

UDP-DEPENDENT GLYCOSYLTRANSFERASE FOR HIGH EFFICIENCY PRODUCTION OF REBAUDIOSIDES

1. CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application No. 62/374,408, filed August 12, 2016, the content of which is hereby incorporated by reference in its entirety.

2. FIELD OF THE INVENTION

[0002] The present disclosure relates to certain uridine diphosphate-dependent glycosyltransferases (UGTs), compositions comprising the same, host cells comprising the same, and methods of their use for the production of rebaudiosides including rebaudioside D and rebaudioside M.

3. BACKGROUND

[0003] Zero-calorie sweeteners derived from natural sources are desired to limit the ill-effects of high-sugar consumption (*e.g.*, diabetes and obesity). Rebaudioside M (RebM), is one of many sweet-tasting compounds produced by the stevia plant (*Stevia rebaudiana Bertoni*). Of all the rebaudiosides, RebM has the highest potency (about 200-300 times sweeter than sucrose) and is the cleanest tasting. However, RebM is only produced in minor quantities by the Stevia plant, and is only a small fraction of the total steviol glycoside content (<1.0%). Ohta *et al.*, 2010, *J. Appl. Glycosci.*, 57, 199-209 (2010). As such it is desirable to produce RebM using biotechnological routes allowing production in large quantities and at high purity.

[0004] To economically produce a product using biotechnology, each step in the bioconversion from feedstock to product advantageously has a high conversion efficiency (ideally >90%). In engineering of yeast to produce RebM, a limitation was identified in the penultimate biosynthetic step, namely the conversion of Rebaudioside A (RebA) to Rebaudioside D (RebD) *See FIG. 1A*. The native enzyme (Ono, EP 2 826 861 A1, UGT91D_like3, or a near homologue) was observed to convert about 3% of RebA to RebD. Two other UGT enzymes have been identified that are capable of converting RebA to RebD. One is Os_UGT_91C1 from *Oryza sativa* (also referred to as EUGT11 in Houghton-Larsen *et al.*, WO 2013/022989 A2), and the other is Sl_UGT_101249881 from *Solanum lycopersicum* (also referred to as UGTSL2 in Markosyan *et al.*, WO2014/193888 A1). However, both Os_UGT_91C1 and Sl_UGT_101249881 were initially observed by the

present inventors to have lower than desired conversion efficiencies of about 53% and 70%, respectively. All three of these enzymes, UGT91D_like3, Os_UGT_91C1, and SL_UGT_101249881, are uridine diphosphate-dependent glycosyltransferases (UGT) which transfer a glucose moiety to the C-2' position of the 19-O-glucose residue via formation of a beta(1->2) linkage (**FIG. 1A**).

[0005] To produce RebM efficiently and at high purity, improved enzymes capable of converting RebA to RebD at high efficiency are needed. The compositions and methods provided herein address this need and provide related advantages as well.

4. SUMMARY

[0006] Provided herein are compositions and methods for the improved conversion of RebA to RebD and for the improved production of RebD and/or RebM. These compositions and methods are based in part on the surprising discovery of certain uridine diphosphate-dependent glycosyltransferases (UGTs) are capable of converting RebA to RebD with remarkably high efficiency. Even a modest improvement in strain performance (*e.g.*, ten percent) with new UGTs can potentially save over ten million dollars in production cost in the future, assuming that the market demand for RebM is 5000 million tons per year.

[0007] Certain UGTs described herein are also capable of producing RebM with little or no non-natural glycosides such as RebM2 side product (*i.e.*, an isomer of RebM). *See* Ceunen S. *et al.* Steviol Glycosides: Chemical Diversity, Metabolism, and Function. *J. Nat. Prod.*, 76, 1201-1228 (2013) for a list of currently-known Stevia glycosides. As such, in certain examples, the compositions and methods described herein can reduce the costs of downstream processing to obtain a composition with high purity RebM.

[0008] Also provided herein are compositions and methods for alternative enzymes which are capable of producing steviol glycosides with different substrate specificities than previously known UGTs. The new alternative enzymes can potentially produce different mixtures or proportions of steviol glycosides compared to those produced by other known enzymes. Compositions with different mixtures or proportions of steviol glycosides can potentially impart alternative, sweet taste profiles, which may be useful in formulating various consumable or food products.

[0009] Thus, provided herein are genetically modified host cells and methods of their use for the production of industrially useful compounds. In one aspect, provided herein is a genetically modified host cell comprising a heterologous nucleic acid encoding a UDP-glycosyltransferase comprising an amino acid sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 11, wherein the genetically modified host cell is capable of converting RebA to RebD at an efficiency of greater than 90%, 95%, 96%, or 97%. In one example, provided herein is a genetically modified host cell comprising: a heterologous nucleic acid encoding a UDP-glycosyltransferase (UGT40087, also refer to as Si_UGT_40087). In some examples, the

genetically modified host cell further comprises one or more enzymatic pathways capable of producing steviol and/or steviol glycosides.

[0010] In certain examples, provided herein are genetically modified host cells comprising a heterologous nucleic acid encoding a UDP-glycosyltransferase comprising an amino acid sequence having at least 80%, 85%, 90%, or 95% sequence identity to the sequence of UGT40087 (*e.g.*, SEQ ID NO:1 or SEQ ID NO:11). In certain examples, the genetically modified host cell is capable of converting RebA to RebD at an efficiency greater than 90%, 95%, 96%, or 97%. In certain examples, the genetically modified host cell comprises a UDP-glycosyltransferase comprising a sugar acceptor domain, wherein the amino acid sequence of the sugar acceptor domain has at least 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of the sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain examples, the genetically modified host cell comprises a UDP-glycosyltransferase which comprises a loop1 amino acid sequence, a variant loop1 amino acid sequence, a loop2 amino acid sequence, a variant loop2 amino acid sequence, a loop3_1 amino acid sequence, a variant loop3_1 amino acid sequence, a loop3_2 amino acid sequence, a variant loop3_2 amino acid sequence, a loop4_1 amino acid sequence, a variant loop4_1 amino acid sequence, a loop4_2 amino acid sequence, or any combination thereof. In certain examples, the genetically modified host cell comprises a UDP-glycosyltransferase comprising an amino acid sequence having at least 61%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity to the sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11, and further comprises the loop4_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11.

[0011] In certain examples, provided herein are genetically modified host cells comprising a heterologous nucleic acid encoding a UDP-glycosyltransferase comprising an amino acid sequence having at least 80%, 85%, 90%, or 95% sequence identity to the sequence of SEQ ID NO:2, 5, or 6. In certain examples, the genetically modified host cell is capable of converting RebA to RebD at an efficiency greater than 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, or 97%. In certain examples, the genetically modified host cell comprises a UDP-glycosyltransferase comprising a sugar acceptor domain, wherein the amino acid sequence of the sugar acceptor domain has at least 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to the amino acid sequence of the sugar acceptor domain of SEQ ID NO:2, 5, or 6. In certain examples, the genetically modified host cell comprises a UDP-glycosyltransferase which comprises a loop1 amino acid sequence, a variant loop1 amino acid sequence, a loop2 amino

acid sequence, a variant loop2 amino acid sequence, a loop3_1 amino acid sequence, a variant loop3_1 amino acid sequence, a loop3_2 amino acid sequence, a variant loop3_2 amino acid sequence, a loop4_1 amino acid sequence, a variant loop4_1 amino acid sequence, a loop4_2 amino acid sequence, or any combination thereof. In certain examples, the genetically modified host cell comprises a UDP-glycosyltransferase comprising an amino acid sequence having at least 61%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% sequence identity to the sugar acceptor domain of SEQ ID NO:2, 5, or 6, and further comprises the loop4_1 amino acid sequence of SEQ ID NO:2, 5, or 6.

[0012] In another example, provided herein are methods for producing a heterologous steviol glycoside, the method comprising: culturing a population of genetically modified host cells provided herein, capable of producing the steviol glycoside as described herein, in a medium with a carbon source under conditions suitable for making said steviol glycoside compound; and recovering said steviol glycoside from the medium. In some examples, the heterologous steviol glycoside is selected from the group consisting of RebD and RebM.

[0013] In another example, provided herein is a method for increasing the production of a steviol glycoside compound in a host cell, the method comprising: expressing in the host cell a heterologous nucleic acid encoding a UGT40087; and culturing the host cell under conditions suitable for producing the steviol glycoside. In some examples, the host cell does not comprise a UGT91D_like3 enzyme, a Os_UGT_91C1 enzyme, or a Sl_UGT_101249881 enzyme.

[0014] In another example, provided herein are methods for producing RebD, the method comprising: culturing a population of genetically modified host cells provided herein, capable of producing RebD as described herein, in a medium with a carbon source under conditions suitable for making said RebD; and recovering said RebD from the medium.

[0015] In another aspect, provided herein are methods for producing RebM, the method comprising: culturing a population of genetically modified host cells provided herein, capable of producing RebM as described herein, in a medium with a carbon source under conditions suitable for making said RebM; and recovering said RebM from the medium.

[0016] In another example, provided herein are methods for producing RebD, the method comprising: contacting RebA with glucose and a UDP-glycosyltransferase described herein, capable of converting RebA to RebD, under conditions suitable for forming RebD.

[0017] In another example, provided herein are methods for producing RebM, the method comprising: contacting RebA with glucose and a UDP-glycosyltransferase described

herein, capable of converting RebA to RebD, under conditions suitable for forming RebD, and with a UDP-glycosyltransferase described herein, capable of converting RebD to RebM.

[0018] In some examples, the host cell is a yeast cell. In some examples, the yeast is *Saccharomyces cerevisiae*. In some examples, the host cell produces RebD or RebM at high efficiency. In some examples, the host cell produces an increased amount of RebD or RebM compared to a yeast cell not comprising the UGT40087 enzyme. In some examples, the host cell produces an increased amount of RebM relative to RebM2 compared to a yeast cell not comprising the UGT40087 enzyme.

[0019] In another example, other UDG-glycosyltransferases provided herein can be used in addition or in alternative to UGT40087. These include, for example, sr.UGT_9252778, Bd_UGT10840, Hv_UGT_V1, Bd_UGT10850, and Ob_UGT91B1_like.

[0019a] In another aspect, the present invention refers to a fermentation composition comprising the genetically modified host cells as described herein and steviol glycosides produced by the genetically modified host cells, wherein the steviol glycosides comprise RebA, RebD and RebM at a RebA:RebD:RebM ratio of at least 1:7:50.

5. BRIEF DESCRIPTION OF THE FIGURES

[0020] FIG. 1A provides a schematic representation of the conversion of RebA to RebD to RebM.

[0021] FIG. 1B provides the structure of RebM2.

[0022] FIG. 1C provides a schematic diagram of the mevalonate pathway.

[0023] FIG. 2A provides an exemplary pathway of farnesyl pyrophosphate (FPP) to steviol.

[0024] FIG. 2B provides an exemplary pathway of steviol to RebM.

[0025] FIG. 2C provides an exemplary pathway for the enzymatic production of RebM.

[0026] FIG. 3 provides the ratio of RebA to RebD conversion *in vivo*, as measured by micromoles of Reb (D+M) / micromoles of Reb (A+D+M). The parent control strain is labelled 91D_like3 (from *Stevia rebaudiana*; this strain contains UGT's: 85C2, 74G1, 91D_like3, and 76G1 only, in addition to an empty landing pad. It is noted that 91D_like3 has very low RebA to RebD conversion (~3% *see* Table 5). A single copy of each UGT enzyme was inserted into the parent control strain and screened for improved RebA to RebD conversion. Six UGT enzymes are shown to have RebA to RebD conversion that are at least equivalent to, or better than, the previous known enzymes Os_UGT_91C1 and Sl_UGT_101249881. Three UGT enzymes (Si_UGT_40087, Ob_UGT91B1_like, and Hv_UGT_V1 are better than both previously identified UGT enzymes at converting RebA to RebD. Error bars are standard error.

[0027] FIG. 4 provides a schematic diagram of “landing pad” design used to insert individual UGT enzymes for screening for RebA to RebD conversion in yeast.

[0028] FIGS. 5a-h illustrate chromatograms of RebA, RebD, RebM, RebM2 produced *in vivo* for each UGT gene used to generate data described in Table 5 and FIG. 3. The chromatogram peaks are selected to show peaks associated with RebA, RebD, RebM, and RebM2. Each figure shows the retention time versus % intensity for authentic standards, the control parent strain Y31062, and Y31062 with an additional UGT enzyme. Y31062 is the parent control strain and contains UGT74G1, UGT85C2, UGT91D_like3, and UGT76G1 with an empty landing pad. FIG. 5a: data for Si_UGT_40087; this figure also includes a chromatogram for a RebE authentic standard. FIG. 5b: data for Ob_UGT91B1_like. FIG. 5c: data for Hv_UGT_V1. FIG. 5d: data for Sl_UGT_101249881. FIG. 5e: data for UGT_g252778. FIG. 5f: data for Os_UGT_91C1. FIG. 5g: data for Bd_UGT10850. FIG. 5h: data for Bd_UGT10840. FIG. 5i shows a magnified view the chromatogram for just the RebE peak of Si_UGT_40087 versus the authentic standard to confirm that this peak is RebE.

[0029] FIG. 6 illustrates a homology model of UGT40087 structure. Its N- and C-terminal domains are shown in light and dark gray, and the four loops used in the loop swapping experiment are also labeled on the structure.

[0030] FIG. 7 illustrates a schematic overview of the UGT domain swapping constructs

[0031] FIG. 8 illustrates sequence alignment of four UDP-glycosyltransferases (UGT40087 (SEQ ID NO: 1); Os_UGT_91C1 (SEQ ID NO: 8); 91Dlike3 (SEQ ID NO: 7); Si91Dlike (SEQ ID NO: 12))

[0032] FIG. 9a illustrate a chromatogram of steviol glycosides produced *in vivo* for the parent control cell (comprising UGT74G1, UGT85C2, UGT76G1, and UGT91D_like3). FIG. 9b illustrates a chromatogram of steviol glycosides produced *in vivo* for the parent control strain with UGT40087. FIG. 9c illustrates a chromatogram of steviol glycosides produced *in vivo* for the parent control strain with Ob_UGT91B1_like.

6. DETAILED DESCRIPTION OF THE EMBODIMENTS

6.1 Terminology

[0033] As used herein, the term “heterologous” refers to what is not normally found in nature. The term “heterologous nucleotide sequence” refers to a nucleotide sequence not normally found in a given cell in nature. As such, a heterologous nucleotide sequence may be: (a) foreign to its host cell (*i.e.*, is “exogenous” to the cell); (b) naturally found in the host

cell (*i.e.*, “endogenous”) but present at an unnatural quantity in the cell (*i.e.*, greater or lesser quantity than naturally found in the host cell); or (c) be naturally found in the host cell but positioned outside of its natural locus. The term “heterologous enzyme” refers to an enzyme that is not normally found in a given cell in nature. The term encompasses an enzyme that is: (a) exogenous to a given cell (*i.e.*, encoded by a nucleotide sequence that is not naturally present in the host cell or not naturally present in a given context in the host cell); and (b) naturally found in the host cell (*e.g.*, the enzyme is encoded by a nucleotide sequence that is endogenous to the cell) but that is produced in an unnatural amount (*e.g.*, greater or lesser than that naturally found) in the host cell.

[0034] On the other hand, the term “native” or “endogenous” as used herein with reference to molecules, and in particular enzymes and nucleic acids, indicates molecules that are expressed in the organism in which they originated or are found in nature, independently of the level of expression that can be lower, equal, or higher than the level of expression of the molecule in the native microorganism. It is understood that expression of native enzymes or polynucleotides may be modified in recombinant microorganisms.

[0035] As used herein, the term “parent cell” refers to a cell that has an identical genetic background as a genetically modified host cell disclosed herein except that it does not comprise one or more particular genetic modifications engineered into the modified host cell, for example, one or more modifications selected from the group consisting of: heterologous expression of an enzyme of a steviol pathway, heterologous expression of an enzyme of a steviol glycoside pathway, heterologous expression of a geranylgeranyl diphosphate synthase, heterologous expression of a copalyl diphosphate synthase, heterologous expression of a kaurene synthase, heterologous expression of a kaurene oxidase, heterologous expression of a steviol synthase (kaurenoic acid hydroxylase), heterologous expression of a cytochrome P450 reductase, heterologous expression of a UGT74G1, heterologous expression of a UGT76G1, heterologous expression of a UGT85C2, heterologous expression of a UGT91D, and heterologous expression of a UGT40087.

[0036] As used herein, the term “naturally occurring” refers to what is found in nature. For example, a UDP-glycosyltransferase that is present in an organism that can be isolated from a source in nature and that has not been intentionally modified by a human in the laboratory is naturally occurring UDP-glycosyltransferase. Conversely, as used herein, the

term “non-naturally occurring” refers to what is not found in nature but is created by human intervention.

[0037] The term “medium” refers to a culture medium and/or fermentation medium.

[0038] The term “fermentation composition” refers to a composition which comprises genetically modified host cells and products or metabolites produced by the genetically modified host cells. An example of a fermentation composition is a whole cell broth, which can be the entire contents of a vessel (*e.g.*, a flasks, plate, or fermenter), including cells, aqueous phase, and compounds produced from the genetically modified host cells.

[0039] As used herein, the term “production” generally refers to an amount of steviol or steviol glycoside produced by a genetically modified host cell provided herein. In some embodiments, production is expressed as a yield of steviol or steviol glycoside by the host cell. In other embodiments, production is expressed as a productivity of the host cell in producing the steviol or steviol glycoside.

[0040] As used herein, the term “productivity” refers to production of a steviol or steviol glycoside by a host cell, expressed as the amount of steviol or steviol glycoside produced (by weight) per amount of fermentation broth in which the host cell is cultured (by volume) over time (per hour).

[0041] As used herein, the term “yield” refers to production of a steviol or steviol glycoside by a host cell, expressed as the amount of steviol or steviol glycoside produced per amount of carbon source consumed by the host cell, by weight.

[0042] As used herein, the term “an undetectable level” of a compound (*e.g.*, RebM2, steviol glycosides, or other compounds) means a level of a compound that is too low to be measured and/or analyzed by a standard technique for measuring the compound. For instance, the term includes the level of a compound that is not detectable by the analytical methods described in Example 7.

[0043] As used herein, the term “steviol glycoside(s)” refers to a steviol enzymatically altered by the addition of one or more sugar moieties, such as 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 D, rebaudioside N, rebaudioside O, synthetic steviol glycosides, *e.g.* enzymatically glucosylated steviol glycosides and combinations thereof.

[0044] As used herein, the term “uridine diphosphate (UDP)-glycosyltransferase” or “UDP-dependent glycosyltransferase” refers to an enzyme that has an activity of transferring a monosaccharide moiety from a glycosyl donor to a glycosyl acceptor, in particular, an enzyme that utilizes a UDP-sugar as a glycosyl donor. The term “UDP-glycosyltransferase” may be used interchangeably with “UGT”.

[0045] As used herein, the term “a functional domain” means either “a sugar acceptor domain” or “a sugar donor domain” of a UDP-glycosyltransferase. Plant UDP-glycosyltransferase (UGTs) belong to Family 1 of glycosyltransferase superfamily. They adopt the GT-B structural fold. This shared structural feature by UGTs consists of two domains, a C-terminal and an N-terminal domain with similar Rossmann-like folds, separated by an inter-domain linker. The C-terminal domain binds UDP-glucose (“the sugar donor”) and is thus also termed as the sugar donor domain, while the N-terminal domain binds the non-sugar substrate (“the acceptor”) and is thus also termed as the acceptor domain.

[0046] As used herein, the term “variant” refers to a polypeptide differing from a specifically recited “reference” polypeptide (*e.g.*, a wild-type sequence) by amino acid insertions, deletions, mutations, and/or substitutions, but retains an activity that is substantially similar to the reference polypeptide. For example, a variant UGT40087 retains an activity that is substantially similar to the reference UGT40087 having SEQ ID NO:11 in that a variant UGT40087 is also capable of catalyzing a reaction to convert RebA to RebD and/or stevioside to RebE. In some embodiments, the variant is created by recombinant DNA techniques, such as mutagenesis. In some embodiments, a variant polypeptide differs from its reference polypeptide by the substitution of one basic residue for another (*i.e.* Arg for Lys), the substitution of one hydrophobic residue for another (*i.e.* Leu for Ile), or the substitution of one aromatic residue for another (*i.e.* Phe for Tyr), *etc.* In some embodiments, variants include analogs wherein conservative substitutions resulting in a substantial structural analogy of the reference sequence are obtained. Examples of such conservative substitutions, without limitation, include glutamic acid for aspartic acid and vice-versa; glutamine for asparagine and vice-versa; serine for threonine and vice-versa; lysine for arginine and vice-versa; or any of isoleucine, valine or leucine for each other.

[0047] As used herein, the term “variant loop1” amino acid sequence refers to an amino acid sequence which differs from the reference loop1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 (or a modified loop1 sequence of UGT40087 having the sequence of SEQ

ID NO:28) by, one, two, three, four, five, six, seven, eight, nine, or ten amino acid insertions, deletions, mutations, and/or substitutions, but allows a UDP-glycosyltransferase comprising a variant loop1 amino acid sequence, inserted at a location which corresponds to the loop1 amino acid sequence location of SEQ ID NO:1 or SEQ ID NO:11, respectively, to catalyze conversion of RebA to RebD and /or stevioside to RebE.

