside is rebaudioside *D*.

In another embodiment, the target steviol glycoside is rebaudioside *I*.

In yet another embodiment, the target steviol glycoside is rebaudioside *M*.

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 free form or immobilized to a solid support made from inorganic or organic materials.

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.

The enzymes necessary for converting the starting composition to target steviol glycosides include the steviol biosynthesis enzymes, UDP-glycosyltransferases (UGTs) and/or UDP-recycling enzyme.

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

In another embodiment, the steviol biosynthesis enzymes include non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway (MEP/DOXP) enzymes.

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), l-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), l-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 etc.

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

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.

In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2, UGT76G1, UGT91D2 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.

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.

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside. In a particular embodiment, the UDP-glucosyltransferase is UGT91D2 or a UGT having >85% amino-acid sequence identity with UGT91D2.

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside *A*. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1 (SEQ ID 3).

In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *A* to form rebaudioside *D*. In a particular embodiment, the UDP-glucosyltransferase is UGT91D2 or a UGT having >85% amino-acid sequence identity with UGT91D2. In yet another embodiment the UDP-glucosyltransferase is UGTSL2 or a UGT having >85% amino-acid sequence identity with UGTSL2 (SEQ ID 2).

In yet another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *D* to form rebaudioside *M*. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1 (SEQ ID 3).

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

the steviol glycoside substrate to the target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose.

In one embodiment, the recycling catalyst is sucrose synthase. In another embodiment the sucrose synthase is SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At (SEQ ID 1).

In one embodiment, the recycling substrate is sucrose.

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.

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.

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.

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

Purified target steviol glycosides can be used in consumable products as a sweetener. Suitable consumer products include, but are not limited to, food, beverages, pharmaceutical compositions, tobacco products, nutraceutical compositions, oral hygiene compositions, and cosmetic compositions.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram showing one embodiment of the manufacturing process for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A.

DETAILED DESCRIPTION

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.

One object of the invention is to provide an efficient biocatalytic method for preparing steviol glycosides, particularly stevioside, reb *E*, reb *A*, reb *D*, and reb *M* from various starting compositions. One particular object of the invention is to provide a manufacturing process for producing a blend of steviol glycosides having greater than about 30% reb M, hereinafter referred to as "steviol glycosides with a high reb M content".

As used herein, "biocatalysis" or "biocatalytic" refers to the use of natural or genetically engineered biocatalysts, such as enzymes, or cells comprising one or more enzyme, capable of 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.

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, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N*, rebaudioside *O*, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

Starting Composition

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.

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

The starting composition steviol glycoside is selected from the group consisting of steviolmonoside, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N* or rebaudioside *O*, or other glycoside of steviol occurring in *Stevia rebaudiana* plant and/or combinations thereof.

In one embodiment, the starting composition steviol glycoside is stevioside.

In another embodiment, the starting composition steviol glycoside is rebaudioside *A*. In a particular embodiment, rebaudioside A is extracted from the leaves of *Stevia rebaudiana* plants, such as *Stevia rebaudiana* Bertoni plants, and purified to greater than 95% rebaudioside A.

In still another embodiment, the starting composition steviol glycoside is rebaudioside *E*.

In another embodiment, the starting composition steviol glycoside is rebaudioside *I*.

In yet another embodiment, the starting composition steviol glycoside is rebaudioside *D*.

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.

The term "carbohydrate" refers to aldehyde or ketone compounds substituted with multiple hydroxyl groups, of the general formula $(CH_2O)_n$, 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.

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, gluconolactone, 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.

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

In one embodiment, the starting composition is glycerol.

In another embodiment, the starting composition is glucose.

In still another embodiment, the starting composition is sucrose.

In yet another embodiment, the starting composition is starch.

In another embodiment, the starting composition is maltodextrin.

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

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, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*,

rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N* or rebaudioside *O*, or other glycoside of steviol.

In one embodiment, the target steviol glycoside is stevioside. In another embodiment, the target steviol glycoside is rebaudioside *A* (reb *A*). In still another embodiment, the target steviol glycoside is rebaudioside *E* (reb *E*). In yet another embodiment, the target steviol glycoside is rebaudioside *I* (reb *I*). In yet another embodiment, the target steviol glycoside is rebaudioside *D* (reb *D*). In a further embodiment, the target steviol glycoside is rebaudioside *M* (reb *M*).

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

In one embodiment, the present invention is a biocatalytic process for the production of reb *D*.

In yet another embodiment, the present invention is a biocatalytic process for the production of reb *E*.

In still another embodiment, the present invention is a biocatalytic process for the production of reb *I*.

In a further embodiment, the present invention is a biocatalytic process for the production of reb *M*.

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.

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.

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

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

In yet another embodiment, when the target steviol glycoside is reb *D*, the process described herein provides a composition greater than about 90% reb *D* content by weight on a dried basis. In another particular embodiment, when the target steviol glycoside is reb *D*, the process described herein provides a composition comprising greater than about 95% reb *D* content by weight on a dried basis.

In still another embodiment, when the target steviol glycoside is reb *E*, the process described herein provides a composition greater than about 90% reb *E* content by weight on a dried basis. In another particular embodiment, when the target steviol glycoside is reb *E*, the process described herein provides a composition comprising greater than about 95% reb *E* content by weight on a dried basis.

In a further embodiment, when the target steviol glycoside is reb *A*, the process described herein provides a composition comprising greater than about 90% reb *A* content by weight on a dried basis. In another particular embodiment, when the target steviol glycoside is reb *A*, the process described herein provides a composition comprising greater than about 95% reb *A* content by weight on a dried basis.

In yet a further embodiment, when the target steviol glycoside is stevioside, the process described herein provides a composition comprising greater than about 90% stevioside content by weight on a dried basis. In another particular embodiment, when the target steviol glycoside is stevioside, the process described herein provides a composition comprising greater than about 95% stevioside content by weight on a dried basis.

Microorganisms and enzyme preparations

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.

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 capable of 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.

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.

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

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

In another embodiment, the steviol biosynthesis enzymes include non-mevalonate 2-C-methyl-D-erythritol-4-phosphate pathway (MEP/DOXP) enzymes.

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), l-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), l-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 etc.

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

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. For example, in one embodiment, the enzymes are produced by microbial fermentation of the *E. coli* production strain LE1B109 carrying the expression vector for the corresponding enzyme gene.

In another embodiment, the UDP-glucosyltransferases are synthesized.

