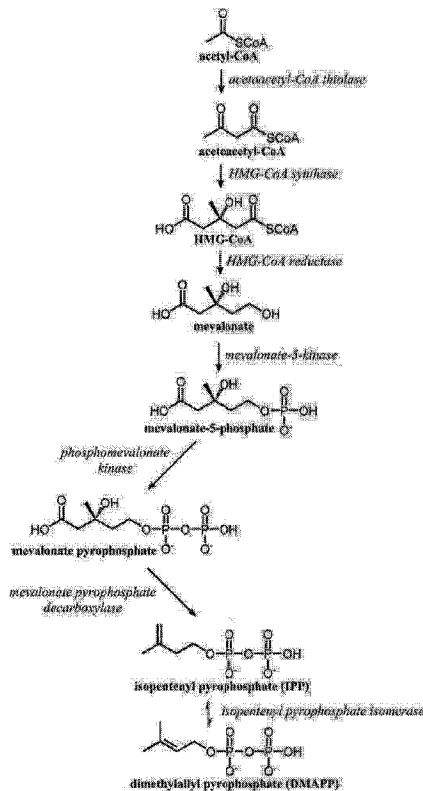




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(57) Abrégé/Abstract:

Disclosed are methods of releasing or enriching for steviol glycosides produced by yeast. The disclosed methods enhance or improve the release or enrichment of the steviol glycosides.

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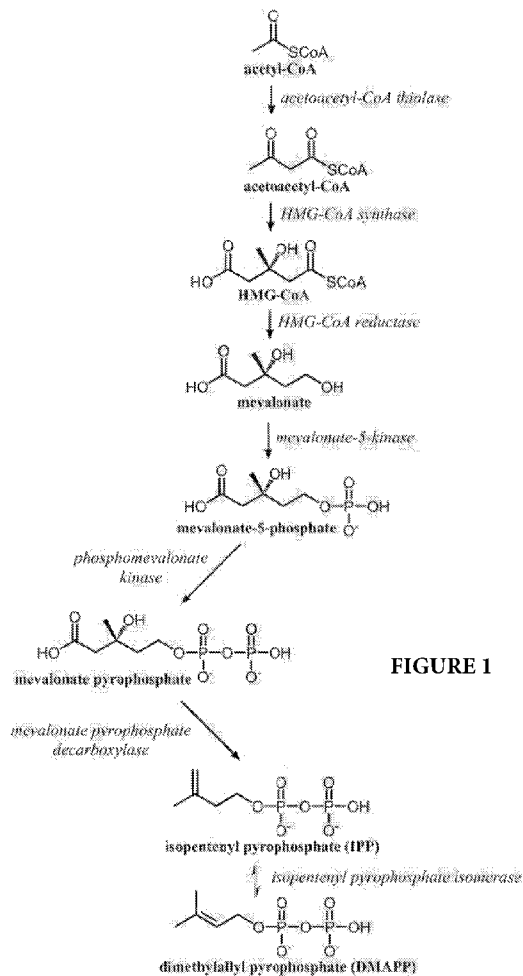
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[Continued on next page]

(54) Title: HEAT TREATMENT TO PRODUCE GLYCOSIDES

(57) Abstract: Disclosed are methods of releasing or enriching for steviol glycosides produced by yeast. The disclosed methods enhance or improve the release or enrichment of the steviol glycosides.



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HEAT TREATMENT TO PRODUCE GLYCOSIDES

Reference to Sequence Listing

[0001] This application contains references to amino acid sequences and/or nucleic acid sequences which have been submitted concurrently herewith as an ASCII text file entitled "CAR0211WO_Sequence_Listing.txt," created on May 27, 2016, and having a size of 92 kilobytes.

Cross-Reference to Related Applications

[0002] This application claims priority to U.S. Provisional Application No. 62/168,142 filed May 29, 2015.

Background

[0003] Sugars, such as sucrose, fructose and glucose, are utilized to provide a pleasant taste to beverages, foods, pharmaceuticals, oral hygienic and cosmetic products. Sucrose, in particular, imparts a taste preferred by most consumers. Although sucrose provides superior sweetness characteristics, it is caloric. Non-caloric or lower caloric sweeteners have been introduced to satisfy consumer demand. Consumers also desire that these sweeteners have favorable taste characteristics.

[0004] Stevia is a genus of about 240 species of herbs and shrubs in the sunflower family (*Asteraceae*), native to subtropical and tropical regions and from western North America to South America. The species *Stevia rebaudiana*, commonly known as sweetleaf, sweet leaf, sugarleaf, or simply stevia, is widely grown for its sweet leaves. Stevia-based sweeteners may be obtained by extracting one or more sweet compounds from the leaves. Many of these compounds are glycosides of steviol, a diterpene compound. These diterpene glycosides are about 150 to 450 times sweeter than sugar. Steviol glycosides differ from each other by sweetness power as well as other sensory features contributing to taste quality such as bitterness, lingering aftertaste and the like. See Kinghorn, A. D., *Stevia: The genus Stevia*, Taylor & Francis, London (2002).

[0005] Examples of steviol glycosides are described in PCT International Patent Application Publication No. WO 2013/096420 (see, e.g., listing in Fig. 1); and in Ohta et. al., "*Characterization of Novel Steviol Glycosides from Leaves of Stevia*

rebaudiana Morita,” J. Appl. Glycosi., 57, 199-209 (2010) (see, e.g., Table 4 at p. 204). Structurally, the diterpene glycosides are characterized by a single base, steviol, and differ by the presence of carbohydrate residues at positions C13 and C19, as presented in Figs. 2a-2k of WO 20013/096420.

[0006] Typically, on a dry weight basis, the four major steviol glycosides found in the leaves of *Stevia* are dulcoside A (0.3%), rebaudioside C (0.6-1.0%), rebaudioside A (3.8%) and stevioside (9.1%). Other glycosides identified in *Stevia* extract include one or more of rebaudioside B, D, E, F, G, H, I, J, K, L, M, N, O, steviolbioside and rubusoside.

[0007] While the major steviol glycoside, rebaudioside A, is commonly used as sweetener in beverage applications it has off-taste issues. More recently, there has been focus on certain minor steviol glycosides which have better taste properties. For example, rebaudioside M has higher sweetness intensity and is more potent than other steviol glycosides (e.g., see Prakash, I., et al. (2013) Nat. Prod. Commun., 8: 1523–1526, and WO 2013/096420). Rebaudioside D tastes about 200–220 times sweeter than sucrose and in a sensory evaluation had a slow onset of sweetness and was very clean, namely sweeter overall than sucrose, less sweet lingering

[0008] aftertaste compared to sucrose (e.g., see Prakash, I., et al. (2012) Int. J. Mol. Sci., 13:15126-15136).

[0009] Recombinant DNA technology has made it possible, and commercially viable, to produce desired steviol glycosides using a wide variety of host cells such as yeast. Recovery of intracellularly expressed products from within the host cells, however, is fraught with difficulties because of the necessity of rupturing or hydrolyzing the tough yeast cell wall. The choice of cell wall disruption can also affect the downstream processing of the recovered product and its subsequent purification from yeast cells or fermentation broths thereby affecting the efficiency and cost effectiveness of the process.

Summary

[0010] Disclosed is a method to efficiently recover desired steviol glycosides produced by recombinant yeast technology in a cost effective manner.

[0011] Disclosed, in one aspect of the invention, is a method of releasing steviol glycosides from a host cell, comprising heating a fermentation medium comprising

steviol glycoside producing engineered host cells to a temperature in the range from 50° C to 95° C for 5 minutes to 48 hours to release one or more steviol glycosides from the engineered host cells into the fermentation medium.

[0012] Disclosed in another aspect of the invention is a method of releasing steviol glycosides from a host cell, comprising the steps of:

- a) providing a fermentation medium comprising a host cell engineered to produce one or more steviol glycosides; and
- b) releasing the one or more steviol glycosides from the engineered host cell by heating the composition to a temperature in the range from 50° C to 95° C for 5 minutes to 48 hours, wherein the one or more steviol glycosides are released from an intracellular portion of the host cell into an extracellular portion of the fermentation medium.

[0013] Disclosed in yet another aspect of the invention is a method of releasing steviol glycosides from a composition, the method comprising the step of heating the composition to a temperature in the range from 50° C to 95° C for 5 minutes to 48 hours to release the steviol glycosides from engineered host cells in the composition.

[0014] Disclosed, in another aspect of the invention, is a method of releasing steviol glycosides, the method comprising the steps of:

- a) providing a composition comprising an engineered host cell to produce one or more steviol glycosides; and
- b) releasing the one or more steviol glycosides from the engineered host cell by heating the composition to a temperature in the range from 50° C to 95° C for 5 minutes to 48 hours.

[0015] Disclosed, in another aspect of the invention, is a composition comprising an engineered host cell to produce one or more steviol glycosides, and one or more steviol glycosides that are released from the engineered host cell by heating the composition to a temperature in the range from 50° C to 95° C for 5 minutes to 48 hours.

[0016] Such methods result in releasing the glycosides into the composition while minimizing cell lysis.

[0016a] In accordance with an aspect of the present invention, there is provided a method of releasing steviol glycosides from a host cell, comprising heating a fermentation medium comprising steviol glycoside-producing engineered host cells to a temperature in the range of from 50°C to 95°C for 5 minutes to 48 hours, wherein one or more steviol glycosides are released from the engineered host cells into the fermentation medium.