[0048] As used herein, the term “variant loop2” amino acid sequence refers to an amino acid sequence which differs from the reference loop2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 by one, two, three, four, five, six, seven, eight, nine, or ten amino acid insertions, deletions, mutations, and/or substitutions, but allows a UDP-glycosyltransferase comprising a variant loop2 amino acid sequence, inserted at a location which corresponds to the loop2 amino acid sequence location of SEQ ID NO:1 or SEQ ID NO:11, respectively, to catalyze conversion of RebA to RebD and /or stevioside to RebE.

[0049] As used herein, the term “variant loop3_1” amino acid sequence refers to an amino acid sequence which differs from the reference loop3_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 by one, two, three, four, five, six, seven, eight, nine, or ten amino acid insertions, deletions, mutations, and/or substitutions, but allows a UDP-glycosyltransferase comprising a variant loop3_1 amino acid sequence, inserted at a location which corresponds to the loop3_1 amino acid sequence location of SEQ ID NO:1 or SEQ ID NO:11, to catalyze conversion of RebA to RebD and/or stevioside to RebE. As used herein, the term “variant loop3_2” amino acid sequence refers to an amino acid sequence which differs from the reference loop3_2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 by one, two, three, four, five, six, seven, eight, nine, or ten amino acid insertions, deletions, mutations, and/or substitutions, but allows a UDP-glycosyltransferase comprising a variant loop3_2 amino acid sequence, inserted at a location that corresponds to the loop3_2 amino acid sequence location of SEQ ID NO:1 or SEQ ID NO:11, respectively to catalyze conversion of RebA to RebD and/or stevioside to RebE. In certain embodiments, a variant loop3_2 amino acid sequence differs from the reference loop3_2 amino acid sequence by, one, two, three, four, five six, seven, eight, nine, ten, or up to thirty amino acid insertions, deletions, mutations, and/or substitutions.

[0050] As used herein, the term “variant loop4_1” amino acid sequence refers to an amino acid sequence which differs from the reference loop4_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 by one, two, three, four, five, six, seven, eight, nine, ten, or up to 30 amino acid insertions, deletions, mutations, and/or substitutions, but allows a UDP-

glycosyltransferase comprising a variant loop4_1 sequence, inserted at a location that corresponds to the loop4_1 amino acid location of SEQ ID NO:11, to catalyze conversion of RebA to RebD and/or stevioside to RebE. As used herein, the term “sequence identity” or “percent identity,” in the context of two or more nucleic acid or protein sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same. For example, the sequence can have a percent identity of at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or higher identity over a specified region to a reference sequence when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using a sequence comparison algorithm or by manual alignment and visual inspection. For example, percent of identity is determined by calculating the ratio of the number of identical nucleotides (or amino acid residues) in the sequence divided by the length of the total nucleotides (or amino acid residues) minus the lengths of any gaps.

[0051] For convenience, the extent of identity between two sequences can be ascertained using computer program and mathematical algorithms known in the art. Such algorithms that calculate percent sequence identity generally account for sequence gaps and mismatches over the comparison region. Programs that compare and align sequences, like Clustal W (Thompson *et al.*, (1994) *Nucleic Acids Res.*, 22: 4673-4680), Clustal Omega (Sievers *et al.*, (2011) *Molecular Systems Biology.*, 7:539), ALIGN (Myers *et al.*, (1988) *CABIOS*, 4: 11-17), FASTA (Pearson *et al.*, (1988) *PNAS*, 85:2444-2448; Pearson (1990), *Methods Enzymol.*, 183: 63-98) and gapped BLAST (Altschul *et al.*, (1997) *Nucleic Acids Res.*, 25: 3389-3402) are useful for this purpose. The BLAST or BLAST 2.0 (Altschul *et al.*, *J. Mol. Biol.* 215:403-10, 1990) is available from several sources, including the National Center for Biological Information (NCBI) and on the Internet, for use in connection with the sequence analysis programs BLASTP, BLASTN, BLASTX, TBLASTN, and TBLASTX. Additional information can be found at the NCBI web site.

[0052] In certain embodiments, the sequence alignments and percent identity calculations can be determined using the BLAST program using its standard, default parameters. For nucleotide sequence alignment and sequence identity calculations, the BLASTN program is used with its default parameters (Gap opening penalty=5, Gap extension penalty=2, Nucleic match=2, Nucleic mismatch=-3, Expectation value = 10.0,

Word size = 11, Max matches in a query range = 0). In certain embodiments, for nucleotide sequence alignment and sequence identity calculations, the BLASTN program is used with these parameters (Gap opening penalty=5, Gap extension penalty=2, Nucleic match=1, Nucleic mismatch=-3, Expectation value = 10.0, Word size = 11). For polypeptide sequence alignment and sequence identity calculations, BLASTP program is used with its default parameters (Alignment matrix = BLOSUM62; Gap costs: Existence=11, Extension=1; Compositional adjustments=Conditional compositional score, matrix adjustment; Expectation value = 10.0; Word size=6; Max matches in a query range = 0. Alternatively, the following program and parameters are used: Align Plus software of Clone Manager Suite, version 5 (Sci-Ed Software); DNA comparison: Global comparison, Standard Linear Scoring matrix, Mismatch penalty=2, Open gap penalty=4, Extend gap penalty=1. In the embodiments described herein, the sequence identity is calculated using BLASTN or BLASTP programs using their default parameters. In the embodiments described herein, the sequence alignment of two or more sequences are performed using Clustal Omega using the suggested default parameters (Dealign input sequences: no; Mbed-like clustering guide-tree: yes; Mbed-like clustering iteration: yes; number of combined iterations: default(0); Max guide tree iterations: default; Max HMM iterations: default; Order: aligned).

6.2 Host Cells

[0053] Provided herein are host cells capable of producing rebaudioside D (RebD) from rebaudioside A (RebA) at high efficiency. In certain embodiments, the host cells can produce RebD from RebA as a starting material. In preferred embodiments, the host cells can produce RebA from a carbon source in a culture medium and can further produce RebD from the RebA. In particular embodiments, the host cells can further produce rebaudioside M (RebM) from the RebD.

[0054] In particular embodiments, the host cells comprise the enzyme activity of uridine diphosphate glycosyltransferase 87 (UGT40087). A UGT40087 enzyme is capable of converting RebA to RebD at high efficiency. In certain embodiments, a UGT40087 enzyme is capable of converting RebA to RebD at an efficiency of greater than 80%. In certain embodiments, a UGT40087 enzyme is capable of converting RebA to RebD at an efficiency of greater than 85%. In certain embodiments, a UGT40087 enzyme is capable of converting RebA to RebD at an efficiency of greater than 90%. In certain embodiments, a UGT40087 enzyme is capable of converting RebA to RebD at an efficiency of greater than 95%. In certain embodiments, a UGT40087 enzyme is capable of converting RebA to RebD at an

efficiency of greater than 96%. In certain embodiments, a UGT40087 enzyme is capable of converting RebA to RebD at an efficiency of about 97%. In certain embodiments, a UGT40087 enzyme is capable of converting stevioside to RebE.

[0055] In certain embodiments, the host cell is capable of converting RebA to RebD at an efficiency of greater than 80%. In certain embodiments, the host cell is capable of converting RebA to RebD at an efficiency of greater than 85%. In certain embodiments, the host cell is capable of converting RebA to RebD at an efficiency of greater than 90%. In certain embodiments, the host cell is capable of converting RebA to RebD at an efficiency of greater than 95%. In certain embodiments, the host cell is capable of converting RebA to RebD at an efficiency of greater than 96%. In certain embodiments, the host cell is capable of converting RebA to RebD at an efficiency of about 97%. In certain embodiments, the host cell is capable of converting stevioside to RebE.

[0056] Efficiency of conversion can be measured by any technique apparent to those of skill in the art. In certain embodiments, efficiency of conversion can be measured by contacting RebA with an enzyme or host cell under suitable conditions for forming RebD. Efficiency can be measured by comparing the molar amount of RebD produced compared to the total amount of RebA and RebD in the resulting composition. Efficiency can also be measured by comparing the total amount of RebD and downstream products of RebD to the total amount of RebA, RebD, and downstream products of RebD in the resulting composition. For instance, efficiency can also be measured by comparing the total amount of RebD, RebM, and RebM2 compared to the total amount of RebA, RebD, RebM, and RebM2 in the resulting composition.

[0057] In certain embodiments, provided herein are host cells comprising a UGT40087 comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 60% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 65% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least

70% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 75% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 96% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 97% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 98% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 60%, at least 99%, or at least any percentage between 60% and 99% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11.

[0058] In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence described herein, and is capable of converting RebA to RebD. In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence described herein, and is capable of beta 1,2 glycosylation of the C2' position of the 19-O glucose of a steviol glycoside. In certain embodiments, provided herein are host cells comprising a UDP-

glycosyltransferase capable of converting RebA to RebD at an efficiency greater than 90%, 95%, 96%, or 97%, and wherein the UDP-glycosyltransferase comprises an amino acid sequence having at least 95% sequence identity to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11.

[0059] In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a UGT40087 comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence substantially identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 60% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 65% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 70% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 75% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 85% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 96% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid

encoding a polypeptide comprising an amino acid sequence that is at least 97% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 98% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 99% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 60%, at least 99%, or any percentage between 60% and 99% identical to the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11.

[0060] In certain embodiments, provided herein are host cells comprising a heterologous nucleic acid comprising a nucleotide sequence of SEQ ID NO:13 which encodes UGT40087 having the sequence of SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a heterologous nucleic acid comprising a nucleotide sequence that is at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to the nucleotide sequence of SEQ ID NO:13. In certain embodiments, provided herein are host cells comprising a heterologous nucleic acid comprising a nucleotide sequence that is at least 90%, at least 99%, or any percentage between 60% and 99% identical to the nucleotide sequence of SEQ ID NO:11.

[0061] In certain embodiments, provided herein are host cells comprising a functional domain of a UGT40087, wherein the UGT40087 comprises the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising the N-terminal sugar acceptor domain of a UGT40087 comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising the C-terminal sugar donor domain of a UGT40087 comprising the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, the sugar acceptor domain of a UGT40087 comprises about amino acid positions 1 to 214 of SEQ ID NO: 11 (which correspond to amino acid positions 1 to 215 of SEQ ID NO:1). In certain embodiments, the sugar donor domain of UGT40087 comprises about amino acid positions 215 to 435 of SEQ ID NO:11 (which correspond to amino acid positions 216 to 436 of SEQ ID NO:1). In

certain embodiments, the sugar acceptor domain of UGT40087 comprises about amino acid positions 1 to 215 of SEQ ID NO:1. In certain embodiments, the sugar donor domain of comprises about amino acid positions of 216 to 436 of SEQ ID NO:1. In certain embodiments, the sugar acceptor domain and the sugar donor domain of a UGT40087 comprises a narrower range of amino acid residues than 1 to 214 or 215 to 435, respectively, in relation to SEQ ID NO:11. In certain embodiments, the sugar acceptor domain and the sugar donor domain of a UGT40087 comprises a narrower range of amino acid residues than 1 to 215 or 216 to 436, respectively, relation to SEQ ID NO:1.

[0062] In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence substantially identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 60% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 65% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 70% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 75% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 85% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID

NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 96% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 97% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 98% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided here are host cells comprising a polypeptide comprising an amino acid sequence that is at least 60%, at least 99%, or any percentage between 60% and 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

[0063] In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a UGT40087 comprising the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence substantially identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 60% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 65% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 70% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 75% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 85% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 96% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 97% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 98% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11.

[0064] In certain embodiments, when three-dimensional modeled structures of UGT40087 and another UDP-glycosyltransferase were compared and analyzed, they revealed four loops (*i.e.*, loop1, loop2, loop3, and loop4) that possess significant conformational differences at the N terminal sugar acceptor domain. *See FIG. 6 and Example 12.* The experimental results from exchanges of corresponding loop sequences between the two UGTs indicated that the loop1, loop2, loop3_1, loop3_2, and loop4_1 of UGT40087 can be substituted with their respective, corresponding loop sequences from other UDP-glycosyltransferases which are capable of converting RebA to RebD. In these

embodiments, two versions of loop3 (*i.e.*, loop3_1 and loop3_2) and loop_4 (*i.e.*, loop4_1 and loop4_2) were designed to account for two possible loop lengths.

[0065] Thus, in certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO: 1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a heterologous nucleic acid encoding a UDP-glycosyltransferase comprising an amino acid sequence that is at least 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO: 1 or SEQ ID NO:11. In certain embodiments, the UDP-glycosyltransferase further comprises a loop1 amino acid sequence of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11), at a location of the UDP-glycosyltransferase that corresponds to the loop1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. In certain embodiments, the loop1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 has the amino acid sequence of SEQ ID NO:30. In certain embodiments, the loop1 amino acid sequence has the sequence of SEQ ID NO:28. In certain embodiments, the UDP-glycosyltransferase further comprises a variant loop1 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. The variant loop1 amino acid sequence refers an amino acid sequence which differs from the reference loop1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 or the loop1 amino acid sequence having SEQ ID NO:28, but allows the UDP-glycosyltransferase comprising the variant loop1 amino acid to retain its activity to convert RebA to RebD and/or to convert stevioside to RebE.

[0066] In certain embodiments, the UDP-glycosyltransferase further comprises loop2 amino acid sequence of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11), at a location of the UDP-glycosyltransferase that corresponds to the loop2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. In certain embodiments, the loop2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 has the amino acid sequence of SEQ ID NO:19. In certain embodiments, the UDP-glycosyltransferase further comprises a variant loop2 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. The variant loop2 amino acid sequence refers to an amino acid sequence which differs from the reference loop2 amino

acid sequence of SEQ ID NO:1 or SEQ ID NO:11, but allows the UDP-glycosyltransferase comprising the variant loop2 amino acid to retain its activity to convert RebA to RebD and/or to convert stevioside to RebE.

[0067] In certain embodiments, the UDP-glycosyltransferase further comprises loop3_1 amino acid sequence of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11), at a location of the UDP-glycosyltransferase that corresponds to the loop3_1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. In certain embodiments, the loop3_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 has the amino acid sequence of SEQ ID NO:20. In certain embodiments, the UDP-glycosyltransferase further comprises a variant loop3_1 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop3_1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. The variant loop3_1 amino acid sequence refers to an amino acid sequence which differs from the reference loop3_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, but allows the UDP-glycosyltransferase comprising the variant loop3_1 amino acid to retain its activity to convert RebA to RebD and/or to convert stevioside to RebE.

[0068] In certain embodiments, the UDP-glycosyltransferase further comprises loop3_2 amino acid sequence of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11), at a location of the UDP-glycosyltransferase that corresponds to the loop3_2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. In certain embodiments, the loop3_2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 has the amino acid sequence of SEQ ID NO:21. In certain embodiments, the UDP-glycosyltransferase further comprises a variant loop3_2 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop3_2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. The variant loop3_2 amino acid sequence refers to an amino acid sequence which differs from the reference loop3_2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, but allows the UDP-glycosyltransferase comprising the variant loop3_2 amino acid to retain its activity to convert RebA to RebD and/or to convert stevioside to RebE.

[0069] In certain embodiments, the UDP-glycosyltransferase further comprises loop4_1 amino acid sequence of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11), at a location of the UDP-glycosyltransferase that corresponds to the loop4_1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. In certain embodiments, the loop4_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 has the amino acid sequence of SEQ ID NO:22. In certain embodiments, the UDP-glycosyltransferase further comprises a variant loop4_1 amino acid

sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop4_1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. The variant loop4_1 amino acid sequence refers to an amino acid sequence which differs from the reference loop4_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, but allows the UDP-glycosyltransferase comprising the variant loop4_1 amino acid to retain its activity to convert RebA to RebD and/or to convert stevioside to RebE.

[0070] In certain embodiments, the UDP-glycosyltransferase further comprises loop4_2 amino acid sequence of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11), at a location of the UDP-glycosyltransferase that corresponds to the loop4_2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively. The loop4_2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 has the amino acid sequence of SEQ ID NO:23.

[0071] In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO: 1 or SEQ ID NO:11, or a heterologous nucleic acid encoding the UDP-glycosyltransferase thereof, and further comprising any combination of the following:

(a) The loop1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, the amino acid sequence of SEQ ID NO:28, or a variant loop1 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively;

(b) the loop2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, or a variant loop2 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively;

(c) the loop3_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, or a variant loop3_1 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop 3_1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively;

(d) the loop3_2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, or a variant loop3_2 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop3_2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively;

(e) the loop4_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, or a variant loop4_1 amino acid sequence, at a location of the UDP-glycosyltransferase that corresponds to the loop4_1 location of SEQ ID NO:1 or SEQ ID NO:11, respectively; and

(f) the loop4_2 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11, at a location of the UDP-glycosyltransferase that corresponds to the loop4_2 location of SEQ ID NO:1 or SEQ ID NO:11, respectively.

[0072] In certain embodiments, when three-dimensional modeled structures of UDP-glycosyltransferases capable of converting RebA to RebD were compared and analyzed, it was discovered that loop4_1 of UGT40087, when incorporated into the corresponding loop4_1 location of another UDP-glycosyltransferase (and replacing its native loop4_1 amino acid sequence) led to superior activity of a variant UDP-glycosyltransferase in terms of its ability to convert RebA to RebD. *See* Example 12. These results indicate that the loop4_1 amino acid sequence of any suitable UDP-glycosyltransferase can be substituted with the loop4_1 amino acid sequence of SEQ ID NO:1 or SEQ ID NO:11 to convert RebA to RebD.

[0073] Therefore, in certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 61%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11, and further comprises the loop4_1 amino acid sequence (*i.e.*, SEQ ID NO:22) of UGT40087 (*i.e.*, SEQ ID NO:1 or SEQ ID NO:11). In certain embodiments, provided herein are host cells comprising a heterologous nucleic acid encoding an UDP-glycosyltransferase comprising an amino acid sequence that is at least 61%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11, and further comprises the loop4_1 amino acid sequence (*e.g.*, SEQ ID NO:22) of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, any suitable UDP-glycosyltransferase which comprises an amino acid sequence that is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% to SEQ ID NO:1 or SEQ ID NO:11 can be used to integrate the loop4_1 amino acid sequence from SEQ ID NO:1 or SEQ ID NO:11 at its corresponding loop4_1 location (replacing its native loop4_1 amino acid sequence). For example, Ob_UGT91B_like, Hv_UGT_V1, Sl_UGT_101249881, Sr.UGT_g252778, Os_UGT_91C1, Bd_UGT10840, Bd_UGT10850, or Si91Dlike can be used as a base to integrate the loop4_1 amino acid sequence from SEQ ID NO:1 or SEQ ID NO:11 at its corresponding loop4_1 location. In certain embodiments, the UDP-glycosyltransferase comprises an amino acid sequence of SEQ ID NO:25.

[0074] In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence substantially identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 60% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 65% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 70% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 75% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 85% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 96% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 97% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 98% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID

NO:11. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 99% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11.

[0075] In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a UGT40087 comprising the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence substantially identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 60% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 65% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 70% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 75% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 80% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 85% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 90% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 95% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein

are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 96% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 97% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 98% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a nucleic acid encoding a polypeptide comprising an amino acid sequence that is at least 99% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO:1 or SEQ ID NO:11.

[0076] In certain embodiments, the N-terminal sugar acceptor domains and the C-terminal sugar donor domains were recombined to either alter substrate specificity or catalytic activity. As described in detail in Example 11, it was determined that when a sugar donor domain from another UDP-glycosyltransferase capable of converting RebA to RebD was recombined with the sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11, the chimeric UDP-glycosyltransferases retained their ability to convert RebA to RebD.

[0077] Thus, in certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO: 1 or SEQ ID NO:11. In certain embodiments, provided herein are host cells comprising a heterologous nucleic acid encoding a UDP-glycosyltransferase comprising an amino acid sequence that is that least 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the amino acid sequence of the C-terminal sugar donor domain of SEQ ID NO: 1 or SEQ ID NO:11. As shown in Example 11, the C-terminal sugar donor domain is relatively exchangeable with other UDP-glycosyltransferase comprising a sequence identity of at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%. In certain embodiments, the UDP-glycosyltransferase further comprises a C-terminal sugar donor domain from other UDP-glycosyltransferase. Examples of other UDP-glycosyltransferases with suitable C-terminal sugar donor domains include Ob_UGT91B_like, Hv_UGT_V1, SI_UGT_101249881, Sr.UGT_g252778, Os_UGT_91C1, Bd_UGT10840, Bd_UGT10850, or Si91Dlike.

[0078] In certain embodiments, it was discovered that certain amino acid residues in the N-terminal sugar acceptor domain can restore the catalytic activity of a non-functional, putative UDP-glycosyltransferase into an active UDP-glycosyltransferase. Therefore, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of the N-terminal sugar acceptor domain of SEQ ID NO:1 or SEQ ID NO:11, and further comprises one or more of the following amino acid residues:

(a) valine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 11 of SEQ ID NO:11;

(b) isoleucine at an amino acid position of UDP-glycosyltransferase that corresponds to amino acid position 12 of SEQ ID NO:11;

(c) proline at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 55 of SEQ ID NO:11;

(d) glutamic acid at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 90 of SEQ ID NO:11;

(e) serine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 203 of SEQ ID NO:11;

(f) glutamic acid at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 223 of SEQ ID NO:11; or

(g) valine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 413 of SEQ ID NO:11,

wherein the amino acid positions of the UDP-glycosyltransferase that correspond to the amino acid positions of SEQ ID NO:11 are determined by sequence alignment.