In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2, UGT76G1, UGT91D2 and UGTs having substantial (>85%) amino-acid sequence identity to these polypeptides as well as isolated nucleic acid molecules that code for these UGTs.

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.

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside. In a particular

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

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside *A*. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1 (SEQ ID 3).

In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *A* to form rebaudioside *D*. In a particular embodiment, the UDP-glucosyltransferase is UGT91D2 or a UGT having >85% amino-acid sequence identity with UGT91D2. In yet another embodiment the UDP-glucosyltransferase is UGTSL or a UGT having >85% amino-acid sequence identity with UGTSL. In another embodiment, the UDP-glucosyltransferase is EUGT11 or a UGT having >85% amino-acid sequence identity with EUGT11. In yet another embodiment the UDP-glucosyltransferase is UGTSL2 or a UGT having >85% amino-acid sequence identity with UGTSL2 (SEQ ID 2).

In yet another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *D* to form rebaudioside *M*. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1 or a UGT having >85% amino-acid sequence identity with UGT76G1 (SEQ ID 3).

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 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 and the recycling substrate can be sucrose. In one embodiment the sucrose synthase is SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At (SEQ ID 1).

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 more preferably the group presented in Table 2.

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 1

GI number	Accession	Origin
190692175	ACE87855.1	*Stevia rebaudiana*
41469452	AAS07253.1	*Oryza sativa*
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 sativa Japonica Group*
115471071	NP_001059134.1	*Oryza sativa Japonica Group*
116310985	CAH67920.1	*Oryza sativa Indica Group*
116788066	ABK24743.1	*Picea sitchensis*
122209731	Q2V6J9.1	*Fragaria x 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 x 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 sativa 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 2

GI number	Accession	Origin	Internal reference
460409128	XP.004249992.1	*Solanum lycopersicum*	UGTSL
460386018	XP.004238697.1	*Solanum lycopersicum*	-
460409134	XP.004249995.1	*Solanum lycopersicum*	-
460410132	XP.004250485.1	*Solanum lycopersicum*	UGTSL2
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*	-

One embodiment is a microbial cell comprising an enzyme of the present invention, 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.

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.

Suitable microorganisms include, but are not limited to, *E.coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp. etc.

In one embodiment, the microorganism is free when contacted with the starting composition.

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.

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.

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.

Highly purified target glycoside(s) particularly, reb *M*, reb *D*, reb *I* and/or reb *E* obtained according to this invention can be used "as-is" or in combination with other sweeteners, flavors, food ingredients and combinations thereof.

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.

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.

Highly purified target glycoside(s) particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* as a sweetening compound may be employed as the sole sweetener, or it may be used together with at least one naturally occurring high intensity sweeteners such as stevioside, reb *A*, reb *B*, reb *C*, reb *F*, reb *N*, reb *O*, steviolbioside, dulcoside *A*, rubusoside, 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.

In a particular embodiment, reb *M*, reb *D*, reb *I* and/or reb *E* can be used in a sweetener composition comprising a compound selected from the group consisting of reb *A*, reb *B*, reb *O*, NSF-02, Mogroside V, Luo Han Guo, allulose, allose, D-tagatose, erythritol and combinations thereof.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Moreover, highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* can be used in combination with natural sweetener suppressors such as gymnemic acid, hodulcin, ziziphin, lactisole, and others. reb *M*, reb *D*, reb *I* and/or reb *E* may also be combined with various umami taste enhancers. reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* may be combined with reduced calorie sweeteners such as, for example, D-tagatose, L-sugars, L-sorbose, L-arabinose and combinations thereof.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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 $(CH_2O)_n$, 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.

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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

Examples of consumable products in which highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E* 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.

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

Moreover, the highly purified target steviol glycoside(s), reb *M*, reb *D*, reb *I* and/or reb *E* obtained in this invention may be used in dry or liquid forms.

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, reb *M*, reb *D*, reb *I* and/or reb *E* depends on the purpose of usage. As discussed above, it can be added alone or in combination with other compounds.

The present invention is also directed to sweetness enhancement in beverages using reb *M*, reb *D*, reb *I* and/or reb *E*. Accordingly, the present invention provides a beverage comprising a sweetener and reb *M*, reb *D*, reb *I* and/or reb *E* as a sweetness enhancer, wherein reb *M*, reb *D*, reb *I* and/or reb *E* is present in a concentration at or below their respective sweetness recognition thresholds.

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."

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.

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.

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.

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.

In another embodiment, the sweetener is erythritol.

In still another embodiment, the sweetener is a rare sugar. Suitable rare sugars include, but are not limited to, D-allose, D-psicose, L-ribose, D-tagatose, L-glucose, L-fucose, L-arbinose, D-turanose, D-leucrose and combinations thereof.

It is contemplated that a sweetener can be used alone, or in combination with other sweeteners.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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.

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 reb *M*, reb *D*, reb *I* and/or reb *E* or a combination thereof, wherein reb *M*, reb *D*, reb *I* and/or reb *E* are present in a concentration at or below their sweetness recognition thresholds.

Addition of reb *M*, reb *D*, reb *I* and/or reb *E* 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%.

The following examples illustrate preferred embodiments of the invention for the preparation of highly purified target steviol glycoside(s), particularly, reb *M*, reb *D*, reb *I* and/or reb *E*. 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.

EXAMPLE 1

Protein sequences of engineered enzymes used in the biocatalytic process

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:

>UGTSl-0234 (engineered glycosyltransferase; UGTSL2; source of WT gene: *Solanum lycopersicum*)

MATNLRVLMFPWLAYGHISPFLNIAKQLADRGFLIYLCSTRINLESIIKK
IPEKYADSIHLIELQLPELPELPPHYHTTNGLPPHLNPTLHKALKMSKPN
FSRILQNLKPDLLIYDVLQPWAEHVANEQGIPAGKLLVSCAAVFSYFFSF
RKNPGVEFPFPAIHLPEVEKVKIREILAKEPEEGGRLDEGNKQMMLMCTS
RTIEAKYIDYCTELCNWKVVPVGPPFQDLITNDADNKELIDWLGTKPENS
TVFVSFGSEYFLSKEDMEEIAFALEASNVNFIWVVRFPKGEERNLEDALP
EGFLERIGERGRVLDKFAPQPRILNHPSTGGFISHCGWNSVMESIDFGVP
IIAMPIHNDQPINAKLMVELGVAVEIVRDDDGKIHRGEIAEALKSVVTGE
TGEILRAKVREISKNLKSIRDEEMDAVAEELIQLCRNSNKSK