[0016b] In accordance with a further aspect of the present invention, there is provided a method of releasing steviol glycosides from a host cell, comprising the steps of: a) providing a fermentation medium comprising a host cell engineered to produce one or more steviol glycosides; and b) releasing the one or more steviol glycosides from the engineered host cell by heating the fermentation medium to a temperature in the range of 50°C to 95°C for 5 minutes to 48 hours, wherein the one or more steviol glycosides are released from an intracellular portion of the host cell extracellularly into the fermentation medium.

[0016c] In accordance with a further aspect of the present invention, there is provided a method of recovering steviol glycosides from a composition, comprising: heating a composition comprising a host cell engineered to synthesize steviol glycosides to a temperature in the range from 70°C to 95°C for 5 minutes to 2 hours to release the steviol glycosides from the host cells into the composition, wherein extracellular nitrogen content in the composition does not increase to more than 45% of total nitrogen content of the composition.

[0016d] In accordance with a further aspect of the present invention, there is provided a method of recovering steviol glycosides from a composition, comprising: heating a composition comprising a host cell engineered to synthesize steviol glycosides to a temperature in the range from 70°C to 95°C for 5 minutes to 2 hours to release the steviol glycosides from the host cells into the composition, wherein extracellular phosphorus content in the composition does not increase to more than 70% of total phosphorous components of the composition.

[0016e] In accordance with a further aspect of the present invention, there is provided a composition comprising an engineered host cell that produces one or more steviol glycosides that are released from the engineered host cell upon heating

the composition to a temperature in the range of from 50°C to 95°C for 5 minutes to 48 hours.

[0016f] In accordance with a further aspect of the present invention, there is provided a method of enriching a composition with steviol glycosides comprising: heating a composition comprising a host cell engineered to synthesize steviol glycosides to a temperature in the range from 70°C to 95°C for 5 minutes to 2 hours to release the steviol glycosides from the host cells into the composition, wherein extracellular nitrogen content in the composition does not increase to more than 45% of total nitrogen content of the composition, and/or wherein extracellular phosphorus content in the composition does not increase to more than 70% of total phosphorous components of the composition.

Description of the Drawings

[0017] FIG. 1 shows a representative mevalonate pathway.

[0018] FIG. 2 shows a representative non-mevalonate pathway.

[0019] FIG. 3 shows a representative pathway for steviol production.

[0020] FIG. 4 shows representative pathways for the biosynthesis of steviol glycosides from steviol.

[0021] FIG. 5A is a digital image of a bright field microscopy image before heat treatment.

[0022] FIG. 5B is a digital image of a bright field microscopy image after heat treatment.

Detailed Description

[0023] Embodiments of the disclosure described herein are not intended to be exhaustive or to limit the invention to the precise forms disclosed in the following detailed description. Rather a purpose of the embodiments chosen and described is so that the appreciation and understanding by others skilled in the art of the principles and practices of the present invention can be facilitated.

[0024] Host cells such as yeast and fungus can be engineered for the synthesis of steviol glycosides. During fermentation only a fraction of the glycosides produced by the host cell may be released from within the cells into the fermentation media. To increase the glycoside amount released from the host cell such as yeast, mechanical and chemical disruption methods may be used. Mechanical disruption, namely the breaking of the cell, releases the intracellular components into the surrounding medium. The result is a complex mixture of not only the desired recombinant product but also other products such as protein, nucleic acids, cellular metabolites, cell fragments, complicating the recovery of the desired product. Chemical methods entail chemically altering or making permeable the cell membrane and/or wall structure to allow diffusion of the product from within the cell. Chemical treatments, however, have to be compatible with the organism and the release of the desired product.

[0025] Disclosed are methods of efficiently recovering steviol glycosides after fermentation using heat treatments. Heat applied for a given time period and temperature to a fermentation broth or fermentation medium that includes host cells (e.g. yeast and fungus). The host cells can produce the desired steviol glycosides. When the fermentation medium containing the host cells are heated, the desired glycosides can be released from the host cells with minimal lysis of the cells thereby enriching the fermentation media or composition for the desired glycoside.

[0026] In some embodiments, the disclosed methods use engineered yeast capable of producing steviol glycosides. An engineered yeast capable of producing steviol glycosides can include one or more exogenous nucleic acids that encode enzyme(s) that promote formation of one or more steviol glycosides in the cell.

[0027] As used herein, the term “steviol glycoside(s)” refers to glycosides of steviol. Exemplary steviol glycoside, include, but are not limited to, rebaudioside A, rebaudioside B, rebaudioside C, rebaudioside D, rebaudioside E, rebaudioside F, rebaudioside G, rebaudioside H, rebaudioside I, rebaudioside J, rebaudioside K, rebaudioside L, rebaudioside M, rebaudioside N, rebaudioside O, stevioside, steviolbioside, dulcoside A, and/or rubusoside. Engineered yeast can produce steviol glycosides that are naturally found (“naturally occurring”) or ones that are not found in nature (non-naturally occurring). As used herein, the term “total steviol glycosides” (TSG) is calculated as the sum of the content of all steviol glycosides in a composition on a dry (anhydrous) basis.

[0028] Structurally, steviol glycosides have a central molecular moiety, which is a single steviol base, and glucopyranosyl residues, or other sugar residuals attached to the C₁₃ and C₁₉ atoms of the steviol base, according to the atom numbering on the base shown below. That is, glucopyranosyl residues represent groups R₁ and R₂ in the following formula:

[0030] The disclosed steviol glycosides may be produced in a fermentation process. The fermentation process can use a genetically modified organism that is engineered for the production of one or more steviol glycosides, such as rebaudioside A (Reb A), rebaudioside M (Reb M) and/ or rebaudioside D (Reb D). Production of steviol glycosides can be carried out using an engineered microbial strain having a set of enzymes that provide a pathway for the synthesis of steviol glycosides.

[0031] Various yeast or fungal host cells can be engineered to provide a pathway to one or more steviol glycosides. Such cells can be transformed with one or more DNA construct encoding enzymes for steviol glycoside synthesis. Exemplary yeast and fungus that can be used for hosts for exogenous DNA constructs encoding steviol glycoside pathway enzymes, include, but are not limited to the genus of, *Candida*, *Pichia* (*Hansenula*), *Kloeckera* (*Hanseniaspora*), *Kluyveromyces*, *Rhodotorula*, *Torulopsis*, *Zygosaccharomyces*, *Saccharomycete*, *Yarrowia*, and *Saccharomyces*. Exemplary species include *Candida albicans*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Further, host cells can also include genetic modifications other than those of the steviol glycoside pathway that may provide improved performance during fermentation.

[0032] An “engineered host cell” refers to yeast and fungal cells having at least one exogenous DNA sequence that is introduced into the cell, either integrated into the cell’s genome or present on an extrachromosomal construct, such as a plasmid in bacteria or episomes. While the specification may describe yeast cells in detail, it should be understood that the present disclosure may also be adapted to fungal cells.

[0033] The term “exogenous” refers to a molecule, such as a nucleic acid, or an activity, such as an enzyme activity. The exogenous molecule is introduced into the host yeast or fungus. An exogenous nucleic acid can be introduced in to the host organism by well-known techniques and can be maintained external to the hosts chromosomal material (e.g., maintained on a non-integrating vector), or can be integrated into the host’s chromosome, such as by a recombination event. Generally, the genome of an engineered yeast or fungus is augmented through the stable introduction of one or more recombinant genes. An exogenous nucleic acid can encode an enzyme, or portion thereof, that is either homologous or heterologous to the host organism. An exogenous nucleic acid can be in the form of a

“recombinant gene or DNA construct” referring to a nucleic acid that is in one or more ways manipulated through molecular techniques to be in a form that does not naturally exist. The term “non-natural” may be used to characterize a molecule, such as a nucleic acid or protein, or an organism that does not naturally exist in nature.

[0034] The term “heterologous” (e.g., “non-native”) refers to a molecule or activity that is from a source that is different than the referenced molecule or organism.

Accordingly, a gene or protein that is heterologous to a referenced organism is a gene or protein not found in that organism. In the context of the disclosure, a “heterologous glycosyltransferase” refers to a glycosyltransferase polypeptide that is different from any glycosyltransferase polypeptide that may be native to the host organism. For example, a specific glycosyltransferase gene found in a first species and exogenously introduced into a host yeast or fungal organism that is different than the first species is “heterologous” to the host yeast or fungal organism.

[0035] The engineered yeast or fungus can use an auxotrophic marker suitable for selecting for a transformant. The host cell can include modifications (e.g. deletions) in one or more genes that control auxotrophies, such as LYS2, LEU2, HIS3, URA3, URA5, and TRP1. Using a host cell having a desired genetic background for introduction of one or more exogenous genes, one or more gene construct(s) is introduced into a cell to integrate into the genome, or to be stably maintained and allow for expression. Methods for introducing a gene construct into a host cell include transformation, transduction, transfection, co-transfection, electroporation. In particular, yeast transformation can be carried out using the lithium acetate method, the protoplast method, and the like. The gene construct to be introduced may be incorporated into a chromosome in the form of a plasmid, or by insertion into the gene of a host, or through homologous recombination with the gene of a host. The transformed yeast into which the gene construct has been introduced can be selected with a selectable marker (for example, an auxotrophic marker as mentioned above). Further confirmation can be made by measuring the activity of the expressed protein.