[0079] In certain embodiments, provided herein are host cells comprising a UDP-glycosyltransferase comprising an amino acid sequence of SEQ ID NO:24.

[0080] In certain embodiments, the host cell comprises a variant of the UGT40087 polypeptide described above. In certain embodiments, the variant can comprise up to 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions relative to the UGT40087 polypeptide. In certain embodiments, the variant can comprise up to 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 conservative amino acid substitutions relative to the UGT40087 polypeptide. In certain embodiments, any of the nucleic acids described herein can be optimized for the host cell, for instance codon optimized.

[0081] In embodiments described herein, any suitable method can be used to determine corresponding amino acid positions or corresponding loop locations of two polypeptides. In certain embodiments, the sequences of a UDP-glycosyltransferase and the reference sequence SEQ ID NO:11 can be aligned using Clustal Omega using its default parameters. In other embodiment, the sequences of a UDP-glycosyltransferase and the reference sequence SEQ ID NO:11 can be aligned using structural alignments such as SWISS-MODEL, which is a protein structure homology-modelling server, accessible via the ExPASy web server, or from the program DeepView (Swiss Pdb-Viewer).

[0082] While SEQ ID NO:11 is referred to as the reference sequence for determining corresponding amino acid positions or loop locations for a UDP-glycosyltransferase, in certain embodiments, SEQ ID NO:1 can be also used as a reference sequence for sequence alignment.

[0083] In certain embodiments, RebA is as shown in **FIG. 1A**. In certain embodiments, a UGT40087 or a variant UGT40087 is capable of catalyzing the reaction of a sugar residue, forming the β linkage, to the C-2' position of the 19-O-glucose of RebA to produce RebD as shown in **FIG. 1A**. In certain embodiments, the UGT40087 or variant UGT40087 is capable of catalyzing the reaction of a hexose residue, in the β formation, to the C-2' of the 19-O-glucose of RebA. In certain embodiments, the UGT40087 is capable of catalyzing the reaction of a glucose residue, in the β formation, to the C-2' of the 19-O-glucose of RebA.

[0084] In certain embodiments, RebE is as shown in **FIG. 2B**. In certain embodiments, a UGT40087 or a variant UGT40087 is capable of catalyzing the reaction of a sugar residue, forming the β linkage, to the C-2' position of the 19-O-glucose of stevioside to produce RebE as shown in **FIG. 2B**. In certain embodiments, the UGT40087 or variant UGT40087 is capable of catalyzing the reaction of a hexose residue, in the β formation, to the C-2' of the 19-O-glucose of stevioside. In certain embodiments, the UGT40087 or variant UGT40087 is capable of catalyzing the reaction of a glucose residue, in the β formation, to the C-2' of the 19-O-glucose of stevioside.

[0085] In certain embodiments, a UGT40087 or a variant UGT40087 does not catalyze the reaction of adding a second sugar moiety to steviolmonoside (*i.e.*, 13-O-steviol glycoside) at a detectable level. In certain embodiments, the UGT40087 or variant UGT40087 does not catalyze the reaction of adding a second sugar moiety to rubusoside (*i.e.*, 19-O-steviol glycoside) at a detectable level.

[0086] In certain embodiments, RebD is as shown in **FIG. 1A**. In certain embodiments, the host cell further comprises one or more enzymes capable of converting RebA to RebD. In certain embodiments, the host cell comprises a UGT40087 and/or a variant UGT40087 for conversion of RebA to RebD.

[0087] In certain embodiments, a UGT76G1 is capable of catalyzing the reaction of a sugar residue to convert RebD to RebM as shown in **FIG. 1A**. In certain embodiments, the host cells further comprise one or more enzymes capable of converting RebD to RebM. In certain embodiments, the host cell further comprises a UGT76G1 capable of converting RebD to RebM.

[0088] In certain embodiments, RebM2 is shown in **FIG. 1B**. RebM2 is an isomer of RebM with a single wrong glucose linkage as shown in **FIG. 1B**. RebM2 has Glc β (1-2)[Glc β (1-6)]Glc β 1- at the 19 carbon position (COOH) instead of the desired Glc β (1-2)[Glc β (1-3)]Glc β 1- for RebM.

[0089] Those of skill will recognize that in certain applications, RebM2 might be an undesired side product. Advantageously, in certain embodiments, the UGT40087 (or variant UGT40087) and host cells provided herein are capable of producing little or no RebM2. The amount of RebM2 can be expressed as a ratio of RebM to RebM2. In certain embodiments, the ratio of RebM to RebM2 is at least 2:1. In certain embodiments, the ratio of RebM to RebM2 is at least 3:1. In certain embodiments, the ratio of RebM to RebM2 is at least 4:1. In certain embodiments, the ratio of RebM to RebM2 is at least 5:1. In certain embodiments, the ratio of RebM to RebM2 is at least 10:1. In certain embodiments, the ratio of RebM to RebM2 is at least 100:1. In certain embodiments, the ratio of RebM to RebM2 is at least 1000:1. In certain embodiments, the ratio of RebM to RebM2 is at least 10000:1. In certain embodiments, the UGT40087 (or variant UGT40087) and host cells provided herein produce an undetectable level of RebM2.

[0090] While the UGT40087 or any variant UGT40087 of the host cells accepts RebA as a substrate, the source of RebA can be any source deemed suitable to those of skill. In certain embodiments, the UGT40087 or any variant UGT40087 can be contacted with RebA. In certain embodiments, the host cell can be contacted with RebA. In certain embodiments, the UGT40087 or any variant of UGT40087 can be contacted with a composition comprising one or more steviol glycosides. In certain embodiments, the composition comprises RebA. In certain embodiments, the composition comprises stevioside. In certain embodiments, the composition is derived from natural products

isolated from *Stevia rebaudiana* leaves. In certain embodiments, the composition is microbially derived. In certain embodiments, the host cell can be contacted with a composition comprising one or more steviol glycosides.

[0091] In certain embodiments, any variant UGT40087 suitable for catalyzing a desired reaction can be screened for any suitable methods known in the art. For example, a suitable variant UGT40087 can be assayed *in vivo* by expressing a heterologous nucleic acid encoding a variant UGT40087 and screening cells that produce functional variant UGT40087 capable of adding a sugar at a desired location of a substrate (*e.g.*, the C2' position of the 19-O-glucose of a steviol glycosides or other substrates). Exemplary screening methods are described in Examples 3-7 below. In another example, a suitable variant UGT40087 can be screened *in vitro* by contacting a variant UGT40087 with a substrate such as RebA. In this example, assaying for the presence of RebD can be used as a test to determine whether a variant UGT40087 is suitable enzyme. The reaction can be analyzed by LC-MS or other known methods in the art. *See, e.g.* WO 2013/022989.

[0092] In certain embodiments, a variant UGT40087 is considered suitable in converting RebA to RebD (or from any suitable substrate to its product by glycosylation) if it is capable of converting RebA to RebD at an efficiency of greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, or 97% *in vivo*.

[0093] In some embodiments, other suitable UDP-glycosyltransferases discovered in the present application can be used in addition or in alternative to UGT40087. These include, for example, UDP-glycosyltransferases sr.UGT_9252778, Bd_UGT10840, Hv_UGT_V1, Bd_UGT10850, and Ob_UGT91B1_like. In certain embodiments, UDP-glycosyltransferase sr.UGT_9252778 comprises SEQ ID NO: 2. In certain embodiments, UDP-glycosyltransferase Bd_UGT10840 comprises SEQ ID NO: 3. In certain embodiments, UDP-glycosyltransferase Hv_UGT_V1 comprises SEQ ID NO: 4. In certain embodiments, UDP-glycosyltransferase Bd_UGT10850 comprises SEQ ID NO: 5. In certain embodiments, UDP-glycosyltransferase Ob_UGT91B1_like comprises SEQ ID NO: 6. Any discussions related to compositions and methods relevant to UGT40087 described herein may also apply to these other UDP-glycosyltransferases.

[0094] For instance, in some embodiments, the host cells comprise the enzyme activity of uridine diphosphate glycosyltransferase sr.UGT_9252778, Bd_UGT10840, Hv_UGT_V1, Bd_UGT10850, and/or Ob_UGT91B1_like. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 40%. In

certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 45%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 50%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 55%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 60%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 65%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 70%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 75%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 80%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 85%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 90%. In certain embodiments, one or more of these enzymes are capable of converting RebA to RebD at an efficiency of greater than 95%.

[0095] In certain embodiments, provided herein are host cells comprising any one or more of sr.UGT_9252778, Bd_UGT10840, Hv_UGT_V1, Bd_UGT10850, and/or Ob_UGT91B1_like comprising an amino acid sequence of SEQ ID NOS: 2, 3, 4, 5, or 6, respectively. In certain embodiments, provided herein are host cells comprising a polypeptide comprising an amino acid sequence that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to the amino acid sequence of SEQ ID NOS: 2, 3, 4, 5, or 6.

[0096] In advantageous embodiments, the host cell can comprise one or more enzymatic pathways capable of making RebA, said pathways taken individually or together. In certain embodiments, the host cells comprise one or more enzymes capable of converting geranylgeranyl diphosphate to RebA. Useful enzymes and nucleic acids encoding the enzymes are known to those of skill in the art. Particularly useful enzymes and nucleic acids are described in the sections below and further described, for example, in US 2014/0329281 A1, US 2014/0357588 A1, US 2015/0159188, WO 2016/038095 A2, and US 2016/0198748 A1.

[0097] In further embodiments, the host cells further comprise one or more enzymes capable of making geranylgeranyl diphosphate from a carbon source. These include enzymes of the DXP pathway and enzymes of the MEV pathway. Useful enzymes and nucleic acids encoding the enzymes are known to those of skill in the art. Exemplary enzymes of each pathway are described below and further described, for example, in US 2016/0177341 A1. The MEV pathway is also shown in **FIG. 1C**.

[0098] In certain embodiments, the additional enzymes are native. In advantageous embodiments, the additional enzymes are heterologous. In certain embodiments, two enzymes can be combined in one polypeptide.

6.3 Non-Naturally Occurring UDP-Glycosyltransferase Polypeptides and Nucleic Acids

[0099] In another aspect, provided herein are non-naturally occurring, variant UDP-glycosyltransferases which include modification(s) of amino acid residues compared to a reference sequence (*e.g.*, SEQ ID NO:1) and yet still retains the activity as a UDP-glycosyltransferase to convert RebA to RebD and/or from stevioside to RebE. In certain embodiments, non-naturally occurring, variant UDP-glycosyltransferases can include up to 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions, deletions, additions, and/or insertions at certain amino acid positions or locations compared to a reference sequence (*e.g.*, SEQ ID NO:1 or SEQ ID NO:11). In certain embodiments, non-naturally occurring, variant UDP-glycosyltransferases comprise any of the variant UDP-glycosyltransferases described herein, in particular those described in Section 6.2.

[00100] In another aspects, provided herein are non-naturally occurring, variant UDP-glycosyltransferases which include modification(s) of nucleic acid residues compared to a reference sequence (*e.g.*, SEQ ID NO:26), and yet, when translated into a protein, the protein retains the activity as a UDP-glycosyltransferase to convert RebA to RebD and/or to convert stevioside to RebE. In certain embodiments, non-naturally occurring, variant UDP-glycosyltransferases can encode any of the variant UDP-glycosyltransferases described herein, in particular those described in Section 6.2.

6.4 Cell Strains

[00101] Host cells useful compositions and methods provided herein include archae, prokaryotic, or eukaryotic cells.

[00102] Suitable prokaryotic hosts include, but are not limited, to any of a variety of gram-positive, gram-negative, or gram-variable bacteria. Examples include, but are not limited to, cells belonging to the genera: *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azobacter*, *Bacillus*, *Brevibacterium*, *Chromatium*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Phormidium*, *Pseudomonas*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodococcus*, *Salmonella*, *Scenedesmun*, *Serratia*, *Shigella*, *Staphylococcus*, *Streptomyces*, *Synnecoccus*, and *Zymomonas*. Examples of prokaryotic strains include, but are not limited to: *Bacillus subtilis*, *Bacillus amyloliquefacines*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum*, *Clostridium beigerinckii*, *Enterobacter sakazakii*, *Escherichia coli*, *Lactococcus lactis*, *Mesorhizobium loti*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Pseudomonas pudica*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Salmonella enterica*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Staphylococcus aureus*. In a particular embodiment, the host cell is an *Escherichia coli* cell.

[00103] Suitable archae hosts include, but are not limited to, cells belonging to the genera: *Aeropyrum*, *Archaeoglobus*, *Halobacterium*, *Methanococcus*, *Methanobacterium*, *Pyrococcus*, *Sulfolobus*, and *Thermoplasma*. Examples of archae strains include, but are not limited to: *Archaeoglobus fulgidus*, *Halobacterium sp.*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, *Pyrococcus horikoshii*, *Pyrococcus abyssi*, and *Aeropyrum pernix*.

[00104] Suitable eukaryotic hosts include, but are not limited to, fungal cells, algal cells, insect cells, and plant cells. In some embodiments, yeasts useful in the present methods include yeasts that have been deposited with microorganism depositories (e.g. IFO, ATCC, etc.) and belong to the genera *Aciculoconidium*, *Ambrosiozyma*, *Arthroascus*, *Arxiozyma*, *Ashbya*, *Babjevia*, *Bensingtonia*, *Botryoascus*, *Botryozyma*, *Brettanomyces*, *Bullera*, *Bulleromyces*, *Candida*, *Citeromyces*, *Clavispora*, *Cryptococcus*, *Cystofilobasidium*, *Debaryomyces*, *Dekkara*, *Dipodascopsis*, *Dipodascus*, *Eeniella*, *Endomycopsella*, *Eremascus*, *Eremothecium*, *Erythrobasidium*, *Fellomyces*, *Filobasidium*, *Galactomyces*, *Geotrichum*, *Guilliermondella*, *Hanseniaspora*, *Hansenula*, *Hasegawaea*, *Holtermannia*, *Hormoascus*, *Hyphopichia*, *Issatchenkia*, *Kloeckera*, *Kloeckeraspora*, *Kluyveromyces*, *Kondoa*, *Kuraishia*, *Kurtzmanomyces*, *Leucosporidium*, *Lipomyces*, *Lodderomyces*,

Malassezia, Metschnikowia, Mrakia, Myxozyma, Nadsonia, Nakazawaea, Nematospora, Ogataea, Oosporidium, Pachysolen, Phachytichospora, Phaffia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Saitoella, Sakaguchia, Saturnospora, Schizoblastosporion, Schizosaccharomyces, Schwanniomyces, Sporidiobolus, Sporobolomyces, Sporopachydermia, Stephanoascus, Sterigmatomyces, Sterigmatosporidium, Symbiotaphrina, Sympodiomyces, Sympodiomycopsis, Torulaspora, Trichosporiella, Trichosporon, Trigonopsis, Tsuchiyaea, Udeniomyces, Waltomyces, Wickerhamia, Wickerhamiella, Williopsis, Yamadazyma, Yarrowia, Zygoascus, Zygosaccharomyces, Zygowilliopsis, and Zygozima, among others.

[00105] In some embodiments, the host microbe is *Saccharomyces cerevisiae*, *Pichia pastoris*, *Schizosaccharomyces pombe*, *Dekkera bruxellensis*, *Kluyveromyces lactis* (previously called *Saccharomyces lactis*), *Kluveromyces marxianus*, *Arxula adeninivorans*, or *Hansenula polymorpha* (now known as *Pichia angusta*). In some embodiments, the host microbe is a strain of the genus *Candida*, such as *Candida lipolytica*, *Candida guilliermondii*, *Candida krusei*, *Candida pseudotropicalis*, or *Candida utilis*.

[00106] In a particular embodiment, the host microbe is *Saccharomyces cerevisiae*. In some embodiments, the host is a strain of *Saccharomyces cerevisiae* selected from the group consisting of Baker's yeast, CBS 7959, CBS 7960, CBS 7961, CBS 7962, CBS 7963, CBS 7964, IZ-1904, TA, BG-1, CR-1, SA-1, M-26, Y-904, PE-2, PE-5, VR-1, BR-1, BR-2, ME-2, VR-2, MA-3, MA-4, CAT-1, CB-1, NR-1, BT-1, and AL-1. In some embodiments, the host microbe is a strain of *Saccharomyces cerevisiae* selected from the group consisting of PE-2, CAT-1, VR-1, BG-1, CR-1, and SA-1. In a particular embodiment, the strain of *Saccharomyces cerevisiae* is PE-2. In another particular embodiment, the strain of *Saccharomyces cerevisiae* is CAT-1. In another particular embodiment, the strain of *Saccharomyces cerevisiae* is BG-1.

[00107] In some embodiments, the host microbe is a microbe that is suitable for industrial fermentation. In particular embodiments, the microbe is conditioned to subsist under high solvent concentration, high temperature, expanded substrate utilization, nutrient limitation, osmotic stress due to sugar and salts, acidity, sulfite and bacterial contamination, or combinations thereof, which are recognized stress conditions of the industrial fermentation environment.

6.5 The Steviol and Steviol Glycoside Biosynthesis Pathways

[00108] In some embodiments, a steviol biosynthesis pathway and/or a steviol glycoside biosynthesis pathway is activated in the genetically modified host cells provided herein by engineering the cells to express polynucleotides and/or polypeptides encoding one or more enzymes of the pathway. **FIG. 2A** illustrates an exemplary steviol biosynthesis pathway. **FIG. 2B** illustrates an exemplary steviol glycoside biosynthesis pathway starting from steviol to various steviol glycosides.

[00109] Thus, in some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having geranylgeranyl diphosphate synthase (GGPPS) activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having copalyl diphosphate synthase or ent-copalyl pyrophosphate synthase (CDPS; also referred to as ent-copalyl pyrophosphate synthase or CPS) activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having kaurene synthase (KS; also referred to as ent-kaurene synthase) activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having kaurene oxidase (KO; also referred to as ent-kaurene 19-oxidase) activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having steviol synthase (also referred to as ent-kaurenoic acid 13-hydroxylase or KAH) activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having cytochrome P450 reductase (CPR) activity.

[00110] In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having UGT74G1 activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having UGT76G1 activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having UGT85C2 activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having UGT91D activity. In some embodiments, the genetically modified host cells provided herein comprise a heterologous polynucleotide encoding a polypeptide having UGT40087 activity.

[00111] In certain embodiments, the host cell comprises a variant. In certain embodiments, the variant can comprise up to 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid substitutions relative to the relevant polypeptide. In certain embodiments, the variant can comprise up to 15, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 conservative amino acid substitutions relative to the reference polypeptide. In certain embodiments, any of the nucleic acids described herein can be optimized for the host cell, for instance codon optimized.

[00112] Exemplary nucleic acids and enzymes of a steviol biosynthesis pathway and/or a steviol glycoside biosynthesis pathway are described below.

6.5.1 Geranylgeranyl diphosphate synthase (GGPPS)

[00113] Geranylgeranyl diphosphate synthases (EC 2.5.1.29) catalyze the conversion of farnesyl pyrophosphate into geranylgeranyl diphosphate (also known as geranylgeranyl pyrophosphate). Illustrative examples of enzymes include those of *Stevia rebaudiana* (accession no. ABD92926), *Gibberella fujikuroi* (accession no. CAA75568), *Mus musculus* (accession no. AAH69913), *Thalassiosira pseudonana* (accession no. XP_002288339), *Streptomyces clavuligerus* (accession no. ZP_05004570), *Sulfolobus acidocaldarius* (accession no. BAA43200), *Synechococcus sp.* (accession no. ABC98596), *Arabidopsis thaliana* (accession no. NP_195399), *Blakeslea trispora* (accession no. AFC92798.1) and US 2014/0329281 A1. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these GGPPS nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, 95% sequence identity to at least one of these GGPPS enzymes.

6.5.2 Copalyl diphosphate synthase (CDPS)

[00114] Copalyl diphosphate synthases (EC 5.5.1.13) catalyze the conversion of geranylgeranyl diphosphate into copalyl diphosphate. Illustrative examples of enzymes include those of *Stevia rebaudiana* (accession no. AAB87091), *Streptomyces clavuligerus* (accession no. EDY51667), *Bradyrhizobium japonicum* (accession no. AAC28895.1), *Zea mays* (accession no. AY562490), *Arabidopsis thaliana* (accession no. NM_116512), *Oryza sativa* (accession no. Q5MQ85.1) and US 2014/0329281 A1. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%,

or 95% sequence identity to at least one of these CDPS nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 95%, 90%, or 95% sequence identity to at least one of these CDPS enzymes.

6.5.3 Kaurene synthase (KS)

[00115] Kaurene synthases (EC 4.2.3.19) catalyze the conversion of copalyl diphosphate into kaurene and diphosphate. Illustrative examples of enzymes include those of *Bradyrhizobium japonicum* (accession no. AAC28895.1), *Phaeosphaeria* sp. (accession no. O13284), *Arabidopsis thaliana* (accession no. Q9SAK2), *Picea glauca* (accession no. ADB55711.1) and US 2014/0329281 A1. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these KS nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 85%, 90%, or 95% sequence identity to at least one of these KS enzymes.