SEQ ID 3:

>UGTSr-0042 (engineered glycosyltransferase; UGT76G1; source of WT gene: *Stevia rebaudiana*)

MENKTETTVRRRRRIILFPVPFQGHINPILQLANVLYSKGFAITILHTNFNKPKTSNYPH
FTFRFILDNDPQDERISNLPTHGPLAGMRIPIINEHGADELRRELELLMLASEEDEEVSC
LITDALWYFAQDVADSLNLRRLVLMTSSLFNFHAHVSLPQFDELGYLDPDDKTRLEEQAS
GFPMLKVKDIKSAYSNWQIGKEILGKMIKQTKASSGVIWNSFKELEESELETVIREIPAP
SFLIPLPKHLTASSSSLLDHDRTVFEWLDQQAPSSVLYVSFGSTSEVDEKDFLEIARGLV
DSGQSFLWVVRPGFVKGSTWVEPLPDGFLGERGKIVKWVPQQEVLAHPAIGAFWTHSGWN
STLESVCEGVPMIFSSFGGDQPLNARYMSDVLRVGVYLENGWERGEVVNAIRRVMVDEEG
EYIRQNARVLKQKADVSLMKGGSSYESLESLVSYISSL

EXAMPLE 2

Expression and formulation of SuSy_At variant of SEQ ID 1

The gene coding for the SuSy_At variant of SEQ ID 1 (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.

Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/l) 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.

Cells were harvested by centrifugation (3220 x g, 20 min, 4°C) and re-suspended to an optical density of 200 (measured at 600nm (OD_{600})) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM $MgCl_2$, 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 x 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.

For enzymatic active preparations of SuSy_At, activity in Units is defined as follows: 1 mU of SuSy 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_0, 3 mM $MgCl_2$, and 15 mM uridin diphosphate (UDP).

EXAMPLE 3

Expression and formulation of UGTSl variant of SEQ ID 2

The gene coding for the UGTSl variant of SEQ ID 2 (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.

Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/l) 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.

Cells were harvested by centrifugation (3220 x g, 20 min, 4°C) and re-suspended to an optical density of 200 (measured at 600nm (OD_{600})) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM $MgCl_2$, 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 x 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.

For enzymatic active preparations of UGTSl, activity in Units is defined as follows: 1 mU of UGTSl turns over 1 nmol of rebaudioside A (RebA) into rebaudioside D (RebD) in 1 minute. Reaction conditions for the assay are 30°C, 50 mM potassium phosphate buffer pH 7.0, 10 mM RebA at t_0, 500 mM sucrose, 3 mM $MgCl_2$, 0.25 mM uridin diphosphate (UDP) and 3 U/mL of SuSy_At.

EXAMPLE 4

Expression and formulation of UGTSr variant of SEQ ID 3

The gene coding for the UGTSr 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.

Cells were cultivated in ZYM505 medium (F. William Studier, Protein Expression and Purification 41 (2005) 207-234) supplemented with kanamycin (50 mg/l) 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.

Cells were harvested by centrifugation (3220 x g, 20 min, 4°C) and re-suspended to an optical density of 200 (measured at 600nm (OD_{600})) with cell lysis buffer (100 mM Tris-HCl pH 7.0; 2 mM $MgCl_2$, 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 x 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.

For enzymatic active preparations of UGTSr, activity in Units is defined as follows: 1 mU of UGTSr turns over 1 nmol of rebaudioside A (RebA) into rebaudioside I (RebI) in 1 minute. Reaction conditions for the assay are 30°C, 50 mM potassium phosphate buffer pH 7.0, 10 mM RebA at t_0, 500 mM sucrose, 3 mM $MgCl_2$, 0.25 mM uridin diphosphate (UDP) and 3 U/mL of SuSy_At.

EXAMPLE 5

Synthesis of rebaudioside M in a one-pot reaction, adding UGTSl, SuSy_At and UGTSr at the same time

Rebaudioside M (RebM) was synthesized directly from rebaudioside A (RebA) in a one-pot reaction, utilizing the three enzymes (see EXAMPLES 1, 2, 3 and 4): UGTSl (variant of SEQ ID 2), SuSy_At-(variant of SEQ ID 1) and UGTSr (variant of SEQ ID 3). The final reaction solution contained 20 mU/mL UGTSl, 160 mU/mL SuSy_At, 10 mU/mL UGTSr, 25 mM rebaudioside A, 0.5 mM uridin diphosphate (UDP), 1 M sucrose, 4 mM $MgCl_2$ and 50 mM potassium phosphate buffer (buffer stock prepared at pH 7.5), prepared in distilled water to a total volume of 1.6 mL. First, 186.6 µL of distilled water were mixed with 6.4 µL of 1M $MgCl_2$, 800 µL of 2 M sucrose, 16.1 µL of 50 mM UDP, 80 µL of 1 M potassium phosphate buffer (pH 7.5) and 400 µL of 100 mM rebaudioside A. To start the biotransformation, 26.4 µL of 1200 mU/mL UGTSl, 10.4 µL of 24600 mU/mL SuSy_At and 74.1 µL of 220 mU/mL UGTSr were added. The reaction was incubated at 30°C, shaking for 70 h. The content of RebM, RebA, as well as the content of rebaudiosides D (RebD) and rebaudiosides I and M2 (RebI/M2) at several time points was determined by HPLC.

For analysis, biotransformation samples were inactivated by mixing 100 µL of reaction solution with 10 µL 1M H_2SO_4, and adding 90 µL of 60% MeOH (in H_2O). Resulting samples were diluted a further 10-fold in 30% MeOH (in H_2O), centrifuged at 18 x g for 10 min at 4°C, and supernatants were used as samples for HPLC injection. HPLC was carried out on a Shimadzu 20A series unit equipped with two pump units, an auto sampler, and a thermostat column compartment. Mobile phases A (10 mM NaH_2PO_4,

pH 2.6) and B (Acetonitrile, HPLC grade) were mixed on-line in different ratios at different times. Separation started with 26% B, changed to 29% B at 7 min and returned to 26% at 12.5 min run time. Total run time were 17 min. The flow rate was 0.75 mL/min. The column used was a Phenomenex Kinetex 2.6 µm C18 100 A, 150x4.6mm. The column temperature was maintained at 40 °C. The injection volume was 5 µl Rebaudioside species were detected by UV at 210 nm.