[0036] The transformation of exogenous nucleic acid sequences including the steviol pathway genes can be confirmed using methods well known in the art. Such

methods include, for example, nucleic acid analysis such as Northern blots or polymerase chain reaction (PCR) amplification, or immunoblotting for expression of gene products, or other suitable analytical methods to test the expression of the introduced nucleic acid sequences or their corresponding gene product. It is understood by those skilled in the art that the exogenous nucleic acid is expressed in an amount sufficient to produce the desired product, and it is further understood that expression levels can be optimized to obtain sufficient expression using methods well known in the art and as disclosed herein.

[0037] The terpenoid compounds, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), can serve as chemical precursors to steviol glycosides in an engineered host cell (e.g. yeast). Some organisms, including plants, insect, and some microbial species, have a mevalonate (MVA) pathway that converts acetyl-CoA through a series of chemical intermediates to IPP and DMAPP. Some organisms produce IPP and DMAPP through the non-mevalonate pathway (also known as the methyl D-erythritol 4-phosphate or MEP pathway) starting with glyceraldehyde-3-phosphate (G3P) and pyruvate (PYR).

[0038] The yeast *Saccharomyces cerevisiae* naturally expresses genes of the mevalonate pathway. Mevalonate pathway genes include: (a1) acetoacetyl CoA thiolase (EC 2.3.1.9), (b1) 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase (EC 4.1.3.5); (c1) HMG-CoA reductase (EC 1.1.1.34); (d1) mevalonate kinase (EC 2.7.1.36); (e1) phosphomevalonate kinase (EC 2.7.4.2); and (f1) mevalonate diphosphate decarboxylase (EC 4.1.1.33). Mevalonate pathway enzymes convert acetyl-CoA to IPP as follows: acetyl-CoA → acetoacetyl-CoA → 3-hydroxy-3-methylglutaryl-CoA → mevalonate → mevalonate-5-phosphate → mevalonate-5-pyrophosphate → IPP. See also FIG. 1

[0039] In some embodiments, the engineered yeast can include one or more modifications to increase the flux from acetyl-CoA to IPP and/or DMAPP, thereby providing an increased pool of IPP and/or DMAPP for use in a pathway to steviol. The modifications can include, for example, increasing expression or activity of one or more mevalonate pathway enzymes (a1)–(f1), such as by placing a nucleic acid encoding an enzyme that is homologous or heterologous to the yeast cell under the control of a promoter that provides increased expression, using multiple copies of

the nucleic acid, and/or using a heterologous enzyme, a variant enzyme (e.g., including one or more amino acid substitutions), or a variant heterologous enzyme that provides a higher level of enzymatic activity as compared to the native enzyme.

[0040] Alternatively, the non-mevalonate (MEP) pathway can be used to provide IPP and DMAPP as precursors to steviol glycoside production. The yeast *Saccharomyces cerevisiae* does not naturally express genes of the MEP pathway, but can optionally be engineered to provide MEP pathway genes. Theoretically, the MEP pathway is more energetically efficient generally because it loses less carbon as CO₂ as compared to the MVA pathway (MEP pathway: 1 CO₂/IPP; MVA pathway: 4 CO₂/IPP; sugar as carbon source).

[0041] In particular, the non-mevalonate (MEP) pathway compounds, isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), are generated through a series of intermediates leading from glyceraldehydes-3-phosphate (G3P) and pyruvate (PYR), and a number of enzymes are responsible for this conversion. Enzymes involved in a biosynthetic pathway from G3P and PYR to IPP and DMAPP include (a2) 1-deoxy-D-xylulose-5-phosphate synthase (DXS), (b2) 1-Deoxy-D-xylulose-5-phosphate reductoisomerase (ispC)-, (c2) 4-diphosphocytidyl-2C-methyl- D-erythritol synthase (IspD), (d2) 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (IspE), (e2) 2C- Methyl-D-erythritol-2,4-cyclodiphosphate Synthase (IspF), (f2) 1-hydroxy-2-methyl-2-(E)-butenyl-4- diphosphate synthase (IspG), (g2) 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase (IspH), and (h2) isopentenyl-diphosphate isomerase (IDI). See FIG. 2

[0042] The engineered yeast used to produce steviol glycosides by fermentation can have one or more genetic modifications to increase the flux from G3P and PYR to IPP and/or DMAPP, thereby providing an increased pool of IPP and/or DMAPP for use in a pathway to steviol. The modifications can include, for example, increasing expression or activity of one or more enzymes (a2) – (h2), such as by placing a nucleic acid encoding an enzyme that is heterologous to the yeast cell under the control of a promoter that provides increased expression, using multiple copies of the nucleic acid, and/or using a heterologous enzyme, a variant enzyme (e.g., one including one or more amino acid substitutions), or a variant heterologous enzyme that provides a high levels of enzymatic activity.

[0043] The engineered yeast used to produce steviol glycosides by fermentation can also include a pathway to convert IPP and/or DMAPP to steviol. For example, in some aspects the engineered yeast can include exogenous nucleic acids expressing the following enzymes: (a3) geranyl geranyldiphosphate synthase (GGPPS), (b3) copalyl diphosphate synthase (CPS), (c3) kaurene synthase (KS), (d3) kaurene oxidase (KO), and (e3) kaurenoic acid 13-hydroxylase (KAH). Enzymes of the mevalonate pathway converts IPP and/or DMAPP to steviol as follows: IPP/DMAPP → geranyl geranyldiphosphate → copalyl diphosphate → kaurene → kaurenoic acid → steviol. (See FIG. 3) Exogenous nucleic acids encoding enzymes (a3) – (e3) that are heterologous to the yeast cell can be placed under the control of a promoter that provides increased expression, using multiple copies of the nucleic acid, and/or using a variant enzyme (e.g., one including one or more amino acid substitutions), or a variant heterologous enzyme that provides a high levels of enzymatic activity.

[0044] The methods of the disclosure for producing steviol glycoside(s) by fermentation can use engineered yeast including one or more steviol biosynthesis enzymes selected from the group of geranylgeranyl diphosphate synthase, copalyl diphosphate synthase, kaurene synthase, kaurene oxidase, kaurenoic acid 13-hydroxylase (KAH), steviol synthetase, deoxyxylulose 5-phosphate synthase (DXS), D-1-deoxyxylulose 5-phosphate reductoisomerase (DXR), 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase (CMS), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (CMK), 4-diphosphocytidyl-2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), 1-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate reductase (HDR), acetoacetyl-CoA thiolase, truncated HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase, cytochrome P450 reductase etc.

[0045] The engineered yeast used to produce steviol glycosides by fermentation can have any pathway to convert steviol to a steviol glycoside. If more than one steviol glycoside pathway enzymes are present in the engineered yeast, the yeast may be able to produce different steviol glycosides. For example, the yeast may be able to produce two, three, four, five, six, seven, eight, nine, ten, or more than 10 different

steviol glycoside species. In some embodiments, Reb A, Reb D and/or Reb M are produced by the engineered yeast cells.

[0046] The steviol glycoside pathway can include one or more uridine diphosphate (UDP) glycosyltransferases (UGTs) that mediate the transfer of glycosyl residues from activated nucleotide sugars to acceptor molecules. In the case of a steviol glycoside pathway, a monosaccharide unit can be transferred to a hydroxyl or carboxyl moiety on a steviol or steviol glycoside molecule, or to a hydroxyl group on a glucose group that is attached to the steviol base. See FIG. 4. UGTs have been classified into families and subfamilies based on sequence homology. See Li et al., 2001, J. Biol. Chem. 276:4338-4343. A superfamily of over 100 genes encoding UGTs, each containing a 42 amino acid consensus sequence, has been identified in the model plant *Arabidopsis thaliana*, and genes encoding UGTs have also been identified in several other higher plant species.

[0047] Exemplary UDP-glycosyltransferase can be any UDP-glycosyltransferase capable of adding at least one glucose unit to the steviol and or steviol glycoside substrate to provide the desired steviol glycoside. In one embodiment, the engineered yeast can include one or more UDP-glycosyltransferase selected from group UGT74G1 (SEQ ID NO: 1), UGT85C2 (SEQ ID NO: 2), UGT76G1 (SEQ ID NO: 3), UGT91D2 (SEQ ID NO: 4), and also UGTs having substantial (e.g. >85%, >75%, >65%, >55%, >45% and >35%) identity to these polypeptides. An engineered yeast can include one or more exogenous nucleic acid molecule(s) that code for these UGTs.

[0048] The engineered yeast can also include one or more UGT and UDP-glucose recycling enzyme(s). An exemplary UDP-glycosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside is UGT91D2 (SEQ ID NO: 4). An exemplary UDP-glycosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside A is UGT76G1 (SEQ ID NO: 3). An exemplary UDP-glycosyltransferase capable of adding at least one glucose unit to rebaudioside A to form rebaudioside D is UGT91D2 (SEQ ID NO: 4). An exemplary UDP-glycosyltransferase capable of adding at least one glucose unit to rebaudioside D to form rebaudioside M is UGT76G1 (SEQ ID NO: 3).

[0049] Exemplary publications that describe engineered microorganisms for steviol glycoside production include, for example, U.S. Patent Application Publication No. 2014/0357588, International Patent Application Publication Nos. WO 2014/193934, WO 2014/193888, and WO 2014/122227.