6.5.4 Bifunctional copalyl diphosphate synthase (CDPS) and kaurene synthase (KS)

[00116] CDPS-KS bifunctional enzymes (EC 5.5.1.13 and EC 4.2.3.19) also can be used. Illustrative examples of enzymes include those of *Phomopsis amygdali* (accession no. BAG30962), *Physcomitrella patens* (accession no. BAF61135), *Gibberella fujikuroi* (accession no. Q9UVY5.1), and US 2014/0329281 A1, US 2014/0357588 A1, US 2015/0159188, and WO 2016/038095 A2. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these CDPS-KS nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these CDPS-KS enzymes.

6.5.5 Ent-Kaurene oxidase (KO)

[00117] Ent-kaurene oxidases (EC 1.14.13.78; also referred to as kaurene oxidases herein) catalyze the conversion of kaurene into kaurenoic acid. Illustrative examples of enzymes include those of *Oryza sativa* (accession no. Q5Z5R4), *Gibberella fujikuroi*

(accession no. O94142), *Arabidopsis thaliana* (accession no. Q93ZB2), *Stevia rebaudiana* (accession no. AAQ63464.1), *Pisum sativum* (Uniprot no. Q6XAF4) and US 2014/0329281 A1, US 2014/0357588 A1, US 2015/0159188, and WO 2016/038095 A2. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these KO nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these KO enzymes.

6.5.6 Steviol synthase (KAH)

[00118] Steviol synthases, or kaurenoic acid hydroxylases (KAH), (EC 1.14.13) catalyze the conversion of kaurenoic acid into steviol. Illustrative examples of enzymes include those of *Stevia rebaudiana* (accession no. ACD93722), *Stevia rebaudiana* (SEQ ID NO:10) *Arabidopsis thaliana* (accession no. NP_197872), *Vitis vinifera* (accession no. XP_002282091), *Medicago trunculata* (accession no. ABC59076), and US 2014/0329281 A1, US 2014/0357588 A1, US 2015/0159188, and WO 2016/038095 A2. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these KAH nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these KAH enzymes.

6.5.7 Cytochrome P450 reductase (CPR)

[00119] Cytochrome P450 reductases (EC 1.6.2.4) are capable of assisting or facilitating the activity of KO and/or KAH above. Illustrative examples of enzymes include those of *Stevia rebaudiana* (accession no. ABB88839) *Arabidopsis thaliana* (accession no. NP_194183), *Gibberella fujikuroi* (accession no. CAE09055), *Artemisia annua* (accession no. ABC47946.1) and US 2014/0329281 A1, US 2014/0357588 A1, US 2015/0159188, and WO 2016/038095 A2. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these CPR nucleic acids. In certain embodiments, provided herein are cells and methods

using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these CPR enzymes.

6.5.8 UDP glycosyltransferase 74G1 (UGT74G1)

[00120] A UGT74G1 is capable of functioning as a uridine 5'-diphospho glucosyl:steviol 19-COOH transferase and as a uridine 5'-diphospho glucosyl:steviol-13-O-glucoside 19-COOH transferase. As shown in **FIG. 2B**, a UGT74G1 is capable of converting steviol to 19-glycoside. A UGT74G1 is also capable of converting steviolmonoside to rubusoside. A UGT74G1 may be also capable of converting steviolbioside to stevioside. Illustrative examples of enzymes include those of *Stevia rebaudiana* (e.g., those of Richman *et al.*, 2005, *Plant J.* 41: 56-67 and US 2014/0329281 and WO 2016/038095 A2 and accession no. AAR06920.1). Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT74G1 nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT74G1 enzymes.

6.5.9 UDP glycosyltransferase 76G1 (UGT76G1)

[00121] A UGT76G1 is capable of transferring a glucose moiety to the C-3' of the C-13-O-glucose of the acceptor molecule, a steviol 1,2 glycoside. Thus, a UGT76G1 is capable of functioning as a uridine 5'-diphospho glucosyl: steviol 13-O-1,2 glucoside C-3' glucosyl transferase and a uridine 5'-diphospho glucosyl: steviol-19-O-glucose, 13-O-1,2 bioside C-3' glucosyl transferase. As shown in **FIG. 2A**, a UGT76G1 is capable of converting steviolbioside to RebB. A UGT76G1 is also capable of converting stevioside to RebA. A UGT76G1 is also capable of converting RebD to RebM. Illustrative examples of enzymes include those of *Stevia rebaudiana* (e.g., those of Richman *et al.*, 2005, *Plant J.* 41: 56-67 and US 2014/0329281 A1 and WO 2016/038095 A2 and accession no. AAR06912.1). Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT76G1 nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT76G1 enzymes.

6.5.10 UDP glycosyltransferase 85C2 (UGT85C2)

[00122] A UGT85C2 is capable of functioning as a uridine 5'-diphospho glucosyl:steviol 13-OH transferase, and a uridine 5'-diphospho glucosyl:steviol-19-O-glucoside 13-OH transferase. Thus, as shown in **FIG. 2B**, a UGT85C2 is capable of converting steviol to steviolmonoside, and is also capable of converting 19-glycoside to rubusoside. Illustrative examples of enzymes include those of *Stevia rebaudiana* (e.g., those of Richman *et al.*, 2005, *Plant J.* 41: 56-67 and US 2014/0329281 A1 and WO 2016/038095 A2 and accession no. AAR06916.1). Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT85C2 nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT85C2 enzymes.

6.5.11 UDP-glycosyltransferase 91D (UGT91D)

[00123] A UGT91D is capable of functioning as a uridine 5'-diphosphoglucosyl:steviol-13-O-glucoside transferase, transferring a glucose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, steviol-13-O-glucoside (steviolmonoside) to produce steviobioside. A UGT91D is also capable of functioning as a uridine 5'-diphospho glucosyl:rubusoside transferase, transferring a glucose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, rubusoside, to provide stevioside as shown in **FIG. 2B**. UGT91D is also capable of transferring a glucose moiety to the C-2' position of the 19-O-glucose of RebA to produce RebD as shown in **FIG. 2B**. A UGT91D is also referred to as UGT91D2, UGT91D2e, or UGT91D-like3. Illustrative examples of UGT91D enzymes include those of *Stevia rebaudiana* (e.g., those of UGT sequence with accession no. ACE87855.1, US 2014/0329281 A1, WO 2016/038095 A2, and SEQ ID NO:7). Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT91D nucleic acids. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT91D enzymes.

6.5.12 UDP-glycosyltransferase 40087 (UGT40087)

[00124] A UGT40087 is capable of transferring a glucose moiety to the C-2' position of the 19-O-glucose of RebA to produce RebD as shown in **FIG. 2B**. A UGT40087 is also capable of transferring a glucose moiety to the C-2' position of the 19-O-glucose of stevioside to produce RebE. Illustrative examples of UGT40087 are described above in Section 5.2. Any UGT40087 variant described herein can be used in the compositions and methods described herein. Nucleic acids encoding these enzymes are useful in the cells and methods provided herein. In certain embodiments, provided herein are cells and methods using a nucleic acid having at least 80%, 85%, 90%, or 95% sequence identity to at least one of the UGT40087 enzymes. In certain embodiments, provided herein are cells and methods using a nucleic acid that encodes a polypeptide having at least 80%, 85%, 90%, or 95% sequence identity to at least one of these UGT40087 enzymes. In certain embodiments, provided herein are a nucleic acid that encodes a UGT40087 variant described herein.

6.6 MEV Pathway FPP and/or GGPP Production

[00125] In some embodiments, a genetically modified host cell provided herein comprises one or more heterologous enzymes of the MEV pathway, useful for the formation of FPP and/or GGPP. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that condenses acetoacetyl-CoA with acetyl-CoA to form HMG-CoA. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts HMG-CoA to mevalonate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that phosphorylates mevalonate to mevalonate 5-phosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-phosphate to mevalonate 5-pyrophosphate. In some embodiments, the one or more enzymes of the MEV pathway comprise an enzyme that converts mevalonate 5-pyrophosphate to isopentenyl pyrophosphate.

[00126] In some embodiments, the one or more enzymes of the MEV pathway are selected from the group consisting of acetyl-CoA thiolase, acetoacetyl-CoA synthetase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase

and mevalonate pyrophosphate decarboxylase. In some embodiments, with regard to the enzyme of the MEV pathway capable of catalyzing the formation of acetoacetyl-CoA, the genetically modified host cell comprises either an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, *e.g.*, acetyl-CoA thiolase; or an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA, *e.g.*, acetoacetyl-CoA synthase. In some embodiments, the genetically modified host cell comprises both an enzyme that condenses two molecules of acetyl-CoA to form acetoacetyl-CoA, *e.g.*, acetyl-CoA thiolase; and an enzyme that condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA, *e.g.*, acetoacetyl-CoA synthase.

[00127] In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding more than one enzyme of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding two enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding an enzyme that can convert HMG-CoA into mevalonate and an enzyme that can convert mevalonate into mevalonate 5-phosphate. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding three enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding four enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding five enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding six enzymes of the MEV pathway. In some embodiments, the host cell comprises one or more heterologous nucleotide sequences encoding seven enzymes of the MEV pathway. In some embodiments, the host cell comprises a plurality of heterologous nucleic acids encoding all of the enzymes of the MEV pathway.

[00128] In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an enzyme that can convert isopentenyl pyrophosphate (IPP) into dimethylallyl pyrophosphate (DMAPP). In some embodiments, the genetically modified host cell further comprises a heterologous nucleic acid encoding an enzyme that can condense IPP and/or DMAPP molecules to form a polyprenyl compound. In some embodiments, the genetically modified host cell further comprise a heterologous nucleic acid encoding an enzyme that can modify IPP or a polyprenyl to form an isoprenoid compound such as farnesene.

6.6.1 Conversion of Acetyl-CoA to Acetoacetyl-CoA

[00129] In some embodiments, the genetically modified host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense two molecules of acetyl-coenzyme A to form acetoacetyl-CoA, *e.g.*, an acetyl-CoA thiolase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (NC_000913 REGION: 2324131.2325315; *Escherichia coli*), (D49362; *Paracoccus denitrificans*), and (L20428; *Saccharomyces cerevisiae*).

[00130] Acetyl-CoA thiolase catalyzes the reversible condensation of two molecules of acetyl-CoA to yield acetoacetyl-CoA, but this reaction is thermodynamically unfavorable; acetoacetyl-CoA thiolysis is favored over acetoacetyl-CoA synthesis. Acetoacetyl-CoA synthase (AACS) (alternately referred to as acetyl-CoA:malonyl-CoA acyltransferase; EC 2.3.1.194) condenses acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. In contrast to acetyl-CoA thiolase, AACS-catalyzed acetoacetyl-CoA synthesis is essentially an energy-favored reaction, due to the associated decarboxylation of malonyl-CoA. In addition, AACS exhibits no thiolysis activity against acetoacetyl-CoA, and thus the reaction is irreversible.

[00131] In host cells comprising acetyl-CoA thiolase and a heterologous ADA and/or phosphotransacetylase (PTA), the reversible reaction catalyzed by acetyl-CoA thiolase, which favors acetoacetyl-CoA thiolysis, may result in a large acetyl-CoA pool. In view of the reversible activity of ADA, this acetyl-CoA pool may in turn drive ADA towards the reverse reaction of converting acetyl-CoA to acetaldehyde, thereby diminishing the benefits provided by ADA towards acetyl-CoA production. Similarly, the activity of PTA is reversible, and thus, a large acetyl-CoA pool may drive PTA towards the reverse reaction of converting acetyl-CoA to acetyl phosphate. Therefore, in some embodiments, in order to provide a strong pull on acetyl-CoA to drive the forward reaction of ADA and PTA, the MEV pathway of the genetically modified host cell provided herein utilizes an acetoacetyl-CoA synthase to form acetoacetyl-CoA from acetyl-CoA and malonyl-CoA.

[00132] In some embodiments, the AACS is from *Streptomyces* sp. strain CL190 (Okamura *et al.*, *Proc Natl Acad Sci USA* 107(25):11265-70 (2010). Representative AACS nucleotide sequences of *Streptomyces* sp. strain CL190 include accession number AB540131.1. Representative AACS protein sequences of *Streptomyces* sp. strain CL190 include accession numbers D7URV0, BAJ10048. Other acetoacetyl-CoA synthases useful for the compositions and methods provided herein include, but are not limited to, *Streptomyces* sp. (AB183750; KO-3988 BAD86806); *S. anulatus* strain 9663 (FN178498;

CAX48662); *Streptomyces* sp. KO-3988 (AB212624; BAE78983); *Actinoplanes* sp. A40644 (AB113568; BAD07381); *Streptomyces* sp. C (NZ_ACEW010000640; ZP_05511702); *Nocardiopsis dassonvillei* DSM 43111 (NZ_ABUI01000023; ZP_04335288); *Mycobacterium ulcerans* Agy99 (NC_008611; YP_907152); *Mycobacterium marinum* M (NC_010612; YP_001851502); *Streptomyces* sp. Mg1 (NZ_DS570501; ZP_05002626); *Streptomyces* sp. AA4 (NZ_ACEV01000037; ZP_05478992); *S. roseosporus* NRRL 15998 (NZ_ABYB01000295; ZP_04696763); *Streptomyces* sp. ACTE (NZ_ADFD01000030; ZP_06275834); *S. viridochromogenes* DSM 40736 (NZ_ACEZ01000031; ZP_05529691); *Frankia* sp. CcI3 (NC_007777; YP_480101); *Nocardia brasiliensis* (NC_018681; YP_006812440.1); and *Austwickia chelonae* (NZ_BAGZ01000005; ZP_10950493.1). Additional suitable acetoacetyl-CoA synthases include those described in U.S. Patent Application Publication Nos. 2010/0285549 and 2011/0281315, the contents of which are incorporated by reference in their entireties.

[00133] Acetoacetyl-CoA synthases also useful in the compositions and methods provided herein include those molecules which are said to be “derivatives” of any of the acetoacetyl-CoA synthases described herein. Such a “derivative” has the following characteristics: (1) it shares substantial homology with any of the acetoacetyl-CoA synthases described herein; and (2) is capable of catalyzing the irreversible condensation of acetyl-CoA with malonyl-CoA to form acetoacetyl-CoA. A derivative of an acetoacetyl-CoA synthase is said to share “substantial homology” with acetoacetyl-CoA synthase if the amino acid sequences of the derivative is at least 80%, and more preferably at least 90%, and most preferably at least 95%, the same as that of acetoacetyl-CoA synthase.

6.6.2 Conversion of Acetoacetyl-CoA to HMG-CoA

[00134] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense acetoacetyl-CoA with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), *e.g.*, a HMG-CoA synthase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (NC_001145, complement 19061.20536; *Saccharomyces cerevisiae*), (X96617; *Saccharomyces cerevisiae*), (X83882; *Arabidopsis thaliana*), (AB037907; *Kitasatospora griseola*), (BT007302; *Homo sapiens*), and (NC_002758, Locus tag SAV2546, GeneID 1122571; *Staphylococcus aureus*).

6.6.3 Conversion of HMG-CoA to Mevalonate

[00135] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert HMG-CoA into mevalonate, *e.g.*, a HMG-CoA reductase. In some embodiments, HMG-CoA reductase is an NADH-using hydroxymethylglutaryl-CoA reductase-CoA reductase. HMG-CoA reductases (EC 1.1.1.34; EC 1.1.1.88) catalyze the reductive deacylation of (S)-HMG-CoA to (R)-mevalonate, and can be categorized into two classes, class I and class II HMGRs. Class I includes the enzymes from eukaryotes and most archaea, and class II includes the HMG-CoA reductases of certain prokaryotes and archaea. In addition to the divergence in the sequences, the enzymes of the two classes also differ with regard to their cofactor specificity. Unlike the class I enzymes, which utilize NADPH exclusively, the class II HMG-CoA reductases vary in the ability to discriminate between NADPH and NADH. *See, e.g., Hedl et al., Journal of Bacteriology* 186 (7): 1927-1932 (2004). Co-factor specificities for select class II HMG-CoA reductases are provided below.

Table 1. Co-factor specificities for select class II HMG-CoA reductases

Source	Coenzyme specificity	K_m^{NADPH} (μM)	K_m^{NADH} (μM)
<i>P. mevalonii</i>	NADH		80
<i>A. fulgidus</i>	NAD(P)H	500	160
<i>S. aureus</i>	NAD(P)H	70	100
<i>E. faecalis</i>	NADPH	30	

[00136] Useful HMG-CoA reductases for the compositions and methods provided herein include HMG-CoA reductases that are capable of utilizing NADH as a cofactor, *e.g.*, HMG-CoA reductase from *P. mevalonii*, *A. fulgidus* or *S. aureus*. In particular embodiments, the HMG-CoA reductase is capable of only utilizing NADH as a cofactor, *e.g.*, HMG-CoA reductase from *P. mevalonii*, *S. pomeroyi* or *D. acidovorans*.

[00137] In some embodiments, the NADH-using HMG-CoA reductase is from *Pseudomonas mevalonii*. The sequence of the wild-type *mvaA* gene of *Pseudomonas mevalonii*, which encodes HMG-CoA reductase (EC 1.1.1.88), has been previously described. *See* Beach and Rodwell, *J. Bacteriol.* 171:2994-3001 (1989). Representative *mvaA* nucleotide sequences of *Pseudomonas mevalonii* include accession number M24015.

Representative HMG-CoA reductase protein sequences of *Pseudomonas mevalonii* include accession numbers AAA25837, P13702, MVAA_PSEMV.

[00138] In some embodiments, the NADH-using HMG-CoA reductase is from *Silicibacter pomeroyi*. Representative HMG-CoA reductase nucleotide sequences of *Silicibacter pomeroyi* include accession number NC_006569.1. Representative HMG-CoA reductase protein sequences of *Silicibacter pomeroyi* include accession number YP_164994.

[00139] In some embodiments, the NADH-using HMG-CoA reductase is from *Delftia acidovorans*. A representative HMG-CoA reductase nucleotide sequences of *Delftia acidovorans* includes NC_010002 REGION: complement (319980..321269).

Representative HMG-CoA reductase protein sequences of *Delftia acidovorans* include accession number YP_001561318.

[00140] In some embodiments, the NADH-using HMG-CoA reductases is from *Solanum tuberosum* (Crane *et al.*, *J. Plant Physiol.* 159:1301-1307 (2002)).

[00141] NADH-using HMG-CoA reductases also useful in the compositions and methods provided herein include those molecules which are said to be “derivatives” of any of the NADH-using HMG-CoA reductases described herein, *e.g.*, from *P. mevalonii*, *S. pomeroyi* and *D. acidovorans*. Such a “derivative” has the following characteristics: (1) it shares substantial homology with any of the NADH-using HMG-CoA reductases described herein; and (2) is capable of catalyzing the reductive deacylation of (S)-HMG-CoA to (R)-mevalonate while preferentially using NADH as a cofactor. A derivative of an NADH-using HMG-CoA reductase is said to share “substantial homology” with NADH-using HMG-CoA reductase if the amino acid sequences of the derivative is at least 80%, and more preferably at least 90%, and most preferably at least 95%, the same as that of NADH-using HMG-CoA reductase.

[00142] As used herein, the phrase “NADH-using” means that the NADH-using HMG-CoA reductase is selective for NADH over NADPH as a cofactor, for example, by demonstrating a higher specific activity for NADH than for NADPH. In some embodiments, selectivity for NADH as a cofactor is expressed as a $k_{\text{cat}}^{\text{(NADH)}} / k_{\text{cat}}^{\text{(NADPH)}}$ ratio. In some embodiments, the NADH-using HMG-CoA reductase has a $k_{\text{cat}}^{\text{(NADH)}} / k_{\text{cat}}^{\text{(NADPH)}}$ ratio of at least 5, 10, 15, 20, 25 or greater than 25. In some embodiments, the NADH-using HMG-CoA reductase uses NADH exclusively. For example, an NADH-using HMG-CoA reductase that uses NADH exclusively displays some activity with NADH supplied as the sole cofactor *in vitro*, and displays no detectable activity when NADPH is

supplied as the sole cofactor. Any method for determining cofactor specificity known in the art can be utilized to identify HMG-CoA reductases having a preference for NADH as cofactor, including those described by Kim *et al.*, *Protein Science* 9:1226-1234 (2000); and Wilding *et al.*, *J. Bacteriol.* 182(18):5147-52 (2000), the contents of which are hereby incorporated in their entireties.

[00143] In some embodiments, the NADH-using HMG-CoA reductase is engineered to be selective for NADH over NADPH, for example, through site-directed mutagenesis of the cofactor-binding pocket. Methods for engineering NADH-selectivity are described in Watanabe *et al.*, *Microbiology* 153:3044-3054 (2007), and methods for determining the cofactor specificity of HMG-CoA reductases are described in Kim *et al.*, *Protein Sci.* 9:1226-1234 (2000), the contents of which are hereby incorporated by reference in their entireties.