Table 3 shows for each time point the conversion of rebA into identified rebaudioside species (percentages calculated from molarities).

Table 3

Biotransformation of RebA to RebM, (addition of UGTSl, SuSy_At and UGTSr at reaction start)

	% conversion from RebA					
time / h	RebA	RebD	RebM	RebM2	RebI	unknown
0	100.0	0.0	0.0	0.00	0.0	0.0
6	69.0	6.1	12.5	0.00	9.0	3.4
22	33.5	4.2	39.4	0.02	18.8	4.1
32	21.1	3.2	49.5	0.05	21.6	4.5
47	8.3	1.8	62.3	0.06	24.7	2.9
71	1.5	0.5	66.9	0.16	25.3	5.6

EXAMPLE 6

Synthesis of rebaudioside M in a one-pot reaction, adding UGTSl, SuSy_At at reaction start, but UGTSr only after 22 h

Rebaudioside M (RebM) was synthesized directly from rebaudioside A (RebA) in a one-pot reaction, utilizing the three enzymes (see EXAMPLES 1, 2, 3 and 4); UGTSl (variant of SEQ ID 2), SuSy_At (variant of SEQ ID 1) and UGTSr (variant of SEQ ID 3). The final reaction solution contained 20 mU/mL UGTSl, 160 mU/mL SuSy_At, 10 mU/mL UGTSr, 25 mM rebaudioside A, 0.5 mM uridin diphosphate (UDP), 1 M sucrose, 4 mM $MgCl_2$ and 50 mM potassium phosphate buffer (buffer stock prepared at pH 7.5), prepared in distilled water to a total volume of 1.6 mL. First, 186.6 µL of distilled water were mixed with 6.4 µL of 1M $MgCl_2$, 800 µL of 2 M sucrose, 16.1 µL of 50 mM UDP, 80 µL of 1 M potassium phosphate buffer (pH 7.5) and 400 µL of 100 mM rebaudioside A. To start the biotransformation, 26.4 µL of 1200 mU/mL UGTSl and 10.4 µL of 24600 mU/mL SuSy_At were added. The reaction was incubated at 30°C, shaking for 22 h. Then, 74.1 µL of 220 mU/mL UGTSr was added to the reaction, and the reaction was

incubated at 30°C, shaking for another 49 h. The content of RebM, RebA, as well as the content of rebaudiosides D (RebD) and rebaudioside I and M2 (RebI/M2) at several time points was determined by HPLC.

For analysis, biotransformation samples were inactivated by mixing 100 µL of reaction solution with 10 µL 1M H_2SO_4, and adding 90 µL of 60% MeOH (in H_2O). Resulting samples were diluted a further 10-fold in 30% MeOH (in H_2O), centrifuged at 18 x g for 10 min at 4°C, and supernatants were used as samples for HPLC injection. HPLC was carried out on a Shimadzu 20A series unit equipped with two pump units, an auto sampler, and a thermostat column compartment. Mobile phases A (10 mM NaH_2PO_4, pH 2.6) and B (Acetonitrile, HPLC grade) were mixed on-line in different ratios at different times. Separation started with 26% B, changed to 29% B at 7 min and returned to 26% at 12.5 min run time. Total run time were 17 min. The flow rate was 0.75 mL/min. The column used was a Phenomenex Kinetex 2.6 µm C18 100 A, 150x4.6mm. The column temperature was maintained at 40 °C. The injection volume was 5 µl Rebaudioside species were detected by UV at 210 nm.

Table 4 shows for each time point the conversion of RebA into identified rebaudioside species (percentages calculated from molarities).

Table 4

Biotransformation of RebA to RebM, (addition of UGTSl and SuSy_At at reaction start, addition of UGTSr after 22 h)

	% conversion from RebA					
time / h	RebA	RebD	RebM	RebM2	RebI	unknown
0	100.0	0.0	0.0	0.0	0.0	0.0
6	71.7	28.4	0.0	0.03	0,0	0.1
22	25.2	65.9	0.0	0.12	0.0	8.8
32	15.8	55.9	22.6	0.20	1.3	4.3
47	9.3	32.1	54.9	0.28	2.0	1.5
71	0.9	2.2	90.6	0.36	2.6	3.4

EXAMPLE 7

Construction of the Enzyme Production Microorganisms

The production strain LE1B109 is a genetically modified derivative strain of the laboratory strain *E. coli* K-12 W3110. The parental strain *E. coli* K-12 W3110 has been modified by site-directed recombination at different chromosomal loci to suit production purposes in terms of genetic stability, especially plasmid stability, and efficiency of expression and biotransformation. The expression of a number of proteases has been eliminated by deletion of the corresponding genes. Antibiotic-free selection of target clones has been enabled through deletion of one gene. One further gene has been deleted to prevent unwanted recombination effects. The gene coding for the T7 RNA polymerase from *E. coli* T7 phage and another gene copy of lacI, a repressor naturally present in *E. coli* K-12 W3110, have been inserted into the genome of W3110 to achieve a strong and regulated enzyme expression. Furthermore, the strain might carry certain deletions of endogenous enzyme genes connected to the degradation of biotransformation reactants in order to avoid side reactions. Insertions and deletions of chromosomal DNA are in general performed by integration of plasmid-based fragments carrying antibiotic resistance genes. After selection of the correct chromosomal mutants, resistance genes are excised and all plasmids are removed. No residual vector sequences or antibiotic resistance genes are left in the final cell.

The final production strain used for manufacturing each enzyme is created from the LE1B109 recipient strain by introducing an expression vector carrying the specific gene for one of the enzymes listed in Table 5. The plasmids used to transform the *E. coli* recipient strain are based on the well-known vector pRSF-1b (Merck KGaA, Darmstadt, Germany). The plasmids have been fully sequenced and do not carry antibiotic resistance genes or any other sequences of concern. The production strain LE1B109 has been sequenced to confirm absence of antibiotic resistance genes or any other sequences of concern.

Table 5

Enzyme	Function	Source Organism
Sucrose synthase	Catalyzes the formation of UDP-glucose	*Arabidopsis thaliana*
UDP-glucosyltransferase UGT-Sr	Catalyzes the addition of glucose to steviol glycosides	*Stevia rebaudiana*
UDP-glucosyltransferase UGT-Sl	Catalyzes the addition of glucose to steviol glycosides	*Solanum lycopersicum*

EXAMPLE 8

Synthesis of rebaudioside M in a one-pot reaction

One embodiment of the manufacturing process for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A is shown in Fig. 1. The steviol glycoside purification processes utilized prior to and following the enzymatic conversion are consistent with the methodologies for the manufacture of steviol glycosides as described in the Chemical and Technical Assessment published by FAO/JECFA (FAO, 2016).

