SUMMARY

The present disclosure 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 example, 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 example, 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 example, the target steviol glycoside is rebaudioside *A*.

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

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

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

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

In an aspect, there is provided a method for producing reb M, comprising the steps of:

a. providing a starting composition comprising reb *A*;

b. providing an enzyme preparation or microorganism containing the enzymes Susy_At of SEQ ID 1, and UGTSr of SEQ ID 3, or a mutant variant thereof having >85% amino-acid sequence identity, and UGTSI of SEQ 2, or a mutant variant thereof having >98% amino-acid sequence;

c. contacting the enzyme preparation or microorganism with a medium containing the starting composition to produce a medium comprising at least reb *M*.

In some preferred examples 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 examples, a microbial cell comprises the necessary enzymes and genes encoding thereof for converting the starting composition to target steviol glycosides.

Accordingly, the present disclosure 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 example, the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.

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

In one example 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 example, 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 example, the UDP-glucosyltransferases are synthesized.

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

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

In another example, 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 example, the UDP-glucosyltransferase is UGT91D2 or a UGT having >85% amino-acid sequence identity with UGT91D2. In yet another example the UDP-glucosyltransferase is UGTSL2 or a UGT having >85% amino-acid sequence identity with UGTSL2 (SEQ ID 2).

In yet another example, 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 example, 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 disclosure further comprises recycling UDP to provide UDP-glucose. In one example, 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 example, the recycling catalyst is sucrose synthase. In another example the sucrose synthase is SuSy_At or a sucrose synthase having >85% amino-acid sequence identity with SuSy_At (SEQ ID 1).

In one example, the recycling substrate is sucrose.

Optionally, the method of the present disclosure 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 example, the target steviol glycoside can be produced within the microorganism. In another example, the target steviol glycoside can be secreted out in the medium. In one another example, the released steviol glycoside can be continuously removed from the medium. In yet another example, the target steviol glycoside is separated after the completion of the conversion reaction.

In one example, 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 example, separation produces a composition comprising greater than about 90% by weight of the target steviol glycoside. In particular examples, the composition comprises greater than about 95% by weight of the target steviol glycoside. In other examples, 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.

CLAIMS

We claim:

1. A method for producing reb *M*, comprising the steps of:

   a. providing a starting composition comprising reb *A*;

   b. providing an enzyme preparation or microorganism containing the enzymes Susy_At of SEQ ID 1, and UGTSr of SEQ ID 3, or a mutant variant thereof having >85% amino-acid sequence identity, and UGTSI of SEQ 2, or a mutant variant thereof having >98% amino-acid sequence identity;

   c. contacting the enzyme preparation or microorganism with a medium containing the starting composition to produce a medium comprising at least reb *M*.

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

   d. separating the reb *M* from the medium to provide a highly purified reb *M*.

3. The method of any one of claims 1 to 2, wherein the microorganism is selected from the group consisting of *E.coli*, *Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., and *Yarrowia* sp.

4. The method of claim 2, wherein the reb *M* content is greater than about 95% by weight on a dry basis.