[00144] In some embodiments, the NADH-using HMG-CoA reductase is derived from a host species that natively comprises a mevalonate degradative pathway, for example, a host species that catabolizes mevalonate as its sole carbon source. Within these embodiments, the NADH-using HMG-CoA reductase, which normally catalyzes the oxidative acylation of internalized (R)-mevalonate to (S)-HMG-CoA within its native host cell, is utilized to catalyze the reverse reaction, that is, the reductive deacylation of (S)-HMG-CoA to (R)-mevalonate, in a genetically modified host cell comprising a mevalonate biosynthetic pathway. Prokaryotes capable of growth on mevalonate as their sole carbon source have been described by: Anderson *et al.*, *J. Bacteriol.*, 171(12):6468-6472 (1989); Beach *et al.*, *J. Bacteriol.* 171:2994-3001 (1989); Bensch *et al.*, *J. Biol. Chem.* 245:3755-3762; Fimongnari *et al.*, *Biochemistry* 4:2086-2090 (1965); Siddiqi *et al.*, *Biochem. Biophys. Res. Commun.* 8:110-113 (1962); Siddiqi *et al.*, *J. Bacteriol.* 93:207-214 (1967); and Takatsuji *et al.*, *Biochem. Biophys. Res. Commun.* 110:187-193 (1983), the contents of which are hereby incorporated by reference in their entireties.

[00145] In some embodiments of the compositions and methods provided herein, the host cell comprises both a NADH-using HMGr and an NADPH-using HMG-CoA reductase. Illustrative examples of nucleotide sequences encoding an NADPH-using HMG-CoA reductase include, but are not limited to: (NM_206548; *Drosophila melanogaster*), (NC_002758, Locus tag SAV2545, GeneID 1122570; *Staphylococcus aureus*), (AB015627; *Streptomyces sp.* KO 3988), (AX128213, providing the sequence encoding a truncated

HMG-CoA reductase; *Saccharomyces cerevisiae*), and (NC_001145: complement (115734.118898; *Saccharomyces cerevisiae*).

6.6.4 Conversion of Mevalonate to Mevalonate-5-Phosphate

[00146] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert mevalonate into mevalonate 5-phosphate, *e.g.*, a mevalonate kinase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (L77688; *Arabidopsis thaliana*), and (X55875; *Saccharomyces cerevisiae*).

6.6.5 Conversion of Mevalonate-5-Phosphate to Mevalonate-5-Pyrophosphate

[00147] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert mevalonate 5-phosphate into mevalonate 5-pyrophosphate, *e.g.*, a phosphomevalonate kinase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (AF429385; *Hevea brasiliensis*), (NM_006556; *Homo sapiens*), and (NC_001145: complement 712315.713670; *Saccharomyces cerevisiae*).

6.6.6 Conversion of Mevalonate-5-Pyrophosphate to IPP

[00148] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can convert mevalonate 5-pyrophosphate into isopentenyl diphosphate (IPP), *e.g.*, a mevalonate pyrophosphate decarboxylase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (X97557; *Saccharomyces cerevisiae*), (AF290095; *Enterococcus faecium*), and (U49260; *Homo sapiens*).

6.6.7 Conversion of IPP to DMAPP

[00149] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding an enzyme that can convert IPP generated via the MEV pathway into dimethylallyl pyrophosphate (DMAPP), *e.g.*, an IPP isomerase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (NC_000913, 3031087.3031635; *Escherichia coli*), and (AF082326; *Haematococcus pluvialis*).

6.6.8 Polyprenyl Synthases

[00150] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding a polyprenyl synthase that can condense IPP and/or DMAPP molecules to form polyprenyl compounds containing more than five carbons.

[00151] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense one molecule of IPP with one molecule of DMAPP to form one molecule of geranyl pyrophosphate (“GPP”), *e.g.*, a GPP synthase. Illustrative examples of nucleotide sequences encoding such an enzyme include, but are not limited to: (AF513111; *Abies grandis*), (AF513112; *Abies grandis*), (AF513113; *Abies grandis*), (AY534686; *Antirrhinum majus*), (AY534687; *Antirrhinum majus*), (Y17376; *Arabidopsis thaliana*), (AE016877, Locus AP11092; *Bacillus cereus*; ATCC 14579), (AJ243739; *Citrus sinensis*), (AY534745; *Clarkia breweri*), (AY953508; *Ips pini*), (DQ286930; *Lycopersicon esculentum*), (AF182828; *Mentha x piperita*), (AF182827; *Mentha x piperita*), (MPI249453; *Mentha x piperita*), (PZE431697, Locus CAD24425; *Paracoccus zeaxanthinifaciens*), (AY866498; *Picrorhiza kurrooa*), (AY351862; *Vitis vinifera*), and (AF203881, Locus AAF12843; *Zymomonas mobilis*).

[00152] In some embodiments, the host cell comprises a heterologous nucleotide sequence encoding an enzyme that can condense two molecules of IPP with one molecule of DMAPP, or add a molecule of IPP to a molecule of GPP, to form a molecule of farnesyl pyrophosphate (“FPP”), *e.g.*, a FPP synthase. Illustrative examples of nucleotide sequences that encode such an enzyme include, but are not limited to: (ATU80605; *Arabidopsis thaliana*), (ATHFPS2R; *Arabidopsis thaliana*), (AAU36376; *Artemisia annua*), (AF461050; *Bos taurus*), (D00694; *Escherichia coli* K-12), (AE009951, Locus AAL95523; *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586), (GFFPPSGEN; *Gibberella fujikuroi*), (CP000009, Locus AAW60034; *Gluconobacter oxydans* 621H), (AF019892; *Helianthus annuus*), (HUMFAPS; *Homo sapiens*), (KLPFPSQCR; *Khuyveromyces lactis*), (LAU15777; *Lupinus albus*), (LAU20771; *Lupinus albus*), (AF309508; *Mus musculus*), (NCFPPSGEN; *Neurospora crassa*), (PAFPS1; *Parthenium argentatum*), (PAFPS2; *Parthenium argentatum*), (RATFAPS; *Rattus norvegicus*), (YSCFPP; *Saccharomyces cerevisiae*), (D89104; *Schizosaccharomyces pombe*), (CP000003, Locus AAT87386; *Streptococcus pyogenes*), (CP000017, Locus AAZ51849; *Streptococcus pyogenes*), (NC_008022, Locus YP_598856; *Streptococcus pyogenes* MGAS10270), (NC_008023, Locus YP_600845; *Streptococcus pyogenes* MGAS2096), (NC_008024, Locus YP_602832;

Streptococcus pyogenes MGAS10750), (MZEFPS; *Zea mays*), (AE000657, Locus AAC06913; *Aquifex aeolicus* VF5), (NM_202836; *Arabidopsis thaliana*), (D84432, Locus BAA12575; *Bacillus subtilis*), (U12678, Locus AAC28894; *Bradyrhizobium japonicum* USDA 110), (BACFDPS; *Geobacillus stearothermophilus*), (NC_002940, Locus NP_873754; *Haemophilus ducreyi* 35000HP), (L42023, Locus AAC23087; *Haemophilus influenzae* Rd KW20), (J05262; *Homo sapiens*), (YP_395294; *Lactobacillus sakei* subsp. *sakei* 23K), (NC_005823, Locus YP_000273; *Leptospira interrogans* serovar *Copenhageni* str. *Fiocruz* L1-130), (AB003187; *Micrococcus luteus*), (NC_002946, Locus YP_208768; *Neisseria gonorrhoeae* FA 1090), (U00090, Locus AAB91752; *Rhizobium* sp. NGR234), (J05091; *Saccharomyces cerevisiae*), (CP000031, Locus AAV93568; *Silicibacter pomeroyi* DSS-3), (AE008481, Locus AAK99890; *Streptococcus pneumoniae* R6), and (NC_004556, Locus NP_779706; *Xylella fastidiosa* Temecula1).

[00153] In some embodiments, the host cell further comprises a heterologous nucleotide sequence encoding an enzyme that can combine IPP and DMAPP or IPP and FPP to form geranylgeranyl pyrophosphate (“GGPP”). Illustrative examples of nucleotide sequences that encode such an enzyme include, but are not limited to: (ATHGERPYRS; *Arabidopsis thaliana*), (BT005328; *Arabidopsis thaliana*), (NM_119845; *Arabidopsis thaliana*), (NZ_AAJM01000380, Locus ZP_00743052; *Bacillus thuringiensis* serovar *israelensis*, ATCC 35646 sq1563), (CRGGPPS; *Catharanthus roseus*), (NZ_AABF02000074, Locus ZP_00144509; *Fusobacterium nucleatum* subsp. *vincentii*, ATCC 49256), (GFGGPPSGN; *Gibberella fujikuroi*), (AY371321; *Ginkgo biloba*), (AB055496; *Hevea brasiliensis*), (AB017971; *Homo sapiens*), (MCI276129; *Mucor circinelloides* f. *lusitanicus*), (AB016044; *Mus musculus*), (AABX01000298, Locus NCU01427; *Neurospora crassa*), (NCU20940; *Neurospora crassa*), (NZ_AAKL01000008, Locus ZP_00943566; *Ralstonia solanacearum* UW551), (AB118238; *Rattus norvegicus*), (SCU31632; *Saccharomyces cerevisiae*), (AB016095; *Synechococcus elongates*), (SAGGPS; *Sinapis alba*), (SSOGDS; *Sulfolobus acidocaldarius*), (NC_007759, Locus YP_461832; *Syntrophus aciditrophicus* SB), (NC_006840, Locus YP_204095; *Vibrio fischeri* ES114), (NM_112315; *Arabidopsis thaliana*), (ERWCRTE; *Pantoea agglomerans*), (D90087, Locus BAA14124; *Pantoea ananatis*), (X52291, Locus CAA36538; *Rhodobacter capsulatus*), (AF195122, Locus AAF24294; *Rhodobacter sphaeroides*), and (NC_004350, Locus NP_721015; *Streptococcus mutans* UA159).

[00154] While examples of the enzymes of the mevalonate pathway are described above, in certain embodiments, enzymes of the DXP pathway can be used as an alternative or additional pathway to produce DMAPP and IPP in the host cells, compositions and methods described herein. Enzymes and nucleic acids encoding the enzymes of the DXP pathway are well-known and characterized in the art. WO 2012/135591 A2.

6.7 Methods of Producing Steviol Glycosides

[00155] In another aspect, provided herein is a method for the production of a steviol glycoside by converting one steviol glycoside to another steviol glycoside using any UDP-glycosyltransferases described herein (*e.g.*, UGT40087 or any variant UGT40087). In certain embodiments, provided herein is a method for the production of RebD comprising converting RebA to RebD using any of the UDP-glycosyltransferases described herein, capable of converting RebA to RebD. In certain embodiments, provided herein is a method for the production of RebM comprising: converting RebA to RebD using any of the UDP-glycosyltransferases described herein, capable of converting RebA to RebD; and converting RebD to RebM using a UDP-glycosyltransferase capable of converting RebD to RebM.

[00156] In certain embodiments, a steviol glycoside (*e.g.*, RebA or RebD) or a composition comprising a steviol glycoside can be contacted, under suitable conditions, with any of the UDP-glycosyltransferase described herein and a UDP-sugar to produce a desired steviol glycoside (*e.g.*, RebM). Such methods can be performed *in vivo* or *in vitro*.

Exemplary UDP-sugars include UDP-glucose, UDP-xylose or UDP-rhamnose.

[00157] In another aspect, provided herein is a method for the production of a steviol glycoside, the method comprising the steps of: (a) culturing a population of any of the genetically modified host cells described herein that are capable of producing a steviol glycoside in a medium with a carbon source under conditions suitable for making the steviol glycoside compound; and (b) recovering said steviol glycoside compound from the medium.

[00158] In some embodiments, the genetically modified host cell produces an increased amount of the steviol glycoside compared to a parent cell not comprising the one or more modifications, or a parent cell comprising only a subset of the one or more modifications of the genetically modified host cell, but is otherwise genetically identical. In some embodiments, the increased amount is at least 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 100% or greater than 100%, as measured, for example, in yield, production, productivity, in grams per liter of cell

culture, milligrams per gram of dry cell weight, on a per unit volume of cell culture basis, on a per unit dry cell weight basis, on a per unit volume of cell culture per unit time basis, or on a per unit dry cell weight per unit time basis.

[00159] In some embodiments, the host cell produces an elevated level of a steviol glycoside that is greater than about 10 grams per liter of fermentation medium. In some such embodiments, the steviol glycoside is produced in an amount from about 10 to about 50 grams per liter of cell culture, more than about 15 grams per liter of cell culture, more than about 20 grams per liter of cell culture, more than about 25 grams per liter of cell culture, more than about 30 grams per liter of cell culture, more than about 35 grams per liter of cell culture, more than about 40 grams per liter of cell culture, more than about 45 grams per liter of cell culture, or more than about 50 grams per liter of cell culture.

[00160] In some embodiments, the host cell produces an elevated level of a steviol glycoside that is greater than about 50 milligrams per gram of dry cell weight. In some such embodiments, the steviol glycoside is produced in an amount from about 50 to about 1500 milligrams, more than about 100 milligrams, more than about 150 milligrams, more than about 200 milligrams, more than about 250 milligrams, more than about 500 milligrams, more than about 750 milligrams, or more than about 1000 milligrams per gram of dry cell weight.

[00161] In some embodiments, the host cell produces an elevated level of a steviol glycoside that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of steviol glycoside produced by a parent cell, on a per unit volume of cell culture basis.

[00162] In some embodiments, the host cell produces an elevated level of a steviol glycoside that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, at least

about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of steviol glycoside produced by the parent cell, on a per unit dry cell weight basis.

[00163] In some embodiments, the host cell produces an elevated level of a steviol glycoside that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of steviol glycoside produced by the parent cell, on a per unit volume of cell culture per unit time basis.

[00164] In some embodiments, the host cell produces an elevated level of a steviol glycoside that is at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 30-fold, at least about 40-fold, at least about 50-fold, at least about 75-fold, at least about 100-fold, at least about 200-fold, at least about 300-fold, at least about 400-fold, at least about 500-fold, or at least about 1,000-fold, or more, higher than the level of steviol glycoside produced by the parent cell, on a per unit dry cell weight per unit time basis.

[00165] In most embodiments, the production of the elevated level of steviol glycoside by the host cell is controlled by a repressing compound. Such a host cell can be manipulated with ease in the presence of the repressing compound. The repressing compound is then removed to induce the production of the elevated level of steviol glycoside by the host cell. In other embodiments, production of the elevated level of steviol glycoside by the host cell is inducible by changing culture conditions, such as, for example, the growth temperature, media constituents, and the like.

6.8 Culture Media and Conditions

[00166] Materials and methods for the maintenance and growth of microbial cultures are well known to those skilled in the art of microbiology or fermentation science (*see*, for example, Bailey *et al.*, Biochemical Engineering Fundamentals, second edition, McGraw Hill, New York, 1986). Consideration must be given to appropriate culture medium, pH, temperature, and requirements for aerobic, microaerobic, or anaerobic conditions, depending on the specific requirements of the host cell, the fermentation, and the process.

[00167] The methods of producing steviol glycosides provided herein may be performed in a suitable culture medium (*e.g.*, with or without pantothenate supplementation) in a suitable container, including but not limited to a cell culture plate, a flask, or a fermenter. Further, the methods can be performed at any scale of fermentation known in the art to support industrial production of microbial products. Any suitable fermenter may be used including a stirred tank fermenter, an airlift fermenter, a bubble fermenter, or any combination thereof. In particular embodiments utilizing *Saccharomyces cerevisiae* as the host cell, strains can be grown in a fermenter as described in detail by Kosaric, *et al.*, in Ullmann's Encyclopedia of Industrial Chemistry, Sixth Edition, Volume 12, pages 398-473, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

[00168] In some embodiments, the culture medium is any culture medium in which a genetically modified microorganism capable of producing a steviol glycoside can subsist, *i.e.*, maintain growth and viability. In some embodiments, the culture medium is an aqueous medium comprising assimilable carbon, nitrogen and phosphate sources. Such a medium can also include appropriate salts, minerals, metals and other nutrients. In some embodiments, the carbon source and each of the essential cell nutrients, are added incrementally or continuously to the fermentation media, and each required nutrient is maintained at essentially the minimum level needed for efficient assimilation by growing cells, for example, in accordance with a predetermined cell growth curve based on the metabolic or respiratory function of the cells which convert the carbon source to a biomass.

[00169] Suitable conditions and suitable media for culturing microorganisms are well known in the art. In some embodiments, the suitable medium is supplemented with one or more additional agents, such as, for example, an inducer (*e.g.*, when one or more nucleotide sequences encoding a gene product are under the control of an inducible promoter), a repressor (*e.g.*, when one or more nucleotide sequences encoding a gene product are under

the control of a repressible promoter), or a selection agent (*e.g.*, an antibiotic to select for microorganisms comprising the genetic modifications).

[00170] In some embodiments, the carbon source is a monosaccharide (simple sugar), a disaccharide, a polysaccharide, a non-fermentable carbon source, or one or more combinations thereof. Non-limiting examples of suitable monosaccharides include glucose, mannose, fructose, xylose, ribose, and combinations thereof. Non-limiting examples of suitable disaccharides include sucrose, lactose, maltose, galactose, trehalose, cellobiose, and combinations thereof. Non-limiting examples of suitable polysaccharides include starch, glycogen, cellulose, chitin, and combinations thereof. Non-limiting examples of suitable non-fermentable carbon sources include acetate and glycerol.

[00171] The concentration of a carbon source, such as glucose, in the culture medium should promote cell growth, but not be so high as to repress growth of the microorganism used. Typically, fermentation cultures are run with a carbon source, such as glucose, being added at levels to achieve the desired level of growth and biomass, but at undetectable levels (with detection limits being about <0.1 g/l). In other embodiments, the concentration of a carbon source, such as glucose, in the culture medium is greater than about 1 g/L, preferably greater than about 2 g/L, and more preferably greater than about 5 g/L. In addition, the concentration of a carbon source, such as glucose, in the culture medium is typically less than about 100 g/L, preferably less than about 50 g/L, and more preferably less than about 20 g/L. It should be noted that references to culture component concentrations can refer to both initial and/or ongoing component concentrations. In some cases, it may be desirable to allow the culture medium to become depleted of a carbon source during culture.

[00172] Sources of assimilable nitrogen that can be used in a suitable culture medium include, but are not limited to, simple nitrogen sources, organic nitrogen sources and complex nitrogen sources. Such nitrogen sources include anhydrous ammonia, ammonium salts and substances of animal, vegetable and/or microbial origin. Suitable nitrogen sources include, but are not limited to, protein hydrolysates, microbial biomass hydrolysates, peptone, yeast extract, ammonium sulfate, urea, and amino acids. Typically, the concentration of the nitrogen sources in the culture medium is greater than about 0.1 g/L, preferably greater than about 0.25 g/L, and more preferably greater than about 1.0 g/L. Beyond certain concentrations, however, the addition of a nitrogen source to the culture medium is not advantageous for the growth of the microorganisms. As a result, the concentration of the nitrogen sources, in the culture medium is less than about 20 g/L,

preferably less than about 10 g/L and more preferably less than about 5 g/L. Further, in some instances it may be desirable to allow the culture medium to become depleted of the nitrogen sources during culture.

[00173] The effective culture medium can contain other compounds such as inorganic salts, vitamins, trace metals or growth promoters. Such other compounds can also be present in carbon, nitrogen or mineral sources in the effective medium or can be added specifically to the medium.

[00174] The culture medium can also contain a suitable phosphate source. Such phosphate sources include both inorganic and organic phosphate sources. Preferred phosphate sources include, but are not limited to, phosphate salts such as mono or dibasic sodium and potassium phosphates, ammonium phosphate and mixtures thereof. Typically, the concentration of phosphate in the culture medium is greater than about 1.0 g/L, preferably greater than about 2.0 g/L and more preferably greater than about 5.0 g/L. Beyond certain concentrations, however, the addition of phosphate to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the concentration of phosphate in the culture medium is typically less than about 20 g/L, preferably less than about 15 g/L and more preferably less than about 10 g/L.

[00175] A suitable culture medium can also include a source of magnesium, preferably in the form of a physiologically acceptable salt, such as magnesium sulfate heptahydrate, although other magnesium sources in concentrations that contribute similar amounts of magnesium can be used. Typically, the concentration of magnesium in the culture medium is greater than about 0.5 g/L, preferably greater than about 1.0 g/L, and more preferably greater than about 2.0 g/L. Beyond certain concentrations, however, the addition of magnesium to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the concentration of magnesium in the culture medium is typically less than about 10 g/L, preferably less than about 5 g/L, and more preferably less than about 3 g/L. Further, in some instances it may be desirable to allow the culture medium to become depleted of a magnesium source during culture.

[00176] In some embodiments, the culture medium can also include a biologically acceptable chelating agent, such as the dihydrate of trisodium citrate. In such instance, the concentration of a chelating agent in the culture medium is greater than about 0.2 g/L, preferably greater than about 0.5 g/L, and more preferably greater than about 1 g/L. Beyond certain concentrations, however, the addition of a chelating agent to the culture medium is

not advantageous for the growth of the microorganisms. Accordingly, the concentration of a chelating agent in the culture medium is typically less than about 10 g/L, preferably less than about 5 g/L, and more preferably less than about 2 g/L.

[00177] The culture medium can also initially include a biologically acceptable acid or base to maintain the desired pH of the culture medium. Biologically acceptable acids include, but are not limited to, hydrochloric acid, sulfuric acid, nitric acid, phosphoric acid and mixtures thereof. Biologically acceptable bases include, but are not limited to, ammonium hydroxide, sodium hydroxide, potassium hydroxide and mixtures thereof. In some embodiments, the base used is ammonium hydroxide.