In the embodiment shown in Fig. 1, in stage 1, *S. rebaudiana* leaves are placed in hot water at 50 to 60°C for 1 to 2 hours in continuous countercurrent extractors. The filtrate is separated using mesh screens, collected in a holding tank, and treated with flocculant (calcium hydroxide) to remove the mechanical particles, proteins, polysaccharides, and coloring agents. A plate-and-frame filter press is used to separate the resulting precipitate from the filtrate, and the filtrate is deionized by ion-exchange resins in (H+) and (OH-) form. The deionized filtrate is fed to a column system packed with macroporous adsorption resin that retains the glycosides. The column is washed with deionized water to remove impurities that did not adsorb to the resin and then the glycosides are desorbed using aqueous ethanol. The obtained glycoside solution is treated with activated carbon and the carbon is separated from the solution by plate-and-frame filter press. A standard evaporator is used to remove the ethanol, and the resulting aqueous solution is deionized again by ion-exchange resins in (H+) and (OH-) forms. The refined solution is concentrated using a nanofiltration membrane and the concentrated solution is spray dried to yield stevia extract powder containing >50% reb A (RA50). The

RA50 powder is further purified by dissolving in aqueous ethanol and incubating at low temperature for several hours to allow for reb A to crystallize. The reb A crystals containing >95% reb A are separated by conventional centrifugation and dried in a rotary drum vacuum dryer at 110°C and 10 mbar. The obtained powder is sifted through US 80 mesh stainless steel screens and passed through metal detectors to be packed in aluminum foil bags.

In stage 2 of the manufacturing process shown in Fig. 1, *E. coli* production strain LE1B109 carrying the expression vector for the corresponding enzyme is inoculated in sterilized culture medium composed of the ingredients listed in Table 6, and fermented.

Table 6

Raw Material	Technological Function	Regulatory Status
Glucose	Fermentation Nutrient	Permitted for use in food as ingredient with no limitations apart from cGMP, 21 CFR §184.1857
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	*Inducer for enzyme expression*	
Defined mineral components	Fermentation Nutrient	Permitted for use in food as food additive, food substance, ingredient, flavor enhancer, flavoring agent, processing aid or nutrient supplement, with no limitations apart from cGMP, each being selected from 21 CFR Parts §184, §172, §573, §182, §582.
Suitable antifoam agent	Processing aid	Listed in the FDA September 11, 2003 letter to ETA as acceptable for use in enzyme manufacturing
Nuclease (*i.e.*, NuCLEANase, food-grade)	Processing aid	

The fermentation conditions are a pH of between 6 to 8 and a temperature of between 25 to 37°C. The fermentation process is continued until laboratory test data shows the desired enzyme production yield. Usually, after at least 15 hours, the fermentation is stopped. In a subsequent recovery process, the enzyme is isolated from the biomass. In a first solid/liquid separation, the biomass is separated from the culture broth by standard techniques (e.g., is centrifuged and/or filtered). The biomass is homogenized to disrupt the bacterial cells and treated with a nuclease (e.g., NuCLEANase, c-LEcta, Leipzig, Germany) to degrade the DNA/RNA nucleic acids released upon cell disruption. This is followed by solid/liquid separation steps to further remove cell debris and other insoluble matter. The cell-free supernatant is filtered to obtain the purified enzyme

preparation. All raw materials used for fermentation and recovery are of food-grade quality or have been assessed to be fit for their intended use.

The obtained UGTSl, SuSy_At, and UGTSr enzyme preparation specifications are provided in Tables 7-9.

UGTSl Table 7

Specification Parameter	**Specification**	**Manufacturing Lot**		
		SK4-14-001	**SK4-18-001**	**SK4-19-001**
Activity	≥7 U/mL	9.6	12.0	9.2
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
E. coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead	≤5 mg/kg	0.12	0.06	0.09
TOS (%)	NS	10.47	13.47	11.41

CFU = colony-forming unit; NS = not specified; TOS = total organic solids; U = units [1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0]

SuSy_At Table 8

Specification Parameter	**Specification**	**Manufacturing Lot**		
		PM2-34-001	**PM-39-001**	**PM-40-001**
Activity	≥400 U/mL	413	547	512
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead	≤5 mg/kg	0.11	0.14	0.11
TOS (%)	NS	9.48	10.49	9.62

CFU = colony-forming unit; NS = not specified; TOS = total organic solids; U = units [1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0]

UGTSr Table 9

Specification Parameter	Specification	Manufacturing Lot		
		FAH-a-U3D1	FAH-a-U4D1	FAH3-002
Activity	≥1 U/mL	1.22	1.66	2.00
Total viable count	<50,000 CFU/g	<100	<100	<100
Salmonella spp.	Absent in 25 g	Conforms	Conforms	Conforms
Escherichia coli	Absent in 25 g	Conforms	Conforms	Conforms
Total coliforms	≤30 CFU/g	<10	<10	<10
Antimicrobial activity	Negative	Negative	Negative	Negative
Lead	≤5 mg/kg	0.08	0.07	0.08
TOS (%)	NS	10.53	13.61	14.17

CFU = colony-forming unit; NS = not specified; TOS = total organic solids; U = units [1 unit corresponds to the conversion of 1 µmol reb A/minute at 30°C and pH 7.0]

In stage 3, the products of stage 1 (reb A, >95%) and stage 2 (UGTSr, UGTSl, and SuSy_At enzymes) are mixed to initiate the enzymatic conversion process. First, the reb A (>95%) powder and sucrose are dissolved in reverse-osmosis water. Next, 5'-UDP-Na2 and UGTSr, UGTSl, and SuSy_At enzymes are added to formulate the reaction mixture. The reaction mixture is incubated at 40 to 50°C for 10 to 48 hours. The use of different reaction times yields steviol glycoside mixtures with different ratios of starting glycoside reb A, intermediate glycosides such as reb D, and the primary final glycoside product reb M. The resulting reaction mixture containing a mixture of steviol glycosides, including those listed in Table 2.2-1, is heated to 80 to 100°C and for 10 minutes to inactivate the enzymes.