[0050] In one embodiment, an engineered yeast useful for the production of steviol glycosides expresses the following enzymes: geranylgeranyl diphosphate synthase (GGPPS), ent-copalyl diphosphate synthase (CDPS), kaurene oxidase (KO), kaurene synthase (KS); steviol synthase (KAH), cytochrome P450 reductase (CPR), UGT74G1, UGT76G1, UGT91D2, UGT85C2 and a EUGT11. WO2014/122227 describes an engineered yeast strain that express these enzymes. The UDP-glucosyltransferases can be a gene encoding a polypeptide for example, UGT74G1 (SEQ ID NO: 1), UGT85C2 (SEQ ID NO: 2), UGT76G1 (SEQ ID NO: 3), UGT91D2 (SEQ ID NO: 4), and a EUGT11 (SEQ ID NO: 13); these genes encode polypeptides capable of carrying out a number of reactions such as a) a gene encoding a polypeptide capable of beta 1,2 glucosylation of the C2' of the 19-*O*-glucose of a steviol glycoside; (b) a gene encoding a polypeptide capable of beta 1,2 glucosylation of the C2' of the 13-*O*-glucose of a steviol glycoside; (c) a gene encoding a polypeptide capable of beta 1,3 glucosylation of the C3' of the 19-*O*-glucose of a steviol glycoside; (d) a gene encoding a polypeptide capable of beta 1,3 glucosylation of the C3' of the 13-*O*-glucose of a steviol glycoside; (i) a gene encoding a polypeptide capable of glucosylation of the 13-OH of steviol or a steviol glycoside; (j) a gene encoding a polypeptide capable of glucosylation of the C-19 carboxyl of steviol or a steviol glycoside. For example, UGT85C2 carries out reaction (i); UGT74G1 carries out reaction (j); UGT91D2 carries out reactions (a; weakly), (b); UGT76G1 carries out reactions (c) and (d) EUGT11 carries out reactions (a), (b; less well).

[0051] In one embodiment the engineered host cell expresses one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the host cell: GGPPS polypeptide, an enf-copalyl diphosphate synthase (CDPS) polypeptide, a kaurene oxidase (KO) polypeptide, a kaurene synthase (KS) polypeptide; a steviol synthase (KAH) polypeptide, a cytochrome

P450 reductase (CPR) polypeptide, a UGT74G1 polypeptide, a UGT76G1 polypeptide, a UGT91 d2 polypeptide, and a EUGT11 polypeptide.

[0052] In another embodiment, the engineered yeast expresses one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the host cell: a GGPPS polypeptide, a truncated *Zea mays* CDPS polypeptide, an *Arabidopsis thaliana* KS polypeptide a *Stevia rebaudiana* KO polypeptide, an *Arabidopsis thaliana* ATR2 polypeptide, an *Oryza sativa* EUGT 11 polypeptide, a SrKAHe1 polypeptide, a *Stevia rebaudiana* CPR8 polypeptide, an *Stevia rebaudiana* UGT85C2 polypeptide, an *Stevia rebaudiana* UGT74G1 polypeptide, a *Stevia rebaudiana* UGT76G1 polypeptide, a *Stevia rebaudiana* UGT91D2 variant or functional homolog, and a UGT91D2e-b polypeptide.

[0053] Steviol glycoside-producing *S. cerevisiae* strains were constructed using methods as described in WO 2011/153378, WO 2013/022989, WO 2014/122227, and WO 2014/122328. The following sequences were used for construction of a parent strain (Strain A): a recombinant gene encoding a *Synechococcus* sp GGPPS polypeptide (SEQ ID NO:6), a recombinant gene encoding a truncated *Zea mays* CDPS polypeptide (SEQ ID NO:7), a recombinant gene encoding an *Arabidopsis thaliana* KS polypeptide (SEQ ID NO:8), a recombinant gene encoding a recombinant *Stevia rebaudiana* KO polypeptide (SEQ ID NO:9, SEQ ID NO:10), a recombinant gene encoding an *Arabidopsis thaliana* ATR2 polypeptide (SEQ ID NO:11, SEQ ID NO:12), a recombinant gene encoding an *Oryza sativa* EUGT 11 polypeptide (SEQ ID NO:13), a recombinant gene encoding an SrKAHe1 polypeptide (SEQ ID NO:14, SEQ ID NO:15), a recombinant gene encoding an *Stevia rebaudiana* CPR8 polypeptide (SEQ ID NO:16, SEQ ID NO:17), a recombinant gene encoding an *Stevia rebaudiana* UGT85C2 polypeptide (SEQ ID NO:2), a recombinant gene encoding an *Stevia rebaudiana* UGT74G1 polypeptide (SEQ ID NO:1), a recombinant gene encoding an *Stevia rebaudiana* UGT76G1 polypeptide (SEQ ID NO:3), and a recombinant gene encoding an *Stevia rebaudiana* UGT91D2 variant (or functional homolog), UGT91D2e-b, (SEQ ID NO:4) polypeptide produced steviol glycosides.

[0053a] The UGT91D2e-b variant of UGT91D2 (SEQ ID NO:5 from PCT/US2012/050021) includes a substitution of a methionine for leucine at position 211 and a substitution of an alanine for valine at position 286. (Additional variants, except T144S, M152L, L213F, S364P, and G384C variants, described in Table 12 and Example 11 of PCT/US2012/050021.) GeneArt codon-optimized sequence encoding a *Stevia rebaudiana* UGT91D2e-b with the amino acid modifications L211M and V286A (SEQ ID NO:4 for amino acid sequence; codon optimized nucleotide sequence is set forth in SEQ ID NO:5).

[0053b] Strain B is derived from the parent strain described above and additionally includes a codon-optimized CPR1 from *Stevia rebaudiana* (SEQ ID NO:18 corresponding to amino acid SEQ ID NO:19).

[0054] In one embodiment, the engineered yeast expresses one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the yeast: GGPPS polypeptide, an ent-copalyl diphosphate synthase (CDPS) polypeptide, a kaurene oxidase (KO) polypeptide, a kaurene synthase (KS) polypeptide; a steviol synthase (KAH) polypeptide, a cytochrome P450 reductase (CPR) polypeptide, a UGT74G1 polypeptide, a UGT76G1 polypeptide, a UGT91D2 polypeptide, and a EUGT11 polypeptide.

[0055] In another embodiment, the engineered yeast expresses one or more exogenous nucleic acid(s) encoding one or more of the following proteins heterologous to the yeast: a GGPPS polypeptide, a truncated *Zea mays* CDPS polypeptide, an *A. thaliana* KS polypeptide a *S. rebaudiana* KO polypeptide, an *A. thaliana* ATR2 polypeptide, an *O. sativa* EUGT 11 polypeptide, a SrKAHe1 polypeptide, a *S. rebaudiana* CPR8 polypeptide, an *S. rebaudiana* UGT85C2 polypeptide, an *S. rebaudiana* UGT74G1 polypeptide, a *S. rebaudiana* UGT76G1 polypeptide, a *S. rebaudiana* UGT91D2 variant or functional homolog, and a UGT91D2e-b polypeptide

[0056] The engineered host cells (e.g., yeast cells) can be used to produce the desired steviol glycosides such as rebaudioside M, rebaudioside D, and rebaudioside A by fermentation. "Fermentation" as used here refers to the assimilation of the medium such as the carbohydrates to produce the desired steviol glycosides through

aerobic fermentation. The term “medium” or grammatical equivalents such as “media” refers to a liquid composition in which the engineered yeast or fungus can be maintained, grown, or fermented, or combinations thereof. A “medium” may also be referred to as a “broth” or “cell culture,” and terms such as “fermentation” that qualifies the term “medium” and its equivalents may be used to more specifically define the type of cellular activity that is occurring in the medium.

[0057] A medium can be defined with regards to the components present in the medium, and amounts thereof, including, but not limited to: (a) carbon sources, including carbohydrates such as glucose and starch products such as maltodextrin; (b) nitrogen sources, such as yeast nitrogen base, ammonium hydroxide, urea, ammonium sulfate, yeast extract or any combination thereof; (c) salts, such as potassium phosphate (monobasic, dibasic), magnesium sulfate, sodium chloride, and calcium chloride; (d) vitamins, such as biotin, calcium pantothenate, folic acid, (myo)-inositol, nicotinic acid, p-aminobenzoic acid, pyridoxine HCl, riboflavin, thiamine HCl, and citric acid; and/or (e) trace metals such as boric acid, copper sulfate, cobalt chloride, calcium chloride, potassium iodide, ferric chloride, magnesium sulfate, manganese chloride, sodium molybdate, and zinc sulfate. The medium can also be defined with regards to its pH, and biocompatible acids, bases, and buffers that are used to control the pH in the medium.

[0058] Fermentation of the engineered yeast can be performed using starch and/or sugar containing plant material derivable from any plant and plant part, such as tubers, roots, stems, leaves and seeds. Starch and/or sugar comprising plant material can be obtained from cereal, such as barley, wheat, maize, rye, sorghum, millet, barley, potatoes, cassava, or rice, and any combination thereof. The starch-and/or sugar comprising plant material can be processed, such as by methods such as milling, malting, or partially malting. In some embodiments, the fermentation media includes a treated starch. For example, the fermentation media can include a partially hydrolyzed starch. The partially hydrolyzed starch can include high molecular weight dextrans and high molecular weight maltodextrins. A partially hydrolyzed starch product can be used that have amounts of starch and starch degradation products within desired ranges.