[00178] The culture medium can also include a biologically acceptable calcium source, including, but not limited to, calcium chloride. Typically, the concentration of the calcium source, such as calcium chloride, dihydrate, in the culture medium is within the range of from about 5 mg/L to about 2000 mg/L, preferably within the range of from about 20 mg/L to about 1000 mg/L, and more preferably in the range of from about 50 mg/L to about 500 mg/L.

[00179] The culture medium can also include sodium chloride. Typically, the concentration of sodium chloride in the culture medium is within the range of from about 0.1 g/L to about 5 g/L, preferably within the range of from about 1 g/L to about 4 g/L, and more preferably in the range of from about 2 g/L to about 4 g/L.

[00180] In some embodiments, the culture medium can also include trace metals. Such trace metals can be added to the culture medium as a stock solution that, for convenience, can be prepared separately from the rest of the culture medium. Typically, the amount of such a trace metals solution added to the culture medium is greater than about 1 mL/L, preferably greater than about 5 mL/L, and more preferably greater than about 10 mL/L. Beyond certain concentrations, however, the addition of a trace metals to the culture medium is not advantageous for the growth of the microorganisms. Accordingly, the amount of such a trace metals solution added to the culture medium is typically less than about 100 mL/L, preferably less than about 50 mL/L, and more preferably less than about 30 mL/L. It should be noted that, in addition to adding trace metals in a stock solution, the individual components can be added separately, each within ranges corresponding independently to the amounts of the components dictated by the above ranges of the trace metals solution.

[00181] The culture media can include other vitamins, such as pantothenate, biotin, calcium, pantothenate, inositol, pyridoxine-HCl, and thiamine-HCl. Such vitamins can be

added to the culture medium as a stock solution that, for convenience, can be prepared separately from the rest of the culture medium. Beyond certain concentrations, however, the addition of vitamins to the culture medium is not advantageous for the growth of the microorganisms.

[00182] The fermentation methods described herein can be performed in conventional culture modes, which include, but are not limited to, batch, fed-batch, cell recycle, continuous and semi-continuous. In some embodiments, the fermentation is carried out in fed-batch mode. In such a case, some of the components of the medium are depleted during culture, including pantothenate during the production stage of the fermentation. In some embodiments, the culture may be supplemented with relatively high concentrations of such components at the outset, for example, of the production stage, so that growth and/or steviol glycoside production is supported for a period of time before additions are required. The preferred ranges of these components are maintained throughout the culture by making additions as levels are depleted by culture. Levels of components in the culture medium can be monitored by, for example, sampling the culture medium periodically and assaying for concentrations. Alternatively, once a standard culture procedure is developed, additions can be made at timed intervals corresponding to known levels at particular times throughout the culture. As will be recognized by those in the art, the rate of consumption of nutrient increases during culture as the cell density of the medium increases. Moreover, to avoid introduction of foreign microorganisms into the culture medium, addition is performed using aseptic addition methods, as are known in the art. In addition, a small amount of anti-foaming agent may be added during the culture.

[00183] The temperature of the culture medium can be any temperature suitable for growth of the genetically modified cells and/or production of steviol glycoside. For example, prior to inoculation of the culture medium with an inoculum, the culture medium can be brought to and maintained at a temperature in the range of from about 20°C to about 45°C, preferably to a temperature in the range of from about 25°C to about 40°C, and more preferably in the range of from about 28°C to about 32°C.

[00184] The pH of the culture medium can be controlled by the addition of acid or base to the culture medium. In such cases when ammonia is used to control pH, it also conveniently serves as a nitrogen source in the culture medium. Preferably, the pH is maintained from about 3.0 to about 8.0, more preferably from about 3.5 to about 7.0, and most preferably from about 4.0 to about 6.5.

[00185] In some embodiments, the carbon source concentration, such as the glucose concentration, of the culture medium is monitored during culture. Glucose concentration of the culture medium can be monitored using known techniques, such as, for example, use of the glucose oxidase enzyme test or high pressure liquid chromatography, which can be used to monitor glucose concentration in the supernatant, *e.g.*, a cell-free component of the culture medium. As stated previously, the carbon source concentration should be kept below the level at which cell growth inhibition occurs. Although such concentration may vary from organism to organism, for glucose as a carbon source, cell growth inhibition occurs at glucose concentrations greater than at about 60 g/L, and can be determined readily by trial. Accordingly, when glucose is used as a carbon source the glucose is preferably fed to the fermenter and maintained below detection limits. Alternatively, the glucose concentration in the culture medium is maintained in the range of from about 1 g/L to about 100 g/L, more preferably in the range of from about 2 g/L to about 50 g/L, and yet more preferably in the range of from about 5 g/L to about 20 g/L. Although the carbon source concentration can be maintained within desired levels by addition of, for example, a substantially pure glucose solution, it is acceptable, and may be preferred, to maintain the carbon source concentration of the culture medium by addition of aliquots of the original culture medium. The use of aliquots of the original culture medium may be desirable because the concentrations of other nutrients in the medium (*e.g.* the nitrogen and phosphate sources) can be maintained simultaneously. Likewise, the trace metals concentrations can be maintained in the culture medium by addition of aliquots of the trace metals solution.

[00186] Other suitable fermentation medium and methods are described in, *e.g.*, WO 2016/196321.

6.9 Fermentation Compositions

[00187] In another aspect, provided herein are fermentation compositions comprising a genetically modified host cell described herein and steviol glycosides produced from genetically modified host cell. The fermentation compositions may further comprise a medium. In certain embodiments, the fermentation compositions comprise a genetically modified host cell, and further comprise RebA, RebD, and RebM. In certain embodiments, the fermentation compositions provided herein comprise RebM as a major component of the steviol glycosides produced from the genetically modified host cell. In certain embodiments, the fermentation compositions comprise RebA, RebD, and RebM at a ratio of at least 1:7:50.

In other embodiments, the fermentation compositions comprise (RebA + RebD) and RebM at a ratio of at least 8:50. In certain embodiments, the fermentation compositions comprise RebA, RebD, and RebM at a ratio of at least 1:7:50 to 1:100:1000. In other embodiments, the fermentation compositions comprise (RebA + RebD) and RebM at a ratio of at least 8:50 to 101:1000. In certain embodiments, the fermentation compositions comprise a ratio of at least 1:7:50 to 1:200:2000. In other embodiments, the fermentation compositions comprise (RebA + RebD) and RebM at a ratio of at least 8:50 to 201:2000. In certain embodiments, the ratio of RebA, RebD, and RebM are based on the total content of these three steviol glycosides that are associated with the genetically modified host cell and the medium. In certain embodiments, the ratio of RebA, RebD, and RebM are based on the total content of these three steviol glycosides in the medium. In certain embodiments, the ratio of RebA, RebD, and RebM are based on the total content of these three steviol glycosides that are associated with the genetically modified host cell.

[00188] In other embodiments, the fermentation compositions comprise a genetically modified host cell, and further comprise RebA and RebM. In certain embodiments, the fermentation compositions comprise RebA and RebM at a RebA:RebM ratio of at least 1:50. In certain embodiments, the fermentation compositions comprise RebA and RebM at a RebA:RebM ratio of at least 1:50 to 1:1000. In certain embodiments, the fermentation compositions comprise RebA and RebM at a RebA:RebM ratio of at least 1:50 to 1:2000. In certain embodiments, the ratio of RebA and RebM are based on the total content of these two steviol glycosides that are associated with the genetically modified host cell and the medium. In certain embodiments, the ratio of RebA and RebM are based on the total content of these two steviol glycosides in the medium. In certain embodiments, the ratio of RebA and RebM are based on the total content of these two steviol glycosides that are associated with the genetically modified host cell.

[00189] In a further embodiment, the fermentation compositions comprise a genetically modified host cell, and further comprise RebD and RebM. In certain embodiments, the fermentation compositions comprise RebD and RebM at a RebD:RebM ratio of at least 7:50. In certain embodiments, the fermentation compositions comprise RebD and RebM at a RebD:RebM ratio of at least 7:50 to 7:100. In certain embodiments, the fermentation compositions comprise RebD and RebM at a RebD:RebM ratio of at least 7:50 to 7:200. In certain embodiments, the ratio of RebA, RebD, and RebM are based on the total content of these two steviol glycosides that are associated with the genetically modified host cell and

the medium. In certain embodiments, the ratio of RebD and RebM are based on the total content of these two steviol glycosides in the medium. In certain embodiments, the ratio of RebD and RebM are based on the total content of these two steviol glycosides that are associated with the genetically modified host cell.

[00190] In certain embodiments, the fermentation compositions provided herein contain RebM2 at an undetectable level. In certain embodiments, the fermentation compositions provided herein contain non-naturally occurring steviol glycosides at an undetectable level. In certain embodiments, the fermentation compositions provided herein, when subjected to GC-chromatography, does not produce a “steviol + 2 glucose” peak between a RebA peak and a RebB at a detectable level

6.10 Recovery of Steviol Glycosides

[00191] Once the steviol glycoside is produced by the host cell, it may be recovered or isolated for subsequent use using any suitable separation and purification methods, including any suitable steviol glycoside separation and purification methods, known in the art. Auitable methods are described in, *e.g.*, U.S. Patent Nos. 7,838,044 and 8,981,081; U.S. Patent Application Nos. 14/603,941, 14/033,563, 14/362,275, 14/613,615, 14/615,888; PCT Application Nos. PCT/US12/070562, and PCT/US14/031129. The contents of these documents are included herein by reference in their entirety. In some embodiments, an aqueous phase comprising the steviol glycoside is separated from the fermentation by centrifugation. In other embodiments, an aqueous phase comprising the steviol glycoside separates from the fermentation spontaneously. In other embodiments, an aqueous phase comprising the steviol glycoside is separated from the fermentation by adding a demulsifier and/or a nucleating agent into the fermentation reaction. Illustrative examples of demulsifiers include flocculants and coagulants. Illustrative examples of nucleating agents include droplets of the steviol glycoside itself and organic solvents such as dodecane, isopropyl myristate, and methyl oleate.

[00192] The steviol glycoside produced in these cells may be present in the culture supernatant and/or associated with the host cells. In embodiments where the steviol glycoside is associated with the host cell, the recovery of the steviol glycoside may comprise a method of permeabilizing or lysing the cells. Alternatively or simultaneously, the steviol glycoside in the culture medium can be recovered using a recovery process including, but not limited to, chromatography, adsorption chromatography, extraction, solvent extraction,

membrane separation, electrodialysis, reverse osmosis, distillation, chemical derivatization and crystallization.

[00193] In some embodiments, the steviol glycoside is separated from other products that may be present in the aqueous phase. In some embodiments, separation is achieved using adsorption, distillation, gas-liquid extraction (stripping), liquid-liquid extraction (solvent extraction), vacuum extraction, evaporation, ultrafiltration, and standard chromatographic techniques. Other suitable fermentation medium and methods are described in, *e.g.*, U.S. Patent Application Publication Nos. 2016/0185813, 2017/0190728, WO/2017/093895. Other suitable methods are described in, *e.g.*, U.S. Patent Nos. 7,838,044 and 8,981,081; U.S. Patent Application Nos. 14/603,941, 14/033,563, 14/362,275, 14/613,615 and 14/615,888; PCT Application Nos. PCT/US12/070562, and PCT/US14/031129. The contents of these documents are included herein by reference in their entirety

[00194] In certain embodiments, the recovered steviol glycoside(s) can be used in a variety of end products. For example, a purified RebM compound or a composition comprising RebM can be used in any consumable products, such as food products, pharmaceutical products, dietary supplements, or nutritional supplements. In particular embodiments, a consumable comprising RebM is made by the process of culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and recovering said RebM compound from the medium.

[0195] Sweetener compositions, as used herein, mean compositions that contain at least one sweet component in combination with at least one other substance, such as, for example, another sweetener or an additive.

[0196] Sweetenable compositions, as used herein, mean substances which are contacted with the mouth of man or animal, including substances which are taken into and subsequently ejected from the mouth and substances which are drunk, eaten, swallowed or otherwise ingested, and are safe for human or animal consumption when used in a generally acceptable range.

[0197] Sweetened compositions, as used herein, mean substances that contain both a sweetenable composition and a sweetener or sweetener composition.

For example, a beverage with no sweetener component is a type of sweetenable composition. A sweetener composition comprising RebM produced by method of present invention, and erythritol can be added to the un-sweetened beverage, thereby providing a

sweetened beverage. The sweetened beverage is a type of sweetened composition. Suitable sweetener compositions, sweetened compositions and methods of making thereof are described in, *e.g.*, PCT application PCT/US12/070562, the contents of which is included herein by reference in its entirety.

[0198] In one embodiment, a sweetener comprising RebM is made by the process of culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and recovering said RebM compound from the medium. In another embodiment, a sweetener composition comprising at least one other substance and RebM is made by the process of culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and recovering said RebM compound from the medium. In another embodiment, a sweetened composition comprising RebM is made by the process of culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and recovering said RebM compound from the medium. One embodiment comprises a method of making a sweetener composition comprising combining at least one other substance with RebM made by the process of culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and recovering said RebM compound from the medium. Another embodiment comprises a method of making a sweetened composition comprising combining at least one sweetenable composition with RebM made by the process of culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and recovering said RebM compound from the medium.

6.11 Methods of Making Genetically Modified Cells

[00199] Also provided herein are methods for producing a host cell that is genetically engineered to comprise one or more of the modifications described above, *e.g.*, one or more heterologous nucleic acids encoding UGT40087, and/or biosynthetic pathway enzymes, *e.g.*, for a steviol glycoside compound. Expression of a heterologous enzyme in a host cell can be accomplished by introducing into the host cells a nucleic acid comprising a nucleotide sequence encoding the enzyme under the control of regulatory elements that permit expression in the host cell. In some embodiments, the nucleic acid is an extrachromosomal

plasmid. In other embodiments, the nucleic acid is integrated into the chromosome of the host cell.

[00200] Nucleic acids encoding proteins can be introduced into the host cell by any method known to one of skill in the art without limitation (*see*, for example, Hinnen *et al.* (1978) *Proc. Natl. Acad. Sci. USA* 75:1292-3; Cregg *et al.* (1985) *Mol. Cell. Biol.* 5:3376-3385; Goeddel *et al.* eds, 1990, *Methods in Enzymology*, vol. 185, Academic Press, Inc., CA; Krieger, 1990, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, NY; Sambrook *et al.* , 1989, *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, NY; and Ausubel *et al.*, eds., *Current Edition, Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley Interscience, NY). Exemplary techniques include, but are not limited to, spheroplasting, electroporation, PEG 1000 mediated transformation, and lithium acetate or lithium chloride mediated transformation.

[00201] The activity of an enzyme in a host cell may be altered by modifying the transcription of the gene that encodes the enzyme. This can be achieved for example by modifying the copy number of the nucleotide sequence encoding the enzyme (*e.g.*, by using a higher or lower copy number expression vector comprising the nucleotide sequence, or by introducing additional copies of the nucleotide sequence into the genome of the host cell or by deleting or disrupting the nucleotide sequence in the genome of the host cell), by changing the order of coding sequences on a polycistronic mRNA of an operon or breaking up an operon into individual genes each with its own control elements, or by increasing the strength of the promoter or operator to which the nucleotide sequence is operably linked. Alternatively or in addition, the activity of an enzyme in a host cell may be altered by modifying the level of translation of an mRNA that encodes the enzyme. This can be achieved for example by modifying the stability of the mRNA, modifying the sequence of the ribosome binding site, modifying the distance or sequence between the ribosome binding site and the start codon of the enzyme coding sequence, modifying the entire intercistronic region located “upstream of” or adjacent to the 5’ side of the start codon of the enzyme coding region, stabilizing the 3’-end of the mRNA transcript using hairpins and specialized sequences, modifying the codon usage of enzyme, altering expression of rare codon tRNAs used in the biosynthesis of the enzyme, and/or increasing the stability of the enzyme, as, for example, via mutation of its coding sequence.

[00202] The activity of an enzyme in a host cell can be altered in a number of ways, including, but not limited to, expressing a modified form of the enzyme that exhibits

increased or decreased solubility in the host cell, expressing an altered form of the enzyme that lacks a domain through which the activity of the enzyme is inhibited, expressing a modified form of the enzyme that has a higher or lower K_{cat} or a lower or higher K_m for the substrate, or expressing an altered form of the enzyme that is more or less affected by feed-back or feed-forward regulation by another molecule in the pathway.

[00203] In some embodiments, a nucleic acid used to genetically modify a host cell comprises one or more selectable markers useful for the selection of transformed host cells and for placing selective pressure on the host cell to maintain the foreign DNA.

[00204] In some embodiments, the selectable marker is an antibiotic resistance marker. Illustrative examples of antibiotic resistance markers include, but are not limited to, the *BLA*, *NAT1*, *PAT*, *AURI-C*, *PDR4*, *SMR1*, *CAT*, mouse dhfr, *HPH*, *DSDA*, *KAN^R*, and *SH BLE* gene products. The *BLA* gene product from *E. coli* confers resistance to beta-lactam antibiotics (e.g., narrow-spectrum cephalosporins, cephamycins, and carbapenems (ertapenem), cefamandole, and cefoperazone) and to all the anti-gram-negative-bacterium penicillins except temocillin; the *NAT1* gene product from *S. noursei* confers resistance to nourseothricin; the *PAT* gene product from *S. viridochromogenes* Tu94 confers resistance to bialaphos; the *AURI-C* gene product from *Saccharomyces cerevisiae* confers resistance to Auerobasidin A (AbA); the *PDR4* gene product confers resistance to cerulenin; the *SMR1* gene product confers resistance to sulfometuron methyl; the *CAT* gene product from Tn9 transposon confers resistance to chloramphenicol; the mouse dhfr gene product confers resistance to methotrexate; the *HPH* gene product of *Klebsiella pneumonia* confers resistance to Hygromycin B; the *DSDA* gene product of *E. coli* allows cells to grow on plates with D-serine as the sole nitrogen source; the *KAN^R* gene of the Tn903 transposon confers resistance to G418; and the *SH BLE* gene product from *Streptoalloteichus hindustanus* confers resistance to Zeocin (bleomycin). In some embodiments, the antibiotic resistance marker is deleted after the genetically modified host cell disclosed herein is isolated.

[00205] In some embodiments, the selectable marker rescues an auxotrophy (e.g., a nutritional auxotrophy) in the genetically modified microorganism. In such embodiments, a parent microorganism comprises a functional disruption in one or more gene products that function in an amino acid or nucleotide biosynthetic pathway and that when non-functional renders a parent cell incapable of growing in media without supplementation with one or more nutrients. Such gene products include, but are not limited to, the *HIS3*, *LEU2*, *LYS1*,

LYS2, *MET15*, *TRP1*, *ADE2*, and *URA3* gene products in yeast. The auxotrophic phenotype can then be rescued by transforming the parent cell with an expression vector or chromosomal integration construct encoding a functional copy of the disrupted gene product, and the genetically modified host cell generated can be selected for based on the loss of the auxotrophic phenotype of the parent cell. Utilization of the *URA3*, *TRP1*, and *LYS2* genes as selectable markers has a marked advantage because both positive and negative selections are possible. Positive selection is carried out by auxotrophic complementation of the *URA3*, *TRP1*, and *LYS2* mutations, whereas negative selection is based on specific inhibitors, *i.e.*, 5-fluoro-orotic acid (FOA), 5-fluoroanthranilic acid, and aminoadipic acid (aAA), respectively, that prevent growth of the prototrophic strains but allows growth of the *URA3*, *TRP1*, and *LYS2* mutants, respectively. In other embodiments, the selectable marker rescues other non-lethal deficiencies or phenotypes that can be identified by a known selection method.

[00206] Described herein are specific genes and proteins useful in the methods, compositions and organisms of the disclosure; however it will be recognized that absolute identity to such genes is not necessary. For example, changes in a particular gene or polynucleotide comprising a sequence encoding a polypeptide or enzyme can be performed and screened for activity. Typically such changes comprise conservative mutations and silent mutations. Such modified or mutated polynucleotides and polypeptides can be screened for expression of a functional enzyme using methods known in the art.

[00207] Due to the inherent degeneracy of the genetic code, other polynucleotides which encode substantially the same or functionally equivalent polypeptides can also be used to clone and express the polynucleotides encoding such enzymes.

[00208] As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The genetic code is redundant with 64 possible codons, but most organisms typically use a subset of these codons. The codons that are utilized most often in a species are called optimal codons, and those not utilized very often are classified as rare or low-usage codons. Codons can be substituted to reflect the preferred codon usage of the host, in a process sometimes called “codon optimization” or “controlling for species codon bias.” Codon optimization for other host cells can be readily determined using codon usage tables or can be performed using commercially available software, such as CodonOp (www.idtdna.com/CodonOptfrom) from Integrated DNA Technologies.

[00209] Optimized coding sequences containing codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, 1989, *Nucl Acids Res.* 17: 477-508) can be prepared, for example, to increase the rate of translation or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, as compared with transcripts produced from a non-optimized sequence. Translation stop codons can also be modified to reflect host preference. For example, typical stop codons for *S. cerevisiae* and mammals are UAA and UGA, respectively. The typical stop codon for monocotyledonous plants is UGA, whereas insects and *E. coli* commonly use UAA as the stop codon (Dalphin *et al.*, 1996, *Nucl Acids Res.* 24: 216-8).