In the last stage of manufacturing, the reaction mixture is treated with a flocculant (calcium hydroxide) to remove the mechanical particles, proteins, polysaccharides, and other impurities. A plate-and-frame filter press is used to separate the resulting precipitate from the filtrate, and the filtrate is deionized by ion-exchange resins in (H+) and (OH-) form. The deionized filtrate is fed to a column system packed with macroporous adsorption resin that retains the reb M and other steviol glycosides. The column is washed with deionized water to remove impurities that did not adsorb to the resin and then the glycosides are desorbed using aqueous ethanol. Next, the filtrate is maintained at low temperatures for several hours to allow reb M to crystallize. The reb M crystals containing >30% reb M are separated by conventional centrifugation and dried in a rotary drum vacuum at 110°C and 10 mbar. The obtained powder is sifted through US 80 mesh stainless steel screens and passed through metal detectors to be packed in aluminum foil

bags. The bags are placed in high-density polyethylene drums sealed with tamper evident seals.

EXAMPLE 9

Product Specifications for Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

The physical and chemical specifications for certain embodiments of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A are based on those established by JECFA for steviol glycosides following their 82nd meeting (JECFA, 2016a). The physical and chemical specifications for steviol glycosides with a high reb M content produced by enzymatic conversion are presented in Table 10. All analytical methods used to measure each specification parameter are internationally-recognized methods (e.g., United States Pharmacopeia [USP], Association of Official Analytical Chemists [AOAC], or JECFA). Total steviol glycoside content is measured using the high-performance liquid chromatography (HPLC) method described in the most recent JECFA specification monograph for steviol glycosides from *S. rebaudiana* Bertoni (JECFA, 2016a).

Table 10

Specification Parameter	Steviol glycosides with a high reb M content	Current JECFA specifications for steviol glycosides (JECFA, 2016a)	Method of analysis
Appearance	White to off-white powder	White to light yellow powder	Sensory Evaluation
Total steviol glycosides (anhydrous basis)	≥95%	≥95% total steviol glycosides[a]	HPLC (JECFA, 2016a)
Loss on drying	≤6.0%	≤6% (105°, 2h)	FAO/JECFA Vol 4[b] (p. 61)
pH (1% solution)	4.5 to 7.0	4.5 to 7.0	FAO/JECFA Vol 4 (p. 36-38)
Residual ethanol	<0.30%	≤0.5%	USP[c] Method 467
Residual methanol	<0.02%	≤0.02%	USP Method 467
Total ash	<1.0%	≤1%	AOAC[d] Method 945.46
Lead (as Pb)	<1.0 ppm	≤1 ppm	AOAC Method 993.14
Arsenic (as As)	<1.0 ppm	≤1 ppm	AOAC Method 993.14
Cadmium (as Cd)	<1.0 ppm	NS	AOAC Method 993.14
Mercury (as Hg)	<1.0 ppm	NS	AOAC Method 993.14
Residual protein	Not detected	NA	SDS-PAGE[e]

Specification Parameter	Steviol glycosides with a high reb M content	Current JECFA specifications for steviol glycosides (JECFA, 2016a)	Method of analysis
Residual DNA	Not detected	NA	PCR[e]

FCC = Food Chemicals Codex; HPLC = high performance liquid chromatography; NA = not applicable; NS = not specified; PCR = polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; USP = United States Pharmacopeia

[a] Where steviol glycosides "consists of a mixture of compounds containing a steviol backbone conjugated to any number or combination of the principal sugar moieties in any of the orientations occurring in the leaves of *Stevia rebaudiana* Bertoni including, glucose, rhamnose, xylose, fructose, deoxyglucose, galactose, and arabinose". (JECFA, 2016a, 2017).

[b] FAO/JECFA (2006). *Combined Compendium of Food Additive Specifications [Online Edition]. General Specifications for Enzymes Analytical Methods, Volume 4: Analytical Methods, Test Procedures and Laboratory Solutions Used by and Referenced in the Food Specifications*. 1st to 65th JECFA Meetings, 1956–2005. (FAO JECFA Monographs 1). Rome, Italy: Food and Agriculture Organization of the United Nations (FAO), Joint FAO/WHO Expert Committee on Food Additives (JECFA). Available at: ftp://ftp.fao.org/docrep/fao/009/a0675e/a0675e00.pdf [Last updated (Web version): August 2011].

[c] USP (2012). United States Pharmacopeia, 35th edition & National Formulary, 30th edition [Online]. Rockville (MD): U.S. Pharmacopeia (USP) Convention Inc. Available at: http://www.uspnf.com/ [Subscription Only].

[d] AOAC (2005). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 18th edition* (Current through Revision 1, 2006). Arlington (VA): Association of Official Analytical Chemists (AOAC).

[e] Method described in Section 3.5.4

The microbiological specification parameters listed in Table 11 have been established for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A to ensure safe use in food and standard microbial tests appropriate for food ingredients are employed .

Table 11

Specification Parameter	Specification	Method of Analysis
Total plate count	<1,000 CFU/g	AOAC[a] Method 966.23
Yeast and mold (CFU/g)	Not detected	Standards Australia[b] Method 1766.2.2
Total coliforms (MPN/g)	Not detected	ISO 4831[c]
Escherichia coli count (MPN/g)	Not detected	ISO 7251[d]
Salmonella sp.	Absent in 25 g	ISO 6579[e]

CFU = colony forming units; MPN = most probable number

[a] AOAC (2005). *Official Methods of Analysis of the Association of Official Analytical Chemists: Vols. 1&2, 18th edition* (Current through Revision 1, 2006). Arlington (VA): Association of Official Analytical Chemists (AOAC).

[b] Standards Australia (1997). *Food microbiology. Method 2.2: Examination for specific organisms—Colony count of yeasts and moulds*. (Australian/New Zealand Standard AS 1766.2.2). Sydney, Australia: Standards Association of Australia/SAI Global.

[c] BSi (1991). *Methods for Microbiological examination of food and animal feeding stuffs — Part 3: Enumeration of coliforms — Most probable number technique*. (British Standard (BS) / International Organization for Standardization (ISO), BS 5763-3:1991 ISO 4831:1991). London, Engl.: British Standards Institution (BSi).

[d] BSi (1993). *Methods for Microbiological examination of food and animal feeding stuffs — Part 8: Enumeration of presumptive Escherichia coli. Most probable number technique*. (British Standard (BS) / International Organization for Standardization (ISO), BS 5763-8:1994 ISO 7251:1993). London, Engl.: British Standards Institution (BSi).