[0059] Fermentation can be carried out under conditions and in medium suitable for production of the desired steviol glycosides such as rebaudioside M, rebaudioside D, and rebaudioside A. The fermentation conditions generally use oxygen (aerobic conditions), a carbon source, and a nutrient (nitrogen) base. Fermentation can be carried out using a fed batch or continuous process. The fermentation minimal medium includes glucose (5g/L), ammonium sulfate (5g/L), potassium dihydrogenphosphate (3g/L), magnesium sulphate (0.5 g/L), trace elements, and vitamins (e.g., see, Verduyn, C. et al. (1992) *Yeast* 8, 501-517). The pH of the fermentation media can be at about pH 4 to pH 5 and the fermentation can be carried out at a temperature at about 30° C. In some embodiments, fermentation can be carried out using a first growth phase in base medium, followed by a longer feeding phase using a glucose-containing defined feed medium (with trace metals, vitamins, and salts).

[0060] In other embodiments, fermentation may be carried out using a pH greater than 5.0. In some embodiments, the pH may range from about 5.5 to 8 or 5.8 to 7.5. In some embodiments, the engineered yeast may be grown initially at a lower pH such as less than 6.0 and fermentation carried out a higher pH such as greater than 5.0. Other methods of fermentation for producing the desired steviol glycosides are described in the application titled “Fermentation Methods for Producing Steviol Glycosides Using High pH and Compositions Obtained Therefrom,” U.S. Pat. App. No. 62/168,345, and in International PCT application titled “Fermentation Methods for Producing Steviol Glycosides,” Attorney Docket No.

N00291WO01 (CAR0210/WO) and filed concurrently with the present application.

[0061] Optionally, fermentation can be carried out in media containing other intermediates such as steviol, rebudioside A, stevioside, rubusoside, steviol monoside, steviolbioside, dulcoside A, rebaudioside C, rebaudioside E, and the like.

[0062] The fermentation step can also be referred to as a “steviol glycoside producing step.” In some embodiments, the fermentation medium is maintained at a temperature less than 40° C during the fermentation step. In some embodiments, the fermentation medium is maintained at a temperature in the range of about 25 to 35° C during the fermentation step.

[0063] After fermentation, but before any treatment (e.g., heat treatment) to release or enrich for the desired steviol glycosides, the fermentation broth may contain only a fraction of the desired glycosides, with the majority of the desired glycosides within the engineered host cell. Disclosed are methods of releasing or enriching for the desired glycoside from the host cell by using heat.

[0064] The heat treatment may be carried out on the whole cell fermentation broth, which contains the engineered yeast capable of producing the desired steviol glycosides and the desired steviol glycoside products. In other embodiments, the engineered yeast after fermentation but before any treatment (e.g. heat treatment) may be separated from the fermentation broth and transferred into any suitable composition such as a liquid media.

[0065] The fermentation broth or composition containing the transferred yeast cells may be heated at temperatures ranging from 50°C to 95°C. In other embodiments, the heating is carried out at temperatures from 70°C to 95°C. Heating is carried out for 5 minutes to 48 hours. In some embodiments, heating is at 70°C to 95°C for 5 minutes to 2 hours, 95° C for 5 minutes to 1 hour, 75°C for 5 minutes to 2 hours, or 50°C for 24 hours. In some embodiments, the heating is at 70°C to 95°C for 5 minutes or from 70°C to 95°C for 2 hours.

[0066] In some embodiments, the heat treatment releases the steviol glycosides into the medium or enriches the steviol glycoside content of the medium such that the extracellular glycoside content is greater than 90% of the total (i.e., both intracellular and extracellular) glycoside content in the fermentation broth or composition. In some embodiments, the heat treatment step releases steviol glycosides into the fermentation medium such that the extracellular steviol glycoside content is greater than 80%, 85%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, of the total (i.e., both intracellular and extracellular) steviol glycoside content in the fermentation broth or composition. In some embodiments, the heat treatment step releases steviol glycosides into the fermentation medium such that the extracellular steviol glycoside content is in the range of about 80 to 100%, 85 to 100%, 90 to 100%, 90 to 99%, 92 to 98%, or 90 to 95% of the total (i.e., both intracellular and extracellular) steviol glycoside content in the fermentation broth or composition.

[0067] In some embodiments, the heat treatment of the fermentation broth or composition releases Reb A, Reb B, Reb M, Reb D or combinations thereof. In some embodiments, the heat treatment enriches for Reb A such that the extracellular Reb A is greater than 50 % of the total (i.e., both intracellular and extracellular) Reb A present in the fermentation broth or composition. In some embodiments, the extracellular Reb A is greater than 40 %, 45%, 48%, 52%, 55%, 57%, 60%, 62%, 65%, 67%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the total (i.e., both intracellular and extracellular) Reb A present in the fermentation broth or composition. In some embodiments, the Reb A amount, can be released extracellularly in the range of about 30% to about 100 %, about 50 % to about 100 %, about 80 % to about 90 % of the total amount of Reb A produced during the fermentation.

[0068] In some embodiments, the heat treatment enriches for Reb D and/or Reb M such that the Reb D and/or Reb M released extracellularly is greater than 33 % of the total (i.e. both intracellular and extracellular) Reb D and/or Reb M present in the fermentation broth or composition. In some embodiments, the extracellular Reb D, Reb M, and/or the total of Reb D and Reb M is greater than 40 %, 45%, 48%, 52%, 55%, 57%, 60%, 62%, 65%, 67%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the total (i.e., both intracellular and extracellular) Reb D, Reb M, and/or the total of Reb D and Reb M present in the fermentation broth or composition. In some embodiments, the combined amount of Reb D and Reb M, released extracellularly can be present in the range of about 30% to about 100%, about 50% to about 100%, about 80% to about 90% of the total amount of Reb D and/or Reb M present in the fermentation broth or composition.

[0069] In other embodiments, the Reb D released, can be present extracellularly in the range of about 30% to about 100%, about 50% to about 100%, about 80% to about 90 % of the total amount of Reb D present in the fermentation broth or composition.

[0070] In other embodiments, the Reb M released, can be present extracellularly in the range of about 30% to about 100%, about 50% to about 100%, about 80% to about 90% of the total amount of Reb M present in the fermentation broth or composition.

[0071] In some embodiments, the Reb B released, can be present extracellularly in the range of about 25% to about 100%, about 30% to about 95%, about 40% to about 80% of the total amount of Reb B present in the fermentation broth or composition. In some embodiments, the extracellular Reb B is greater than 30%. 33%. 35%, 40 %, 45%, 48%, 52%, 55%, 57%, 60%, 62%, 65%, 67%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the total (i.e., both intracellular and extracellular) Reb B present in the fermentation broth or composition. In some embodiments, the disclosed heat treatment does not result in significant degradation from Reb DM and/or Reb A to Reb B.

[0072] In some embodiments, the disclosed heat treatment results in the release of the desired glycosides yet does not result in extensive lysis of the cell. Cell lysis may be determined, for example, by the amount of nitrogen or phosphorus or both released into the fermentation broth (e.g., cell free) or composition after the fermentation and heat treatment. In some embodiments, the heating of the fermentation broth or composition does not increase nitrogen and/or phosphorus content in the cell free fermentation broth or composition. In some embodiments, the extracellular nitrogen content in the cell free fermentation broth or composition after the heat treatment is not increased by more than 45% of the total (i.e. extracellular and intracellular) nitrogen present in the whole cell fermentation broth or composition. In some embodiments, the extracellular phosphorous content in the fermentation broth or composition after the heat treatment is not increased by more than 70% of the total (i.e. extracellular and intracellular) phosphorous present in the cell-containing fermentation broth or composition.

[0073] In still other embodiments, the extracellular nitrogen content is not increased by more than 45% of the total nitrogen present in the cell-containing fermentation broth or composition and/or the extracellular phosphorous content in the fermentation broth or composition after the heat treatment is not increased by more than 70% of the total phosphorous present in the cell-containing fermentation broth or composition when heated at 70°C to 95°C for 5 minutes to 2 hours.

[0074] In some embodiments, when the fermentation medium or composition is heated at 50 to 95°C for 5 minutes to 48 hours, the extracellular nitrogen content is not increased by more than 30%, 33%, 35%, 37%, 40%, 42%, 47%, or 50% of the

total nitrogen present in the cell-containing fermentation broth or composition. In some embodiments, when the fermentation medium or composition is heated at 50 to 95°C for 5 minutes to 48 hours, the extracellular phosphorus content is not increased by more than 50%, 55%, 60%, 63%, 65%, 67%, 73%, 75%, 77%, or 80% of the total phosphorus present in the cell-containing fermentation broth or composition.

[0075] The intracellular and/or extracellular content of various components of the fermentation broth or composition (e.g., steviol glycosides, nitrogen, or phosphorus) can be determined as described in Example 1.

[0076] The heat treatment of the fermentation broth or composition may also be evaluated based on the cell viability. While the desired glycosides may be released from the yeast cell into the fermentation broth or composition, the yeast viability is decreased while limiting cell lysis. Cell lysis may be determined, for example, by plating the whole cell broth on Potato Dextrose Antibiotic Agar or by cell viability counter (e.g., Cellometer™).

[0077] Heating may be carried out by using a heat exchanger in which heat is transferred to hot water or steam. In some embodiments, heating may be carried out using a heat exchanger with a holding loop, direct steam injection, or bulk heating of the material utilizing steam applied to a jacketed vessel. In some embodiments, the yeast may be concentrated for example, by centrifugation or filtration, before a heat treatment. In other embodiments, the centrifuged or filtered yeast may be transferred into other suitable media or composition and then used for heating.