[00210] Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA molecules differing in their nucleotide sequences can be used to encode a given enzyme of the disclosure. The native DNA sequence encoding the biosynthetic enzymes described above are referenced herein merely to illustrate an embodiment of the disclosure, and the disclosure includes DNA molecules of any sequence that encode the amino acid sequences of the polypeptides and proteins of the enzymes utilized in the methods of the disclosure. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The disclosure includes such polypeptides with different amino acid sequences than the specific proteins described herein so long as the modified or variant polypeptides have the enzymatic anabolic or catabolic activity of the reference polypeptide. Furthermore, the amino acid sequences encoded by the DNA sequences shown herein merely illustrate embodiments of the disclosure.

[00211] In addition, homologs of enzymes useful for the compositions and methods provided herein are encompassed by the disclosure. In some embodiments, two proteins (or a region of the proteins) are substantially homologous when the amino acid sequences have at least about 30%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity. To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In one embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, typically at least 40%, more typically at least 50%, even more typically at least 60%, and even more typically at

least 70%, 80%, 90%, 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[00212] When “homologous” is used in reference to proteins or peptides, it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions. A “conservative amino acid substitution” is one in which an amino acid residue is substituted by another amino acid residue having a side chain (R group) with similar chemical properties (*e.g.*, charge or hydrophobicity). In general, a conservative amino acid substitution will not substantially change the functional properties of a protein. In cases where two or more amino acid sequences differ from each other by conservative substitutions, the percent sequence identity or degree of homology may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art (*See, e.g.*, Pearson W. R., 1994, *Methods in Mol Biol* 25: 365-89).

[00213] The following six groups each contain amino acids that are conservative substitutions for one another: 1) Serine (S), Threonine (T); 2) Aspartic Acid (D), Glutamic Acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Alanine (A), Valine (V), and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

[00214] Sequence homology for polypeptides, which is also referred to as percent sequence identity, is typically measured using sequence analysis software. A typical algorithm used comparing a molecule sequence to a database containing a large number of sequences from different organisms is the computer program BLAST. When searching a database containing sequences from a large number of different organisms, it is typical to compare amino acid sequences.

[00215] Furthermore, any of the genes encoding the foregoing enzymes (or any others mentioned herein (or any of the regulatory elements that control or modulate expression

thereof)) may be optimized by genetic/protein engineering techniques, such as directed evolution or rational mutagenesis, which are known to those of ordinary skill in the art. Such action allows those of ordinary skill in the art to optimize the enzymes for expression and activity in yeast.

[00216] In addition, genes encoding these enzymes can be identified from other fungal and bacterial species and can be expressed for the modulation of this pathway. A variety of organisms could serve as sources for these enzymes, including, but not limited to, *Saccharomyces spp.*, including *S. cerevisiae* and *S. uvarum*, *Kluyveromyces spp.*, including *K. thermotolerans*, *K. lactis*, and *K. marxianus*, *Pichia spp.*, *Hansenula spp.*, including *H. polymorpha*, *Candida spp.*, *Trichosporon spp.*, *Yamadazyma spp.*, including *Y. spp. stipitis*, *Torulaspora pretoriensis*, *Issatchenkia orientalis*, *Schizosaccharomyces spp.*, including *S. pombe*, *Cryptococcus spp.*, *Aspergillus spp.*, *Neurospora spp.*, or *Ustilago spp.* Sources of genes from anaerobic fungi include, but are not limited to, *Piromyces spp.*, *Orpinomyces spp.*, or *Neocallimastix spp.* Sources of prokaryotic enzymes that are useful include, but are not limited to, *Escherichia. coli*, *Zymomonas mobilis*, *Staphylococcus aureus*, *Bacillus spp.*, *Clostridium spp.*, *Corynebacterium spp.*, *Pseudomonas spp.*, *Lactococcus spp.*, *Enterobacter spp.*, and *Salmonella spp.*

[00217] Techniques known to those skilled in the art may be suitable to identify additional homologous genes and homologous enzymes. Generally, analogous genes and/or analogous enzymes can be identified by functional analysis and will have functional similarities. Techniques known to those skilled in the art may be suitable to identify analogous genes and analogous enzymes. For example, to identify homologous or analogous UDP glycosyltransferases, PTA, or any biosynthetic pathway genes, proteins, or enzymes, techniques may include, but are not limited to, cloning a gene by PCR using primers based on a published sequence of a gene/enzyme of interest, or by degenerate PCR using degenerate primers designed to amplify a conserved region among a gene of interest. Further, one skilled in the art can use techniques to identify homologous or analogous genes, proteins, or enzymes with functional homology or similarity. Techniques include examining a cell or cell culture for the catalytic activity of an enzyme through *in vitro* enzyme assays for said activity (e.g. as described herein or in Kiritani, K., *Branched-Chain Amino Acids Methods Enzymology*, 1970), then isolating the enzyme with said activity through purification, determining the protein sequence of the enzyme through techniques such as Edman degradation, design of PCR primers to the likely nucleic acid sequence,

amplification of said DNA sequence through PCR, and cloning of said nucleic acid sequence. To identify homologous or similar genes and/or homologous or similar enzymes, analogous genes and/or analogous enzymes or proteins, techniques also include comparison of data concerning a candidate gene or enzyme with databases such as BRENDA, KEGG, or MetaCYC. The candidate gene or enzyme may be identified within the above mentioned databases in accordance with the teachings herein.

7. EXAMPLES

Example 1: Generation of a base yeast strain capable of high flux to farnesylpyrophosphate (FPP) and the isoprenoid farnesene.

[00218] A farnesene production strain was created from a wild-type *Saccharomyces cerevisiae* strain (CEN.PK2) by expressing the genes of the mevalonate pathway (**FIG. 1C**) under the control of GAL1 or GAL10 promoters. This strain comprised the following chromosomally integrated mevalonate pathway genes from *S. cerevisiae*: acetyl-CoA thiolase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, and IPP:DMAPP isomerase. All genes described herein were codon optimized using publicly available or other suitable algorithms. In addition, the strain contained six copies of farnesene synthase from *Artemisinin annua*, also under the control of either GAL1 or GAL10 promoters. The strain also contained a deletion of the GAL80 gene and an additional copy of GAL4 under GAL4oc promoter, wherein the coding sequence of the GAL4 gene of *Saccharomyces cerevisiae* is under regulatory control of an “operative constitutive” version of its native promoter (PGAL4oc; *see, e.g.*, Griggs & Johnston (1991) PNAS 88(19):8597-8601). Lastly the ERG9 gene, encoding squalene synthase, is downregulated by replacing the native promoter with promoter of the yeast gene MET3 (Westfall et al PNAS 2012).

Example 2. Generation of a base yeast strain capable of high flux to RebA.

[00219] **FIG. 2A** shows an exemplary biosynthetic pathway from FPP to the steviol. **FIG. 2B** shows an exemplary biosynthetic pathway from steviol to glycoside RebM. To convert the farnesene base strain described above to have high flux to the C-20 isoprenoid kaurene, six copies of a geranylgeranylpyrophosphate synthase (GGPPS) were integrated into the genome, followed by four copies each of a copalylidiphosphate synthase and kaurene synthase. At this point the six copies of farnesene synthase were removed from the strain. Once the new strain was confirmed to make ent-kaurene, the remaining genes for converting

ent-kaurene to RebA were inserted into the genome. Table 4 lists all genes and promoters used to convert FPP to RebA. Each gene after kaurene synthase was integrated with a single copy, except for the Sr.KAH enzyme which had two copies (Table 4). The strain containing all genes described in Table 1 primarily produced RebA. The enzyme UGT91D_like3 has some low activity to convert RebA to Rebaudioside D (RebD). We measured that a single copy of UGT91D_like3 is able to convert approximately (3%) of the RebA in the strain to RebD *in vivo* in the yeast strain described above (FIG. 3 and Table 5). UGT76G1 then can convert RebD to the final product RebM.

Example 3. Generation of a strain to screen novel UDP-glycosyltransferase (UGT) enzymes to convert RebA to RebD.

[00220] To make a screening strain to rapidly screen for RebA to RebD conversion *in vivo*, a landing pad was inserted into the RebA strain described above. The landing pad consisted of 500 bp of locus-targeting DNA sequences on either end of the construct to the genomic region downstream of the ALG1 open reading frame (FIG. 4). Internally, the landing pad contained a PGAL1 promoter and a yeast terminator flanking an endonuclease recognition site (F-CphI).

Example 4. Screening for UGT genes that convert RebA to RebD with high efficiency *in vivo*

[00221] Over a hundred UGT enzymes obtained from Genbank, were codon optimized for optimal expression in *S. cerevisiae* and synthesized with 60 bp of sequence homologous to the PGAL1 and yeast terminator flanking the F-CphI sequences in the landing pad described above. Each synthesized UGT gene was tested individually, with a single copy, for the ability to convert RebA to RebD *in vivo* in the yeast strain described above. Yeast were transformed with UGT donor DNA and a plasmid containing the endonuclease F-CphI to cut the DNA in the landing pad. Correct integrations were verified by colony PCR using a reverse primer internal to the specific UGT gene in each transformation and a universal forward primer at the end of the ALG1 ORF.

Example 5. Yeast transformation methods

[00222] Each DNA construct was integrated into *Saccharomyces cerevisiae* (CEN.PK2) with standard molecular biology techniques in an optimized lithium acetate (LiAc) transformation. Briefly, cells were grown overnight in yeast extract peptone dextrose (YPD)

media at 30°C with shaking (200 rpm), diluted to an OD₆₀₀ of 0.1 in 100 mL YPD, and grown to an OD₆₀₀ of 0.6 – 0.8. For each transformation, 5 mL of culture was harvested by centrifugation, washed in 5 mL of sterile water, spun down again, resuspended in 1 mL of 100 mM LiAc, and transferred to a microcentrifuge tube. Cells were spun down (13,000 xg) for 30 seconds, the supernatant was removed, and the cells were resuspended in a transformation mix consisting of 240 µL 50% PEG, 36 µL 1 M LiAc, 10 µL boiled salmon sperm DNA, and 74 µL of donor DNA. For transformations that required expression of the endonuclease F-CphI, the donor DNA included a plasmid carrying the F-CphI gene expressed under the yeast TDH3 promoter for expression. This will cut the F-CphI endonuclease recognition site in the landing pad to facilitate integration of the UGT gene. Following a heat shock at 42°C for 40 minutes, cells were recovered overnight in YPD media before plating on selective media. DNA integration was confirmed by colony PCR with primers specific to the integrations.

Example 6. Yeast culturing conditions

[00223] Yeast colonies verified to contain the expected UGT gene were picked into 96-well microtiter plates containing Bird Seed Media (BSM, originally described by van Hoek *et al.*, *Biotechnology and Bioengineering* 68(5), 2000, pp. 517-523) with 20 g/L sucrose and 37.5g/L ammonium sulfate. Cells were cultured at 30°C in a high capacity microtiter plate incubator shaking at 1000 rpm and 80% humidity for 3 days until the cultures reached carbon exhaustion. The growth-saturated cultures were subcultured into fresh plates containing BSM with 40g/L sucrose and 150g/L ammonium sulfate by taking 14.4µl from the saturated cultures and diluting into 360µl of fresh media. Cells in the production media were cultured at 30°C in a high capacity microtiter plate shaker at 1000 rpm and 80% humidity for an additional 3 days prior to extraction and analysis. Upon completion the whole cell broth is diluted with 360uL of 100% ethanol, sealed with a foil seal, and shaken at 1250 rpm for 30 min to extract the steviol glycosides. 490uL of 50:50 ethanol:water is added to a new 1.1mL assay plate and 10uL of the culture/ethanol mixture is added to the assay plate. The mixture is centrifuged to pellet any solids, and 400uL of the solution is transferred to a new 1.1mL plate and assayed by LC-MS.

Example 7. Analytical methods

[00224] Samples were analyzed by LC-MS mass spectrometer (AB QTrap 4000) using a Sigma Ascentis Express Peptide ES-C18 (5cm, 2.1mm, 2.7µm; part #53301-U) with the

following gradient (Mobile phase A: 0.1% Formic Acid in H₂O; Mobile phase B: 0.1% Formic acid in Acetonitrile):

Table 2.

	Time (min)	%B
1	0	25
2	2.50	25
3	10.00	60
4	10.50	100
5	12.50	100
6	12.51	25

[00225] The mass spectrometer was operated in negative ion multiple reaction monitoring mode. Each rebaudioside isomer was identified by retention time, determined from an authentic standard, and MRM transition:

Table 3.

RT (min)	Compound	Q1 Mass (Da)	Q3 Mass (Da)
10.5	Steviol	317.328	317.300
8.2	Steviolmonoside	479.354	317.200
7.9	19-glycoside	479.369	317.100
7.4	Steviolbioside	641.451	479.300
6.9	Rubusoside	641.491	479.400
7.3	RebB	803.612	641.500
6.2	Stevioside	803.550	641.400
3.3	RebE	965.441	479.400
6.2	RebA	965.441	803.700
3.8	RebD	1127.140	803.500
4.5	RebM	1289.540	803.400
2.4	RebM2	1289.540	641.400

[00226] The peak areas from a chromatogram from a mass spectrometer were used to generate the calibration curve. The molar ratio of relevant compounds (*i.e.*, RebA, RebD, RebM) were determined by quantifying the amount in moles of each compound through external calibration using an authentic standard, and then taking the appropriate ratios.

[00227] NMR analysis was performed to confirm that RebM was produced from the genetically modified strains. After fermentation, strains were removed from the fermentation broth. Steviol glycosides were purified from the remaining liquid medium. Any suitable methods for NMR analysis can be used including those described in, *e.g.*, WO/2017/093895 and WO/2016/028899.

Example 8. UGT enzymes found to have high activity to convert RebA to RebD.

[00228] Six UGT enzymes were found to have high activity to convert RebA to RebD (**FIG. 3**). The performance of these enzymes were benchmarked against two other UGT enzymes described in the literature, Os_UGT_91C1 (*i.e.*, EUGT11 described in WO 2013/022989 A2) and Sl_UGT_101249881 (*i.e.*, UGTSL2 in WO2014/193888 A1) as also performing the conversion of RebA to RebD. Table 5 lists the median ratio of [micromoles of Reb (D+M) / micromoles of (A+D+M)]; RebA, RebD, and RebM were measured in *in vivo* as described above. This ratio is a measure of the efficiency of RebA to RebD conversion. The sum of the uM of [Reb (A+D+M)] measures the total RebA that was ever made in the cell. The sum of the uM of [Reb (D+M)] measures the total RebD that was ever made in the cell. **FIG. 5 a-i** show the chromatograms of RebE, RebA, RebD, RebM, and RebM2 for all UGT genes in **FIG. 3** and Table 2. RebM2 is an isomer of RebM with a single wrong glucose linkage. RebM2 has Glc β (1-2)[Glc β (1-6)]Glc β 1- at the 19 carbon position (COOH) instead of the desired Glc β (1-2)[Glc β (1-3)]Glc β 1- for RebM.

[00229] Using the NMR methods described in Example 7, the sample product generated from the strain comprising UGT40087 was compared against a standard RebM by 1D and 2D NMR spectroscopy. Overlay of 1D and 2D NMR spectra recorded in methanol and pyridine confirmed that both compounds are identical. Detailed interpretation of 2D NMR data and comparison with data published in the literature confirmed that both samples were RebM.

[00230] In a fermentation vessel either 0.5 or 2.0 liters, UGT40087, when expressed in a different strain background, produced RebA, RebD, and RebM at a ratio of about RebA:RebD:RebM of about 1:7:50.

Table 4. Genes, promoters, and amino acid sequences of the enzymes used to convert FPP to RebA.

Enzyme name	Accession number or sequence ID	Promoter
Btrisporea.GGPPS	AFC92798.1	PGAL1
ent-CDPS_Os	Q5MQ85.11	PGAL1
KS_Pg	ADB55711.1	PGAL1
Sr.KO	AAQ63464.1	PGAL1
Sr.KAH	SEQ ID:10	PGAL1

Aa.CPR	ABC47946.1	PGAL3
UGT85C2	AAR06916.1	PGAL1
UGT74G1	AAR06920.1	PGAL10
UGT91D_like3	SEQ ID NO:7	PGAL1
UGT76G1	AAR06912.1	PGAL10

¹ First 65 amino acids removed and replaced with methionine

Table 5. Median ratio of RebA to RebD conversion of different UGT enzymes, with protein accession number or sequence ID

UGT name	Median conversion ratio	Protein Accession number or sequence ID	SEQ ID NO.	Organism	% identity to UGT40087 (BLASTP)
UGT40087	0.98	XP_004982059.1	1	<i>Setaria italica</i>	100%
Ob_UGT91B1_like	0.97	XP_006650455.1	6	<i>Oryza brachyantha</i>	53%
Hv_UGT_V1	0.87	BAJ94055.1	4	<i>Hordeum vulgare subsp. vulgare</i>	53%
Sl_UGT_101249881	0.70	XP_004250485.1	9	<i>Solanum lycopersicum</i>	28%
Sr.UGT_g252778	0.60	Sequence ID 3	2	<i>Stevia Rebaudiana</i>	39%
Os_UGT_91C1	0.53	XP_015629141.1	8	<i>Oryza sativa</i>	54%
Bd_UGT10840	0.47	XP_003560669.1	3	<i>Brachypodium distachyon</i>	54%
Bd_UGT10850	0.45	XP_010230871.1	5	<i>Brachypodium distachyon</i>	51%
UGT91D_like3	0.03	ACE87855.1	7	<i>Stevia Rebaudiana</i>	36%

Example 9: Specificity of enzymes in Table 5 that convert RebA to RebD.

[00231] The enzymes listed in Table 5 are able to catalyze the conversion of RebA to RebD by the addition of a glucose sugar onto the C-2' position of the 19-O-glucose of RebA through the formation of a beta-1,2-linked glycosidic bond. In certain embodiments, it is desirable for the biotechnological production of RebM in a heterologous host to produce RebM (1) at high purity and (2) without producing any “non-natural” steviol glycosides. Any product sold into the market will likely require extremely high purity to ensure the best flavor profile and meet multiple human food regulatory standards. The presence of steviol glycosides other than RebM will likely increase the cost of downstream processing to obtain

highly pure RebM. If there are significant amounts of non-RebM steviol glycosides, it could potentially compromise the final purity of the RebM product. In certain embodiments, it could be advantageous that the heterologous enzymes do not produce any “non-natural” steviol glycosides. A “non-natural” steviol glycoside is defined here as any steviol glycoside that is not known to occur naturally in the plant *Stevia rebaudiana*.

[00232] For the reasons described above, all enzymes listed in Table 5 were examined for their impurity profile, to determine if they made any unexpected or non-natural steviol glycosides that are not shown in Figure 2B. Chromatographic traces for the longer retention time for all enzymes listed in Table 2 were further analyzed. Of all the enzymes with conversion efficiencies above 50%, the only enzymes that did not make unexpected products were UGT40087 and Os_UGT_91C1 (EUGT11). As expected, UGT91D_like3 did not produce unexpected peaks in the chromatogram.

[00233] Among enzymes with conversion efficiencies above 50%, the chromatographic traces of these enzymes produced one or more unexpected peaks which are associated with not naturally occurring glycoside. For example, the chromatographic trace of Ob_UGT91B1_like produced an unexpected peak (steviol+2 glucose) between the RebA peak and the RebB peak at retention time of about 6.61 minutes. The intensity of the unexpected peak of Ob_UGT91B1_like was at least 31 times greater than that of the RebA peak. See **FIG. 9c**. The unexpected peak (steviol+2 glucose) is not present in the chromatographic trace from a parent control strain (with UGT74G1, UGT85C2, UGT76G1, and UGT91D_like3) or from a strain comprising UGT40087. See **FIG. 9b** and **9c**. In another example, the chromatographic trace of Hv_UGT_V1 produced an unexpected peak (steviol+2 glucose) between the RebA peak and the RebB peak at retention time of about 6.61 minutes. The intensity of the unexpected peak of Hv_UGT_V1 was 0.6 factor lower than that of the RebA peak. In another example, the chromatographic trace of SI_UGT_101249881 produced an unexpected peak (steviol+2 glucose) between the RebA peak and the RebB peak at retention time of about 6.67 minutes. The intensity of the unexpected peak of SI_UGT_101249881 was about 1.11 times greater than that of the RebA peak. In another example, the chromatographic trace of Sr.UGT_g252778 produced an unexpected peak (steviol+2 glucose) between the RebA peak and RebB peak at retention time of about 6.67 minutes. The intensity of the unexpected peak of Sr.UGT_g252778 was about 0.96 factor lower than that of the RebA peak. Some of these enzymes also produced

additional unexpected peaks. The chromatographic traces for Hv_UGT_V1, SI_UGT_101249881, and Sr.UGT_g252778 are omitted.

[00234] These results indicate that compared to the two enzymes UGT40087 and Os_UGT_91C1 (EUGT11), the other three enzymes with greater than 50% A to D conversion efficiency (*i.e.*, Hv_UGTV1, SI_UGT_101249881, and Sr.UGT_g252778) may catalyze additional reactions, potentially producing steviol glycosides not normally made in the pathway to RebM in the plant *Stevia Rebaudiana*.

Table 6. UGT enzymes specificity.