[e] BSi (2012). Microbiology of Food and Animal Feed. Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella. Enumeration by a miniaturized most probable number technique. (PD CEN ISO/TS 6579-2:2012). London, Engl.: British Standards Institution (BSi). Information available at: http://shop.bsigroup.com/en/ProductDetail/?pid=000000000030255346.

EXAMPLE 10

Product Analysis of Steviol Glycosides with a High Reb M Content Produced by Enzymatic Conversion of Reb A

Physical and chemical analyses of 3 non-consecutive lots of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A demonstrate that the manufacturing process, as described in Section 3.4.1, produces a consistent product that conforms to the defined specification parameters. The results of the batch analyses for the 3 production lots are summarized in Table 12.

Table 12

Specification Parameter	Limit	Manufacturing Lot		
		BM050517	SK-B-U2D1	SK-B-U3D1
Appearance	White to off-white powder	Conforms	Conforms	Conforms
Total steviol glycosides (anhydrous basis)	≥95%	98.88%	97.91%	97.20%
Loss on drying	≤6.0%	1.64%	1.64%	3.85%
pH (1% solution)	4.5 to 7.0	6.32	5.99	5.89
Residual ethanol	<0.30%	0.041%	0.134%	0.133%
Residual methanol	<0.02%	ND	0.001%	0.001%
Total ash	<1.0%	0.05%	<0.005%	0.02
Lead (as Pb)	<1.0 ppm	0.021 ppm	0.035 ppm	0.038 ppm
Arsenic (as As)	<1.0 ppm	<0.005 ppm	<0.005 ppm	<0.005 ppm
Cadmium (as Cd)	<1.0 ppm	<0.005 ppm	<0.005 ppm	<0.005 ppm
Mercury (as Hg)	<1.0 ppm	<0.005 ppm	<0.005 ppm	<0.005 ppm
Residual protein	Not detected	ND	ND	ND
Residual DNA	Not detected	ND	ND	ND

ND = not detected; ppm = parts-per-million

Microbial analyses of 3 non-consecutive lots of steviol glycosides with a high reb M content produced by enzymatic conversion of reb A demonstrate that the microbiological specifications outlined in Example 9 are consistently met. A summary of the microbiological analyses is presented in Table 13.

Table 13

Specification Parameter	Limit	Manufacturing Lot		
		BM050517	SK-B-U2D1	SK-B-U3D1
Total plate count	<1,000 CFU/g	ND	ND	ND
Yeast and mold (CFU/g)	Not detected	ND	ND	ND
Total coliforms (MPN/g)	Not detected	ND	ND	ND
Escherichia coli count (MPN/g)	Not detected	ND	ND	ND
Salmonella sp.	Absent in 25 g	Absent	Absent	Absent

CFU = colony forming units; MPN = most probable number; ND = not detected

The distribution of steviol glycosides in the final product is dependent upon the length of reaction time of the enzymes with starting material reb A extracted from the leaves of *S. rebaudiana*. Example data from 2 production lots (SK BU2D1, SK-BU3D1) presented in Table 13 demonstrates that as the enzyme reaction time proceeds from 10 to 40 hours the steviol glycoside distribution changes, with increasing amounts of reb M being produced as the reaction proceeds. Example intermediate glycosides include rebaudiosides D and I, as reported in Table 14.

Table 14

Steviol Glycoside (%)	Time (hours)					
	0	14	16	18	21	40
Lot SK-BU2D1						
Rebaudioside A	100	30.4	25.6	NM	14.2	2.1
Rebaudioside D	ND	69.2	74.1	NM	43.6	1.7
Rebaudioside I	ND	0	0.1	NM	3.4	6.6
Rebaudioside M2	ND	0.38	0.12	NM	0.14	0.19
Rebaudioside M	ND	ND	ND	NM	38.6	89.4
Total Steviol Glycosides (%)	**100**	**99.98**	**99.92**	**NA**	**99.94**	**99.99**
Lot SK-BU3D1						
Rebaudioside A	100	NM	28.6	21.1	9.4	1.2
Rebaudioside D	ND	NM	71.1	77.3	60.0	1.8
Rebaudioside I	ND	NM	ND	0.3	3.1	4.2
Rebaudioside M2	ND	NM	0.28	0.35	0.34	0.37
Rebaudioside M	ND	NM	ND	0.9	27.1	92.5
Total Steviol Glycosides (%)	**100**	**NA**	**99.98**	**99.95**	**99.94**	**100.1**

NA = not applicable; ND = not detected; NM = not measured

Pursuant to the defined product specifications in Table 9 for steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, the final product contains ≥95% steviol glycosides, comprised of >30% reb M and other steviol glycosides such as those listed in Table 15. The steviol glycoside distribution, measured by HPLC, is provided for 3 non-consecutive lots of final product manufactured with a 40-hour enzyme reaction time is shown in Table 16 and demonstrates that the manufacturing process produces a product with a consistent steviol glycoside distribution and that the total steviol glycosides measured is consistently ≥95%.

Table 15

Common name	Trivial formula	Mol. Wt.	R_1	R_2
Rebaudioside A	SvG4	967	Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside D	SvG5	1,129	Glcβ(1-2)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside I	SvG5	1,129	Glcβ(1-3)Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M	SvG6	1,291	Glcβ(1-2)[Glcβ (1-3)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-
Rebaudioside M2	SvG6	1,291	Glcβ(1-2)[Glcβ (1-6)]Glcβ1-	Glcβ(1-2)[Glcβ(1-3)]Glcβ1-

Table 16

Steviol Glycoside (%)	Manufacturing Lot			Average
	BM050517	**SK-BU2D1**	**SK-BU3D1**	
Rebaudioside D	1.78[a]	0.23	0.41	0.81
Rebaudioside M	95.98	95.71	95.43	95.71
Rebaudioside I	0.91	1.54	0.93	1.13
Rebaudioside A	0.09	0.28	0.12	0.16
Total Steviol Glycosides (%)	**98.76**	**97.76**	**96.89**	**97.80**

[a] Average of 3 duplicates is reported

To confirm the success of the purification techniques and confirm the absence of proteins in steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, the final product is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples of steviol glycosides with a high reb M content are dissolved to a concentration of 1,000 ppm, and about 10 µL from each dissolved sample is stained with 3X protein loading dye and loaded onto a precast polyacrylamide gel (10% Mini-PROTEAN® TGX™ Precast Protein Gels, BIORAD). Electrophoresis is conducted at 60 minutes at 130 V and the gel is stained with 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% water for 1 hour. Gels are destained by soaking for 4 hours in a mixture of 10% acetic acid, 50% methanol, and

40% water. If protein is present in the sample, it will be visually detected on the gel (limit of detection = 0.1 μg protein). No visible protein bands were detected in any batches of final product.