[0078] Following the release of the desired recombinant product (e.g. steviol glycosides) from the host cell into the medium, thereby enriching for the desired product, various procedures can be used for further isolation and purification of the desired recombinant product. Such procedures include centrifugation, ultrafiltration, precipitation and chromatographic procedures.

[0079] After the steviol glycosides are released or enriched, the steviol glycosides may be further purified by preparative HPLC as described, for example in International Patent Application Publication No. WO 2009/140394.

[0080] The fermentation broth or composition after the heat treatment can then be centrifuged or filtered to remove the engineered cells. The fermentation broth can optionally be treated to remove low molecular weight components (glucose, basic nutrients, and salts), such as by membrane dialysis.

[0081] If it is desired to provide a composition with steviol glycosides including Reb A, Reb B, Reb M, Reb D, and combinations thereof in enriched or purified form, or where Reb A, Reb B, Reb M, Reb D, and combinations thereof are separated from other steviol glycosides, or separated from one another, further purification can be carried out. Such enrichment or purification of steviol glycoside components can be carried out on liquid fermentation media, or the fermentation media can then be dried down prior to purification. For example, fermentation media can be dried down using lyophilization to form a dry composition (e.g., powder or flakes) including steviol glycosides with Reb A, Reb B, Reb M, Reb D, and combinations thereof that can be subsequently processed.

[0082] In some embodiments, dried fermentation broth or composition enriched for steviol glycosides including Reb A, Reb B, Reb M, and/or Reb D, may be used as the starting material for purification. For example, a solvent or solvent combination can be added to the dried fermentation broth to dissolve or suspend materials that include the steviol glycosides. An exemplary combination for dissolving the steviol glycosides is a mixture of water and an alcohol (e.g., 50:50 ethanol:water). To facilitate dissolving or resuspending, the dried broth materials can be heated at a temperature above room temperature, such as in the range of 40°C – 60°C. Mechanical disruption of the dried fermentation broth materials can also be performed, such as by sonication. The dissolved or resuspended fermentation broth materials can be filtered using a micron or sub-micron filtration system prior to further purification, such as by preparative chromatography.

[0083] Dried fermentation broth or composition enriched for steviol glycoside compounds can be subjected to other purification processes, such as reverse phase liquid chromatography. A suitable resin can be used to retain steviol glycoside compounds in the column, with removal of hydrophilic compounds which get washed through the column with a liquid such as water. Elution of steviol glycosides including Reb A, Reb B, Reb M, Reb D, and combinations thereof from

the column can be accomplished using a suitable solvent or solvent combination such as acetonitrile or methanol.

[0084] In some embodiments, the steviol glycosides such as Reb A, Reb B, Reb M, Reb D, and combinations thereof can be purified using preparative liquid chromatography, such as high pressure liquid chromatography (HPLC) or ultra high pressure liquid chromatography (UHPLC). A steviol glycoside composition with Reb A, Reb B, Reb M, Reb D, and combinations thereof can be dissolved in a mobile phase, such as a mixture of water and an solvent (e.g., methanol, ethanol, acetonitrile) at a desired ratio (e.g., 60% water, 40% methanol, v/v). The composition can also be heated to enhance dissolution of the steviol glycoside material, such as heating at about 50°C. The solution can also be filtered prior to injection into the column, such as using a 0.2 µm filter. Phenomenex Kinetex XB-C18 5 µm, core-shell silica solid support, and stationary phase of C18 with iso-butyl side chains and TMS endcapping. The flow rate through the column can be based on column properties (such as about 20 mL/min), with a maximum pressure of 400 bar. Reb A, Reb B, Reb M, Reb D, and combinations thereof can be identified by their elution times from the column. In exemplary flow conditions Reb A, Reb B, Reb M, Reb D, and combinations thereof can elute from the column within 60 minutes. One of skill in the art will appreciate that the elution times for the Reb A, Reb B, Reb M, Reb D, and combinations thereof can vary with changes in solvent and/or equipment. One of skill in the art will also understand that although the process described below assumes certain order of the described steps, this order can be altered in some cases.

[0085] In some embodiments, the fermentation broth or composition enriched for steviol glycosides including Reb A, Reb M, and/or Reb D, may be dried and used as the starting material for further purification. A steviol glycoside composition with Reb A, Reb M, Reb D, and combinations thereof can be dissolved in water and passed through adsorption chromatographic column to remove hydrophilic impurities in the composition. Adsorbed steviol glycosides can be desorbed from the column with ethanol/water mixture. Upon removal of the ethanol, the steviol glycoside liquid stream can be further purified with ion exchange chromatography to remove ions and color bodies. Additional treatment with activated carbon could be

performed. To further separate specific steviol glycosides from others, a crystallization step could be used. The crystals may be enriched with Reb M, Reb D, Reb A, or other steviol glycosides. The crystals can be dried under heat and vacuum or can be re-slurried with water and spray dried.

Example 1

Production Of Reb D, Reb M And Reb A In Fed Batch Fermentation With Ammonium Hydroxide As The Primary Nitrogen Source

[0086] For inoculum preparation, a yeast strain designated strain B was cultured in 150 mLs of seed flask medium in 1 liter shake flasks at 250 rpm and 30°C for 20-24 hours.

Table 1 - Seed Flask Medium

Component	Formula	Concentration	Units
Biospringer 0251 yeast extract		7.5	g/L
Glucose monohydrate	C ₆ H ₁₂ O ₆ *H ₂ O	22.0	g/L

[0087] For the fermentation, 75 mL of seed culture was transferred into initial fermentation medium (Tables 2, 3 and 4) with a starting volume of 0.75 liters. Temperature was maintained at 30°C throughout. The air flow rate was 1.75 SLPM and the agitation rate was automatically controlled to increase in a stepwise manner from 400 to 900 rpm during the fermentation. Glucose concentration was kept limiting by controlling flow rates of feed medium (Table 5). A 2-phase feeding strategy involved an initial exponential phase beginning at 10 hours with a growth rate of $\mu=0.12$ 1/h while the second phase of feeding (or feed phase II) started at 33 hours with a constant flow rate of 0.180 ml/minute. Feeding was continued until a final volume of 1.95 liters was obtained by 120 hours.

[0088] pH was maintained at pH 5 with 12% NH₄OH throughout fermentation. Antifoam addition was controlled by utilization of foam control probes with 10 wt% antifoam solution (Ivanhoe 1163B). The medium was based on Verduyn et al (Verduyn C, Postma E, Scheffers WA, Van Dijken JP. Yeast. 1992 Jul; 8(7):501-17) with modifications as described in Tables 2 through 5.

Table 2 - Initial Fermentation Medium

Component	Formula	Concentration	Units
Glucose monohydrate	$C_6H_{12}O_5 \cdot H_2O$	22.0	g/L
Ammonium sulfate	$(NH_4)_2SO_4$	5.0	g/L
Monobasic potassium phosphate	KH_2PO_4	3.0	g/L
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7 H_2O$	0.5	g/L
Trace metals stock		10.0	ml/L
Vitamin stock		12.0	ml/L

Table 3 - Trace Metals Stock Solution

Component	Formula	Concentration	Units
Disodium edetate	$C_{10}H_{14}N_2Na_2O_8 \cdot 2H_2O$	15	g/L
Zinc sulfate heptahydrate	$ZnSO_4 \cdot 7H_2O$	4.5	g/L
Manganese (II) chloride tetrahydrate	$MnCl_2 \cdot 4H_2O$	1.026	g/L
Cobalt (II) chloride hexahydrate	$CoCl_2 \cdot 6H_2O$	0.32	g/L
Copper (II) sulfate heptahydrate	$CuSO_4 \cdot 5H_2O$	0.3	g/L
Sodium molybdate dihydrate	$Na_2MoO_4 \cdot 2H_2O$	0.4	g/L
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	3	g/L
Iron (II) sulfate heptahydrate	$FeSO_4 \cdot 7H_2O$	3	g/L
Boric acid	H_3BO_3	1	g/L
Potassium iodide	KI	0.1	g/L

Table 4 - Vitamin Stock Solution

Component	Formula	Concentration	Units
d-Biotin	$C_{10}H_{16}N_2O_6S$	50	mg/L
Calcium pantothenate	$C_{15}H_{32}CaN_2O_{10}$	1000	mg/L
Nicotinic acid	$C_5H_5NO_2$	1000	mg/L
Thiamine hydrochloride	$C_{12}H_{17}ClN_4OS \cdot HCl$	1000	mg/L
Pyridoxine hydrochloride	$C_8H_{11}NO_3 \cdot HCl$	1000	mg/L
p-aminobenzoic acid	$C_7H_7NO_2$	200	mg/L
myo-inositol	$C_6H_{12}O_5$	25000	mg/L

Table 5 - Fermentation Feed Medium

Component	Formula	Concentration	Units
Glucose monohydrate	$C_5H_{12}O_5 \cdot H_2O$	660	g/L
Urea (in urea treatments only)	NH_2CONH_2	33	g/L
Antifoam		1.3	g/L
Potassium sulfate	K_2SO_4	4.2	g/L
Sodium sulfate	Na_2SO_4	0.336	g/L
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	6.12	g/L
Monobasic potassium phosphate	KH_2PO_4	10.8	g/L
Trace metal stock		14.4	mL/L
Vitamin stock		14.4	mL/L

Example 2**Enrichment or Release and Analysis of Steviol Glycosides**

[0089] The fermentation broth generated from a recombinant yeast fermentation as described in Example 1 was used. The measurement of total steviol glycosides including intracellular and extracellular concentrations as well as the total nitrogen content (intracellular and extracellular) and total phosphorous content (intracellular and extracellular) was carried out by agitating the fermentation samples including the yeast cells to ensure that yeast cells were mixed and did not settle to the bottom of the vial. 100 μ L of the mixed fermentation broth were pipetted into a 2 mL microcentrifuge tube. 900 μ L of 61% methanol (extraction solvent) was added into the 2 ml microcentrifuge tube and agitated by placing on a sample rotator for 10 min to extract the steviol glycosides. The samples were then centrifuged at 10K rpm in a microcentrifuge for 3 min and the clarified supernatant was pipetted into an autosampler vial for analysis.