UGT name	Unexpected steviol glycoside detected in chromatogram	Median conversion ratio
UGT40087	No	0.98
Ob_UGT91B1_like	Yes	0.97
Hv_UGT_V1	Yes	0.87
SI_UGT_101249881 (SL2)	Yes	0.70
Sr.UGT_g252778	Yes	0.60
Os_UGT_91C1 (EUGT11)	No	0.53
Bd_UGT10840	No	0.47
Bd_UGT10850	No	0.45
UGT91D_like3	No (control)	0.03

Example 10: UGT40087 enzyme activity is specific for the C-2' position of a 19-O-glucose steviol glycoside.

[00235] UGT40087 catalyzes the reaction of addition of a second sugar moiety to the C-2' of a 19-O-glucose of either stevioside or RebA. As shown in Figure 5a and 5i, the parent control strain without UGT40087 (as described in Example 2) is able to make RebA, but does not make detectable amounts of RebE, RebD, or RebM. Addition of UGT40087 into the parent cell now results in a detectable amount of RebE, indicating that UGT40087 catalyzes the addition of a second glucose moiety to the C-2' position of a 19-O-glucose in a beta-1,2 linkage of stevioside to make RebE. Also, two new peaks for RebD and RebM appear in the chromatogram for the strain containing UGT40087, while the peak for RebA decreases significantly, compared to the parent strain without UGT40087. Therefore, UGT40087 is able to catalyze the addition of a second glucose moiety to the C-2' position of

a 19-O-glucose in a beta-1,2 linkage of rebaudioside A to rebaudioside D. The presence of UGT76G1 in the strain then catalyzes the final glucose addition to convert RebD to RebM.

[00236] In order to screen of the ability to add a sugar to the C-2' position of a 13-O-glucose in a beta-1,2 linkage of either steviolmonoside or rubusoside, a base strain was made that carries high flux to rubusoside only. The rubusoside base strain is genetically identical to the RebA base strain described in example 2, except that the rubusoside base strain does not contain the enzyme UGT91D_like3. As shown in Table 7, the rubusoside base strain, which has UGT74G1, UGT85C2, and UGT76G1 is only capable of making 19-glycoside, steviolmonoside, and rubusoside. Adding UGT40087 to this strain does not change this profile at all. If UGT40087 were capable of adding a sugar to the C-2' position of a 13-O-glucose in a beta-1,2 linkage of either steviolmonoside or rubusoside, then there should be detectable amounts of either or both steviolbioside or stevioside. Steviolbioside, stevioside, and also RebA are seen when the strain is transformed with UGT91D_like3, which is capable of adding a sugar to the C-2' position of a 13-O-glucose in beta-1,2 linkage of both steviolmonoside and rubusoside.

[00237] EUGT 11 (Os_UGT_91C1) was previously characterized by others to catalyze the addition of a second sugar moiety to the C-2' position of a either a 19-O-steviol glycoside or a 13-O-steviol glycoside. See **FIG. 5** of WO 2013/022989. As noted above, UGT40087 does not appear to catalyze the addition of a sugar to the C-2' position of a 13-O-glucose in a beta-1,2 linkage of either steviolmonoside or rubusoside at a detectable level. These results indicate that UGT40087 is a UDP-glycosyltransferase which is functionally distinguishable and produces different results compared to EUGT11.

Table 7. Titters for all steviol glycoside intermediates in the pathway to RebM are normalized to the uM of steviolmonoside produced in the rubusoside base strain (bolded).

Strain	19-glycoside (uM) normalized	RebA (uM) normalized	RebB (uM) normalized	RebD (uM) normalized	RebM (uM) normalized	Rubusoside (uM) normalized	Steviol- bioside (uM) normalized	Steviol- monoside (uM) normalized	Stevioside (uM) normalized
Rubusoside base strain	2.01	0.00	0.00	0.00	0.00	0.73	0.00	1.00	0.00
Rubusoside base strain + UGT40087	1.60	0.00	0.00	0.00	0.00	0.82	0.00	1.15	0.00
Rubusoside base strain + 91D_like3	2.50	0.25	0.22	0.00	0.00	0.15	0.13	0.35	0.10

Example 11. Engineering of UGT chimeras by swapping the N-terminal domains

[00238] Plant UDP-glycosyltransferases (UGTs) share highly conserved protein structures even though they have relatively low amino acid sequence homology. These UGTs, which adopt a so-called GT-B structural fold, consist of two domains, roughly breaking at the mid-point of their primary amino acid sequences. The N-terminal domain is the sugar acceptor domain that mainly determines the substrate specificity. As expected, this domain is a more variable domain in terms of its amino acid sequence, reflecting the diverse substrates that can be glycosylated by UGTs. The more conserved C-terminal domain is the sugar donor domain, where UDP-glucose is bound. The two domains usually are linked by a flexible linker creating an ideal region for splitting the protein for domain swapping designs (**FIG. 6**). Given the nature of highly conserved domain structure of UGTs, domains from any two UGTs could be recombined to either alter substrate specificity or enhance a desired function such as catalytic activity. In this example, we investigated the role of the N-terminal domain of UGT40087 in conferring substrate specificity, *i.e.*, the activity of converting RebA to RebD by designing several UGT chimeras via domain swapping.

[00239] The general approach one would take to design a domain swapping experiment involves the following steps: 1) select swapping candidate pairs, 2) select domain swapping site for making a chimera between the pair of UGTs (with an option to mutate the C-terminal domain to improve interactions with the targeted substrate and the N-terminal domain), and 3) create, test and evolve chimeric proteins for the desired activity. This approach was taken in this example to perform domain swapping as described below.

[00240] Four UGTs were selected as parents for constructing chimeras: UGT40087 (SEQ ID No. 11), UGT Si91Dlike (SEQ ID No. 12), OS_UGT_91C1 (SEQ ID No. 8), and 91Dlike3 (SEQ ID No. 7). The N-terminal domain of UGT40087 was used to replace the N-terminal domains of Si91Dlike, OS_UGT_91C1 and 91Dlike3, creating three chimeras with 97%, 79% and 71% overall sequence identities to UGT40087, respectively.

[00241] SWISS-MODEL was used to generate structural models for all four UGTs. The exact amino acid positions (“swapping sites”) were selected based on structural alignments and sequence alignments. To minimize the perturbation to the 3-dimensional folding of the chimeric proteins, the flexible region located between the N- and C- terminal domains, which constitute the two distinct halves of the enzyme (**FIG. 6**), were used for swapping sites. Gly215 and Leu216 (amino acid numbers refer to UGT40087) in this region are highly

conserved in all UGTs including the four UGTs in this Example. Therefore, the two domains were split in between these two residues for domain swapping (**FIG. 6**).

[00242] In order to test the effects of domain swaps, the experiments were performed in the yeast strains that are genetically identical, except that the yeast strains include different UGTs. These yeast strains are, however, genetically different from those used in the UGT diversity screening described in Example 4. The general methods described in Examples 4 to 7 were used for strain transformation, culture, and analysis. The RebA to RebD conversion ratios of various domain swap designs are shown in Table 8 below.

Table 8. UGT40087 domain swapping results

Chimera	Median RebA to RebD conversion ratio	Sequence identity to UGT40087 (full length)	Sequence identity to UGT40087 (N-domain)	Sequence identity to UGT40087 (C-domain)
UGT40087	0.97	100%	100%	100%
Si91Dlike	0.07	90%	85%	93%
Os_UGT_91C1	0.88	54%	50%	60%
91Dlike3	0.11	36%	39%	42%
Chimera_UGT40087-Si91Dlike	0.96	97%	100%	93%
Chimera_UGT40087-Os_UGT_91C1	0.28	79%	100%	60%
Chimera_UGT40087-91Dlike3	0.07	71%	100%	42%
Control (no enzyme)	0.07	N/A	N/A	N/A

[00243] UGT40087 shares 90%, 54% and 36% overall sequence identities with Si91Dlike, Os_UGT_91C1 and 91Dlike3, respectively. Except 91Dlike3, which has approximately 40% sequence identities in both the N- and C-terminal sequences to UGT40087, the chimeras of the UGT40087 N-terminal domain and the C-terminal domains of Si91Dlike and Os_UGT_91C1 are active in converting RebA to RebD (Table 8). Even though Si91Dlike has no detectable activity, the replacement of its N-terminal domain with that of UGT40087 conferred all of the UGT40087 activity, indicating that the N-terminal domain of UGT40087 is important for RebA to RebD conversion.

[00244] Alignments of the four parent UGTs also reveal six N-terminal amino acid residues (V11, I12, P55, E90, S203, E223, and V413 in UGT40087; **FIG. 8** – the sequence alignment figure) that are shared by the two active enzymes (UGT40087 and Os_UGT_91C1) for RebA to RebD conversion, but are different from those in the two inactive enzymes (Si91Dlike and 91Dlike3).

Example 12. Engineering of UGT variants by swapping N-terminal loops

[00245] Comparison of modeled structures of UGT40087 and Os_UGT_91C1 revealed four loops that possess significant conformational differences at the N terminal sugar acceptor domain. The loop locations are shown in **FIG. 6**. To identify loops that may contribute to UGT40087's superior activity for RebA to RebD conversion, each of these loops was swapped in the two proteins to generate a total of 12 UGT variants. Two versions of loop_3 and loop_4 were designed to account for two possible loop lengths. Detailed designs are listed in Tables 9 and 10.

Table 9. UGT40087-based loop swapping design (Sequences between amino acids with subscript number are the regions being swapped in from Os_UGT_91C1).

Loop Number	Swapping sequence
UGT40087_loop1	S ₄₂ --- TPRNISRLLPPVPPALAP (SEQ ID NO:27) --- L ₆₀
UGT40087_loop2	V ₇₁ --- EGLPDGAESTNDVPHDRPDMV (SEQ ID NO:14) --- E ₉₀
UGT40087_loop3_1	F ₁₀₃ --- SEFLGTACAD (SEQ ID NO:15) --- W ₁₂₁
UGT40087_loop3_2	F ₁₀₃ --- SEFLGTACADWVIVDVFFH (SEQ ID NO:16) --- W ₁₃₀
UGT40087_loop4_1	Y ₁₅₆ --- ADRLERAETESPAAGQGRPAAAPTFEVARMKLIRTKGSSGM (SEQ ID NO:17) --- S ₁₇₁
UGT40087_loop4_2	A ₁₄₃ --- MMLLGSAHMIASADRLERAETESPAAGQGRPAAAPTFEVARMKLIRTKGSSGM (SEQ ID NO:18) --- S ₁₇₁

[00246] In Table 9, the amino acid sequence with a sequence ID number between two amino acid residues with subscripts is the loop region being swapped in from Os_UGT_91C into the UGT40087 base. The two amino acid residues with subscripts adjacent to the swapped loop region from Os_UGT_91C and the subscript numbers correspond to the amino acid residues and amino acid positions of UGT40087, respectively, prior to incorporation of the swapped loop region. In the new chimeric UGTs listed in Table 9, the swapped loop region from Os_UGT_91C replaced the corresponding loop region of the UGT40087 base.

[00247] It is noted that the amino acid sequence of SEQ ID NO:27, the swapped loop region, integrated into UGT40087_loop1 is a modified version of the original loop1 of Os_UGT_91C1. More specifically, the 12th amino acid residue in the sequence of SEQ ID NO:27 is proline (instead of arginine which is present in the corresponding position in the original loop1 of Os_UGT_91C having SEQ ID NO:8). Thus, in UGT40087_loop1, there is a single amino acid substitution, proline at position 51, compared to the original sequence of

UGT40087 having SEQ ID NO:11. The position of the substituted proline at position 51 in UGT40087_loop1 is shown in boldface in Table 9.

Table 10. Os_UGT_91C1-based loop swapping design (Sequences between amino acids with subscript number are the regions being swapped in from UGT40087).

Loop Number	Swapping sequence
Os_UGT_91C1_loop1	S ₄₉ --- TPRNISRRLRPVRPALAP (SEQ ID NO:28) --- L ₆₇
Os_UGT_91C1_loop2	V ₇₈ --- DGLPDGAEATSDIPPGKT (SEQ ID NO:19) --- E ₁₀₀
Os_UGT_91C1_loop3_1	F ₁₁₃ --- AAFLDAACADGSTNKVD (SEQ ID NO:20) --- W ₁₂₄
Os_UGT_91C1_loop3_2	F ₁₁₃ --- AAFLDAACADGSTNKVDWFLDNFQY (SEQ ID NO:21) --- W ₁₃₃
Os_UGT_91C1_loop4_1	I ₁₅₉ --- GVPRVEPPVDGSTA (SEQ ID NO:22) --- S ₂₀₃
Os_UGT_91C1_loop4_2	A ₁₄₆ --- LNLTFAASTSAEYGVPRVEPPVDGSTA (SEQ ID NO:23) --- S ₂₀₃

[00248] In Table 10, the amino acid sequence with a sequence ID number between two amino acid residues with subscripts is the loop region being swapped in from UGT40087 into the Os_UGT_91C1 base. The two amino acid residues with subscripts adjacent to the swapped loop region from UGT40087 and the subscript numbers correspond to the amino acid residues and amino acid positions of Os_UGT_91C1, respectively, prior to incorporation of the swapped loop region. In the new chimeric UGTs listed in Table 10, the swapped loop region from UGT40087 replaced the corresponding loop region of the Os_UGT_91C1 base.

[00249] It is noted that the amino acid sequence of SEQ ID NO:28, the swapped loop region, integrated into Os_UGT_91C1_loop1 is a modified version of the original loop1 of UGT40087. More specifically, the 12th amino acid residue in the sequence of SEQ ID NO:28 is arginine (instead of proline which is present in the corresponding position in the original loop1 of UGT40087 having SEQ ID NO:11). Thus, in Os_UGT_91C1_loop1, there is a single amino acid substitution, arginine at position 58, compared to the original sequence of Os_UGT_91C1 having SEQ ID NO:8. The position of the substituted arginine at position 58 in Os_UGT_91C1_loop1 is shown in boldface in Table 10.

[00250] The variant proteins were synthesized by Twist Bioscience with 60 bp of sequence homologous to the GAL1 promoter and a yeast terminator flanking the F-CphI sequences in the landing pad described in Example 4. Each synthesized UGT variant was tested individually as a single chromosomal copy for its activity in converting RebA to RebD in the same strain background as in Example 11 for the UGT domain swapping

chimera experiments. To create the strains, the UGT chimera donor DNAs and a plasmid containing the endonuclease F-CphI were transformed into the yeast host. Correct integrations were verified by colony PCR using a reverse primer internal to the specific UGT genes and a universal forward primer at the end of the integration locus. The confirmed strains were tested for RebA to RebD conversion as described above.

Table 11. UGT40087-based loop swapping results

Loop#	Median RebA to RebD conversion ratio	Sequence identity to UGT40087 (full length)	Sequence identity to UGT40087 (N-domain)
UGT40087	0.97	100%	100%
UGT40087_loop1	0.96	99%	99%
UGT40087_loop2	0.43	98%	95%
UGT40087_loop3_1	0.96	97%	95%
UGT40087_loop3_2	0.94	92%	92%
UGT40087_loop4_1	0.41	92%	84%
UGT40087_loop4_2	0.06	90%	80%
Control (no enzyme)	0.07	N/A	N/A

Table 12. Os_UGT_91C1-based loop swapping results

Loop#	Median RebA to RebD conversion ratio	Sequence identity to UGT40087 (full length)	Sequence identity to UGT40087 (N-domain)
Os_UGT_91C1	0.88	54%	50%
Os_UGT_91C1_loop1	0.70	54%	54%
Os_UGT_91C1_loop2	0.41	54%	54%
Os_UGT_91C1_loop3_1	0.89	55%	55%
Os_UGT_91C1_loop3_2	0.12	57%	57%
Os_UGT_91C1_loop4_1	0.97	61%	61%
Os_UGT_91C1_loop4_2	0.07	66%	66%
Control (no enzyme)	0.07	N/A	N/A

[00251] With UGT40087 as the parent, the loop-swapping variants were all active in converting RebA to RebD except for UGT40087_loop4_2 (Table 11). Loop4_2 is a long sequence that is located close to the N- and C-terminal domain interface, and its replacement may create significant perturbation to the overall structure of the variant. Among all the active variants, N-terminal sequence identities to UGT40087 range from 84% (Loop4_1) and 99% (Loop1).

[00252] With Os_UGT_91C1 as the parent, most of the loop-swapping variants also showed activity in converting RebA to RebD (Table 12). Again, Loop4_2 swapping

abolished the activity, supporting the hypothesis that its replacement may impact the structural integrity of the variant. Notably, incorporation of the Loop4_1 led to an increased RebA to RebD conversion (from 88% to 97%), indicating that Loop4_1 is responsible for conferring superior activity of UGT40087. In addition, the results from the loop swapping experiments with both proteins as parents also showed that these loop regions are important for conferring differences in activity and substrate specificity of the UGTs having RebA to RebD activity, and additional swapping of homolog loop variants or mutagenesis of these loop regions may be employed to generate improved UGT variants for converting RebA to RebD.

[00253] All publications, patents and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED:

1. A genetically modified host cell comprising a heterologous nucleic acid encoding a UDP-glycosyltransferase comprising an amino acid sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO: 1 or SEQ ID NO: 11, wherein the genetically modified host cell is capable of converting RebA to RebD at an efficiency of greater than 90%, 95%, 96%, or 97%.
2. The genetically modified host cell of claim 1, wherein the UDP-glycosyltransferase comprises an amino acid sequence having the sequence of SEQ ID NO:1 or SEQ ID NO:11.
3. The genetically modified host cell of any of the preceding claims, wherein the UDP-glycosyltransferase is capable of beta 1,2 glycosylation of the C2' position of the 19-O glucose of a steviol glycoside.
4. The genetically modified host cell of any of the preceding claims, wherein the UDP-glycosyltransferase is encoded by a heterologous nucleic acid, wherein the heterologous nucleic acid comprises a nucleotide sequence having at least 80%, 85%, 90%, or 95% sequence identity to SEQ ID NO:13.
5. The genetically modified host cell of any of the preceding claims, wherein the UDP-glycosyltransferase is encoded by a heterologous nucleic acid having the sequence of SEQ ID NO:13.
6. The genetically modified host cell of any of the preceding claims, that is capable of converting RebA to RebD at an efficiency of greater than 90%, 95%, 96%, or 97%, and wherein the UDP-glycosyltransferase comprises an amino acid sequence having at least 95% sequence identity to SEQ ID NO:1 or SEQ ID NO:11.
7. The genetically modified host cell of any of the preceding claims, wherein the UDP-glycosyltransferase comprises one or more of the following amino acid residues:

- (a) valine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 11 of SEQ ID NO:11;
- (b) isoleucine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 12 of SEQ ID NO:11;
- (c) proline at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 55 of SEQ ID NO:11;
- (d) glutamic acid at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 90 of SEQ ID NO:11;
- (e) serine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 203 of SEQ ID NO:11;
- (f) glutamic acid at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 223 of SEQ ID NO:11; or
- (g) valine at an amino acid position of the UDP-glycosyltransferase that corresponds to amino acid position 413 of SEQ ID NO:11,

wherein the amino acid positions of the UDP-glycosyltransferase that correspond to the amino acid positions of SEQ ID NO:11 are determined by sequence alignment.

8. The genetically modified host cell of any of the preceding claims, wherein the UDP-glycosyltransferase comprises an amino acid sequence having SEQ ID NO:24.

9. The genetically modified host cell of any of the preceding claims, wherein the UDP-glycosyltransferase comprises an amino acid sequence having at least 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO:1 or SEQ ID NO:11.

10. The genetically modified host cell of any of the preceding claims that is capable of producing RebD.

11. The genetically modified host cell of any of the preceding claims that is capable of producing RebM.

12. The genetically modified host cell of any of the preceding claims, wherein the genetically modified host cell further comprises one or more heterologous nucleic acids encoding one or more enzymes of a pathway for making steviol.

13. The genetically modified host cell of any of the preceding claims, wherein the genetically modified host cell further comprises one or more heterologous nucleic acids encoding one or more enzymes of a pathway for making a steviol glycoside.

14. The genetically modified host cell of any of the preceding claims, wherein the one or more enzymes of the pathway comprise a geranylgeranyl diphosphate synthase, a copalyl diphosphate synthase, a ent-kaurene synthase, a kaurene oxidase, a kaurenoic acid hydroxylase, a cytochrome P450 reductase, UGT74G1 (AAR06920.1), UGT76GI (AAR06912.1), UGT85C2 (AAR06916.1), and UGT91D (SEQ ID NO:7).

15. The genetically modified host cell of any one of any of the preceding claims, wherein the cell is selected from the group consisting of a bacterial cell, a fungal cell, an algal cell, an insect cell, and a plant cell.

16. The genetically modified host cell of any of the preceding claims, wherein the cell is a yeast cell.

17. The genetically modified host cell of any of the preceding claims, wherein the yeast is *Saccharomyces cerevisiae*.

18. A method for producing RebM:

- (a) culturing a population of the genetically modified host cells of any of the preceding claims in a medium with a carbon source under conditions suitable for making RebM; and
- (b) recovering said RebM compound from the medium.

19. A fermentation composition comprising the genetically modified host cells of any of claim 1-17 and steviol glycosides produced by the genetically modified host cells, wherein the steviol glycosides comprise RebA, RebD and RebM at a RebA:RebD:RebM ratio of at least 1:7:50.