To confirm the absence of residual DNA in steviol glycosides with a high reb M content produced by enzymatic conversion of reb A, a polymerase chain reaction (PCR) method was developed and primers were designed to amplify the gene of interest. Genomic DNA is extracted using a DNA extraction kit according to manufacturer's protocol. The genomic DNA is quantified using a spectrophotometer and the extracted genomic DNA is evaluated for the presence of the gene of interest. The thermal profile used is 2 minutes at 95°C followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 57°C, and 30 seconds at 72°C. Results of the PCR analysis did not detect any PCR products in any of the batches of final product (limit of detection = 0.00002 ng DNA).

CLAIMS

We claim:

1. A method for producing highly purified target steviol glycosides, comprising the steps of:

a. providing a starting composition comprising an organic compound with at least one carbon atom;

b. providing an enzyme preparation or microorganism containing at least one enzyme selected from steviol biosynthesis enzymes, UDP-glycosyltransferases, and optionally UDP-glucose recycling enzymes;

c. contacting the enzyme preparation or microorganism with a medium containing the starting composition to produce a medium comprising at least one target steviol glycoside.

2. A method for producing highly purified target steviol glycosides, comprising the steps of:

a. providing a starting composition comprising an organic compound with at least one carbon atom;

b. providing a biocatalyst comprising at least one enzyme selected from steviol biosynthesis enzymes, UDP-glycosyltransferases, and optionally UDP-glucose recycling enzymes;

c. contacting the biocatalyst with a medium containing the starting composition to produce a medium comprising at least one target steviol glycoside.

3. The method of claim 1 or 2 further comprising the step of:

d. separating the target steviol glycoside from the medium to provide a highly purified target steviol glycoside composition.

4. The method of claim 1 or 2, wherein the starting composition is selected from the group consisting steviol, steviol glycosides, polyols, carbohydrates, and combinations thereof.

5. The method of claim 1, 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 biocatalyst is an enzyme, or a cell comprising one or more enzyme, capable of converting the starting composition to target steviol glycoside.

7. The method of claim 1 or 2, wherein the target steviol glycoside is selected from the group consisting of reb *M*, reb *D*, reb *I* and/or reb *E* and mixtures thereof.

8. The method of claim 1 or 2, wherein the enzyme is selected from the group consisting of 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), l-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), l-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, UGTSL2, 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.

9. The method of claim 3, wherein the target steviol glycoside content is greater than about 95% by weight on a dry basis.

10. A consumable product comprising a highly purified target glycoside composition produced by the method of claim 3, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition.

11. A consumable product comprising the highly purified target steviol glycoside composition produced by the method of claim 3, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition, and wherein the target steviol glycoside is reb *D*.

12. A consumable product comprising the highly purified target steviol glycoside composition produced by the method of claim 3, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition, and wherein the target steviol glycoside is reb *M*.

13. A consumable product comprising the highly purified target steviol glycoside composition produced by the method of claim 3, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition, and wherein the target steviol glycoside is reb *E*.

14. A consumable product comprising the highly purified target steviol glycoside composition produced by the method of claim 3, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition, and wherein the target steviol glycoside is reb *I*.

15. The consumable product of claim 10, wherein the composition is selected from the group consisting of beverages; 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 and medicinal products.

16. The consumable product of claim 10, further comprising at least one 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, caffeine, flavorants and flavoring ingredients, astringent compounds, proteins or protein hydrolysates, surfactants, emulsifiers, flavonoids, alcohols, polymers and combinations thereof.

17. The consumable product of claim 10, further comprising at least one functional ingredient selected from the group consisting of saponins, antioxidants, dietary fiber sources, fatty acids, vitamins, glucosamine, minerals, preservatives, hydration agents, probiotics, prebiotics, weight management agents, osteoporosis management agents, phytoestrogens, long chain primary aliphatic saturated alcohols, phytosterols and combinations thereof.

18. The consumable product of claim 10, further comprising a compound selected from the group consisting of reb *A*, reb *B*, reb *O*, NSF-02, Mogroside V, Luo Han Guo, allulose, allose, D-tagatose, erythritol and combinations thereof.

19. A method for enhancing the sweetness of a beverage comprising a sweetener comprising:

a.) providing a beverage comprising a sweetener; and

b.) adding a sweetness enhancer selected from highly purified target glycoside composition produced by the method of claim 3,

wherein highly purified target glycoside composition produced by the method of claim 3 is present in a concentration at or below the sweetness recognition threshold.

20. A method for producing rebaudioside M, comprising the steps of:

(a) providing stevia leaves;

(b) extracting rebaudioside A from the stevia leaves;

(c) reacting the rebaudioside A with an enzyme capable of converting rebaudioside A to rebaudioside M;

(d) separating the rebaudioside M.

21. The method of claim 20, wherein the enzyme is selected from the group consisting of: SuSy_At of SEQ ID 1, UGTSl of SEQ ID 2, and UGTSr of SEQ ID 3, 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.

22. A method of producing target steviol glycosides composition, comprising the steps of:

(a) providing starting steviol glycosides;

(b) providing a first polypeptide; wherein the first polypeptide comprises a polypeptide 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 to the amino acid sequence set forth in SEQ ID 1.

(c) providing a second polypeptide capable of beta 1,2 glycosylation of the C2′ of the 13-O-glucose, 19-O-glucose, or both 13-O-glucose and 19-O-glucose of a steviol glycoside; wherein the second polypeptide comprises a polypeptide 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 to the amino acid sequence set forth in SEQ ID 2;

(d) providing a third polypeptide capable of beta 1,3 glycosylation of the C3′ of the 13-O-glucose, 19-O-glucose, or both 13-O-glucose and 19-O-glucose of the steviol glycoside; wherein the third polypeptide comprises a polypeptide 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 to the amino acid sequence set forth in SEQ ID 3;

(e) obtaining target steviol glycosides composition, wherein the target steviol glycosides composition comprises steviol-13-O-glucoside, steviol-19-O-glucoside, rubusoside, stevioside, 1,2-bioside, Rebaudioside A, Rebaudioside B, Rebaudioside D, Rebaudioside I or Rebaudioside E.