[0090] The fermentation broth (including cells) was heated at various temperatures and for various times. Heating was accomplished using a hot water bath and the sample was prepared as follows: 1 ml of the fermentation broth (including cells) was placed into a headspace vial for each temperature and time point studied. The vials were sealed and placed into the water bath and allowed to equilibrate for 5 minutes at each temperature studied prior to maintaining the samples at the desired

temperature for the desired amount of time. At the end of the designated time, the samples were removed from the water bath and were cooled at 4°C. 4 ml of sterile water was added to each vial after which the sample was transferred to a 14 ml sterile tube. Samples were vortexed and 1 ml was removed for determining cell viability by the plating method. The remainder of the sample was centrifuged at 4700 rpm for 30 minutes at room temperature to obtain clear, cell-free supernatant. The cell-free supernatant was removed for further analysis, for example the extracellular content of steviol glycosides, nitrogen, and phosphorus in the fermentation medium (Phosphorous via ICP, Nitrogen via Antek and glycoside via UHPLC).

[0091] The amount of glycosides released into the fermentation broth after the heat treatment was determined. Reb A, Reb D and Reb M were measured by ultra-performance liquid chromatography (UPLC). The cell free supernatant was also used to evaluate nitrogen and phosphorous release into the fermentation broth. Nitrogen levels were analyzed using the combustion method and an ANTEK™ nitrogen analyzer (Antek 9000 nitrogen detector utilizing nitrogen combustion and reaction with ozone to produce metastable nitrogen dioxide, which is detected via chemiluminescence and phosphorous levels were analyzed using inductively coupled plasma spectrometry ICP, Perkin Elmer Optima 3200DV, UV detection at 213.617nm, samples dissolved in 0.5% HNO₃). Yeast viability was determined using the Potato Dextrose Antibiotic Easy gel plates (Microbiology Labs test kits), incubated at room temperature for 5 days.

UPLC Method for glycoside separation

[0092] The steviol glycosides were separated using an Eclipse C18 Plus RRHD column (3mm x 150mm x 1.8um) with Eclipse Plus C18 guard (column 3mm x 5mm x 1.8um). The mobile phase used was channel A: 10mM phosphate buffer, pH 2.6 and channel B acetonitrile. The flow rate was 0.6 ml/min, the column temperature was 40° C and the detection was performed at ultraviolet absorption of 210 nm. The gradient elution profile in which the ratio of phosphate buffer to acetonitrile changed is shown below:

- i. Initial: 80% A/20% B
- ii. 7 minutes: 70% A/30% B

- iii. 12 minutes: 70% A/30% B
- iv. 15 minutes: 45% A/55% B
- v. 18 minutes: 45% A/55% B
- vi. 20 minutes: 20% A/80% B
- vii. 25 minutes: 20% A/80% B
- viii. 27 minutes: 15% A/85% B
- ix. 30 minutes: 15% A/85% B
- x. 30.5 minutes: 80% A/20% B
- xi. 35 minutes: 80% A/20% B

[0093] The UPLC results, shown as the % of extracellular Reb A, Reb D & M released from the yeast cell after the heat treatment is shown in Table 6. The % of the extracellular nitrogen and phosphorus are also shown.

[0094] Total nitrogen in the fermentation broth including cells was determined using a Leco Nitrogen Analyzer (Leco TruMac N, St. Joseph, MI). 1 ml of homogeneously mixed fermentation broth including cells were applied to a nickel lined ceramic combustion container for nitrogen combustion analysis.

[0095] Total phosphorus in the fermentation broth including cells was determined by ashing 5 mL of homogeneously mixed fermentation broth with a propane torch in a ceramic crucible. The resultant ash was held at 525°C overnight prior to dissolving the ash with 10 mls of 0.5% HNO₃. Phosphorus content in the nitric acid solution was determined using ICP.

Table 6.

Heat treatment condition		% extracellular Reb D&M	% extracellular Reb A	% extracellular Reb B	Reb B (% extracellular)	% extracellular N	% extracellular P
Temperature, °C	Time hr						
24	1	34%	48%	20%	28%	20%	45%
	2	34%	48%	21%	28%	21%	45%
	24	43%	52%	25%	30%	25%	51%
	48	47%	58%	30%	34%	30%	56%
	70	48%	59%	31%	34%	31%	55%

Temperature, °C	Time, hr	% extracellular D&M	% extracellular A	% extracellular B	% extracellular N	% extracellular P
50	0.08	44%	54%	30%	23%	46%
	0.5	56%	67%	36%	28%	55%
	1	72%	88%	41%	37%	65%
	1.5	76%	85%	44%	40%	67%
	2	88%	91%	46%	50%	76%
	24	98%	99%	58%	88%	93%
	48	95%	97%	60%	91%	92%
	70	93%	96%	61%	91%	97%
	72	92%	96%	60%	93%	95%
	96	91%	96%		91%	95%
	120	87%	92%		92%	95%

Temperature, °C	Time, hr	% extracellular D&M	Reb B (% extracellular)	% extracellular N	% extracellular P
70	0.08	101%		36%	64%
	0.25	101%	45%	35%	62%
	0.5	101%		36%	64%
	0.75	99%	45%	35%	66%
	1	101%		36%	66%
	1.5	101%	46%	37%	65%
	2	99%		37%	64%

Temperature, °C	Time, hr	% extracellular D&M	% extracellular A	Reb B (% extracellular)	% extracellular N	% extracellular P
75	0.08	96%	92%	44%	35%	65%
	0.5	98%	94%	45%	36%	68%
	1	96%	94%	46%	35%	69%
	2	95%	93%	47%	36%	67%
	24	80%	82%	69%	38%	74%
	48	74%	78%	86%	40%	82%
	70	68%	72%	98%	44%	88%

Temperature, °C	Time, hr	% extracellular D&M	% extracellular A	Reb B (% extracellular)	% extracellular N	% extracellular P
95	0.08	98%	93%	47%	32%	65%
	0.25	100%	99%	49%	33%	65%
	0.5	101%	99%	53%	36%	66%
	1	96%	91%	55%	35%	67%

[0096] As can be seen from Table 6, a heat treatment at a higher temperature and for a shorter time results in an increase in the release of the Reb A, Reb D and Reb M from within the yeast cell while affecting a heat kill but with minimal cell lysis as evaluated by the amount of nitrogen and phosphorus in the medium after the heat treatment. The table also shows that at higher temperatures (50°C, 75°C and 95°C and longer times), Reb DM and RebA can degrade to Reb B resulting in a lower Reb DM content in the broth and an increase in Reb B content.

[0097] Viable yeast counts after heat treatment are shown in Table 7. It takes between 2 and 24 hours at 50°C to kill the yeast while no more than 5 minutes at 75°C or 95°C to kill the yeast.

Table 7

Temperature	Time	YM (CFU/ml)
24C	24 h	1.02E+09
50C	1h	2.03E+04
	2h	600
	24h	< 10
	48h	< 10
	72h	< 10
75C	0.08 h	< 10
	0.5h	< 10
	1h	< 10
	2h	< 10
	24h	< 10
	48h	< 10
	70h	< 10
95C	0.08h	< 10
	1h	< 10

Example 3

[0098] Fermentation broth from a strain designated A in a two-phase process was heat treated at 75°C for one hour. The two phase rates of feeding are shown below:

Strain	pH control	Phase I	Phase II
B	5.0	0.12u	0.166 mL/min

[0099] Yeast cells were stained by the fluorescent dyes based on the Nexcelom Cellometer method. Live cells before heat treatment appeared green under microscope and dead cells after heat treatment appeared red. Bright field microscopy images showed a lack of debris after heat treatment (compare FIG. 5A with FIG. 5B) indicating that the yeast cells were intact even after heat treatment.

What is claimed is:

1. A method of releasing steviol glycosides from a host cell, comprising heating a fermentation medium comprising steviol glycoside-producing engineered host cells to a temperature in the range of from 50°C to 95°C for 5 minutes to 48 hours, wherein one or more steviol glycosides are released from the engineered host cells into the fermentation medium.
2. A method of releasing steviol glycosides from a host cell, comprising the steps of:
 - a) providing a fermentation medium comprising a host cell engineered to produce one or more steviol glycosides; and
 - b) releasing the one or more steviol glycosides from the engineered host cell by heating the fermentation medium to a temperature in the range of 50°C to 95°C for 5 minutes to 48 hours, wherein the one or more steviol glycosides are released from an intracellular portion of the host cell extracellularly into the fermentation medium.
3. The method of claim 1 or 2, wherein the temperature range is 70°C to 95°C for 5 minutes to 2 hours.
4. The method of claim 1 or 2, wherein the temperature range is 70°C to 95°C for 5 minutes.
5. The method of claim 1 or 2, wherein the temperature is about 50°C for 24 hours.
6. The method of claim 1 or 2, wherein the temperature is about 75°C for 5 minutes to 2 hours.
7. The method of claim 1 or 2, wherein the temperature is about 95°C for 5 minutes to 1 hour.

8. The method of any one of claims 1 to 7, further comprising a steviol glycoside production step wherein the fermentation medium is maintained at a temperature of less than 40°C.
9. The method of any one of claims 1 to 8, wherein the engineered host cell is yeast.
10. The method of claim 9, wherein the yeast is selected from a genus selected from the group consisting of *Candida*, *Pichia (Hansenula)*, *Kloeckera (Hanseniaspora)*, *Kluyveromyces*, *Rhodotorula*, *Torulopsis*, *Zygosaccharomyces*, *Saccharomycete*, *Yarrowia*, and *Saccharomyces*.
11. The method of claim 10, wherein the yeast is from the genus *Saccharomycete* or *Saccharomyces*.
12. The method of claim 11, wherein the yeast is *Saccharomyces cerevisiae*.
13. The method of any one of claims 1 to 12, wherein the one or more steviol glycosides released into the fermentation medium is greater than 90% of the total glycosides produced and found both intracellularly and extracellularly in the fermentation medium.
14. The method of any one of claims 1 to 13, wherein the one or more steviol glycosides is rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B or combinations thereof.
15. The method of claim 14, wherein the rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B or combinations thereof released into the fermentation medium is greater than 90% of the rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B total glycosides produced and found both intracellularly and extracellularly in the fermentation medium.
16. The method of claim 14 or 15, wherein the amount of rebaudioside M and/or rebaudioside D released into fermentation medium is greater than 90% of the

rebaudioside M and/or rebaudioside D total glycosides produced and found both intracellularly and extracellularly in the fermentation medium.

17. The method of claim 14, wherein the rebaudioside D and/or rebaudioside M released into the fermentation medium is greater than 33% of the total rebaudioside D and/or rebaudioside M produced and found both intracellularly and extracellularly in the fermentation medium.

18. The method of claim 14, wherein the rebaudioside D released into the fermentation medium is present in the range of about 30% to about 100% of the total rebaudioside D produced and found both intracellularly and extracellularly in the fermentation medium.

19. The method of claim 14, wherein the rebaudioside M released into the fermentation medium is present in the range of about 30% to about 100% of the total amount of rebaudioside M produced and found both intracellularly and extracellularly in the fermentation medium.

20. The method of claim 14, wherein the rebaudioside A released extracellularly into the fermentation medium is in the range of about 30% to about 100 %, about 50 % to about 100%, or about 80% to about 90% of the total amount of rebaudioside A produced during fermentation.

21. The method of claim 14, wherein the rebaudioside B released extracellularly into the fermentation medium is in the range of about 25% to about 100%, about 30% to about 95%, or about 40% to about 80% of the of the total amount of rebaudioside B produced during fermentation.

22. The method of any one of claims 1 to 21, wherein the heating does not increase extracellular nitrogen content in the fermentation medium to more than 40%, more than 45%, or more than 50% of total nitrogen content of the fermentation medium.

23. The method of claim 22, wherein the heating does not increase the extracellular nitrogen content to more than 45% of the total nitrogen components of the fermentation medium.
24. The method of any one of claims 1 to 23, wherein the heating does not increase extracellular phosphorus content to more than 65%, more than 70%, or more than 75% of total phosphorus components of the fermentation medium.
25. The method of claim 24, wherein the heating does not increase the extracellular phosphorus content to more than 70% of the total phosphorus content of the fermentation medium.
26. The method of any one of claims 1 to 25, further comprising separating or purifying the released steviol glycosides in the fermentation medium.
27. A method of recovering steviol glycosides from a composition, comprising:
heating a composition comprising a host cell engineered to synthesize steviol glycosides to a temperature in the range from 70°C to 95°C for 5 minutes to 2 hours to release the steviol glycosides from the host cells into the composition, wherein extracellular nitrogen content in the composition does not increase to more than 45% of total nitrogen content of the composition.
28. A method of recovering steviol glycosides from a composition, comprising:
heating a composition comprising a host cell engineered to synthesize steviol glycosides to a temperature in the range from 70°C to 95°C for 5 minutes to 2 hours to release the steviol glycosides from the host cells into the composition, wherein extracellular phosphorus content in the composition does not increase to more than 70% of total phosphorous components of the composition.
29. The method of claim 27 or 28, wherein the temperature range is 70°C to 95°C for 5 minutes.

30. The method of claim 27 or 28, wherein the temperature is about 75°C for 5 minutes to 2 hours.
31. The method of claim 27 or 28, wherein the temperature is about 95°C for 5 minutes to 1 hour.
32. The method of any one of claims 27 to 31, further comprising a steviol glycoside production step wherein the composition is maintained at a temperature of less than 40°C.
33. The method of any one of claims 27 to 32, wherein the engineered host cell is yeast.
34. The method of claim 33, wherein the yeast is selected from a genus selected from the group consisting of *Candida*, *Pichia (Hansenula)*, *Kloeckera (Hanseniaspora)*, *Kluyveromyces*, *Rhodotorula*, *Torulopsis*, *Zygosaccharomyces*, *Saccharomycete*, *Yarrowia*, and *Saccharomyces*.
35. The method of claim 34, wherein the yeast is from the genus *Saccharomycete* or *Saccharomyces*.
36. The method of claim 35, wherein the yeast is *Saccharomyces cerevisiae*.
37. The method of any one of claims 27 to 36, wherein the steviol glycosides released into the composition are greater than 90% of total glycosides produced and found both intracellularly and extracellularly in the composition.
38. The method of any one of claims 27 to 37, wherein the steviol glycosides are selected from the group consisting of rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B and combinations thereof.
39. The method of claim 38, wherein the rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B and combinations thereof released into the composition are greater than 90% of the rebaudioside M, rebaudioside D,

rebaudioside A, rebaudioside B and combinations thereof total glycosides produced and found both intracellularly and extracellularly in the composition.

40. The method of claim 38, wherein the rebaudioside M and/or rebaudioside D released into the composition are greater than 90% of the rebaudioside M, and/or rebaudioside D total glycosides produced and found both intracellularly and extracellularly in the composition.

41. The method of claim 38, wherein the rebaudioside D and/or rebaudioside M released into the composition is greater than 33% of the total rebaudioside D and/or rebaudioside M produced and found both intracellularly and extracellularly in the composition.

42. The method of claim 38, wherein rebaudioside D released into the composition is present in the range of about 30% to about 100% of the total amount of rebaudioside D produced and found both intracellularly and extracellularly in the composition.

43. The method of claim 38, wherein the rebaudioside M released into the composition is present in the range of about 30% to about 100% of the total amount of rebaudioside M produced and found both intracellularly and extracellularly in the composition.

44. The method of claim 38, wherein the rebaudioside A released extracellularly into the composition is in the range of about 30% to about 100 %, about 50 % to about 100%, or about 80% to about 90% of the total amount of rebaudioside A produced during fermentation.

45. The method of claim 38, wherein the rebaudioside B released extracellularly into the composition in the range of about 25% to about 100%, about 30% to about 95%, or about 40% to about 80% of the of the total amount of rebaudioside B produced during fermentation.

46. The method of any one of claims 27 to 45, wherein the heating does not increase extracellular nitrogen content in the composition to more than 40%, more than 45%, or more than 50% of total nitrogen content of the composition.

47. The method of claim 46, wherein the heating does not increase the extracellular nitrogen content to more than 45% of the total nitrogen components of the composition.

48. The method of any one of claims 27 to 47, wherein the heating does not increase extracellular phosphorus content in the composition to more than 65%, more than 70%, or more than 75% of total phosphorus components of the composition.

49. The method of claim 48, wherein the heating does not increase the extracellular phosphorus content to more than 70% of the total phosphorus content of the composition.

50. The method of any one of claims 27 to 49, further comprising separating or purifying the released steviol glycosides from the composition.

51. A composition comprising steviol glycosides obtained according to the method of any one of claims 1 to 50.

52. A composition comprising an engineered host cell that produces one or more steviol glycosides that are released from the engineered host cell upon heating the composition to a temperature in the range of from 50°C to 95°C for 5 minutes to 48 hours.

53. The composition of claim 52, wherein the steviol glycosides are selected from the group consisting of rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B and combinations thereof.

54. The composition of claim 52, wherein the rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B and combinations thereof are greater than 90% of the total rebaudioside M, rebaudioside D, rebaudioside A, rebaudioside B and

combinations thereof glycosides produced and found both intracellularly and extracellularly in the composition.

55. The composition of claim 53, wherein the rebaudioside M and/or rebaudioside D are greater than 90% of the total rebaudioside M and/or rebaudioside D glycosides produced and found both intracellularly and extracellularly in the composition.

56. The composition of claim 53, wherein the rebaudioside D and/or rebaudioside M are greater than 33 % of the total intracellular and extracellular rebaudioside D and/or rebaudioside M present in the composition.

57. The composition of claim 53, wherein rebaudioside D is present extracellularly in the composition in the range of about 30% to about 100% of the total rebaudioside D present in the composition.

58. The composition of claim 53, wherein the Reb M is present extracellularly in the composition in the range of about 30% to about 100% of the total Reb M present in the composition.

59. The composition of claim 53, wherein the Reb A released extracellularly is in the range of about 30% to about 100 %, about 50 % to about 100 %, or about 80 % to about 90 % of the total amount of Reb A produced.

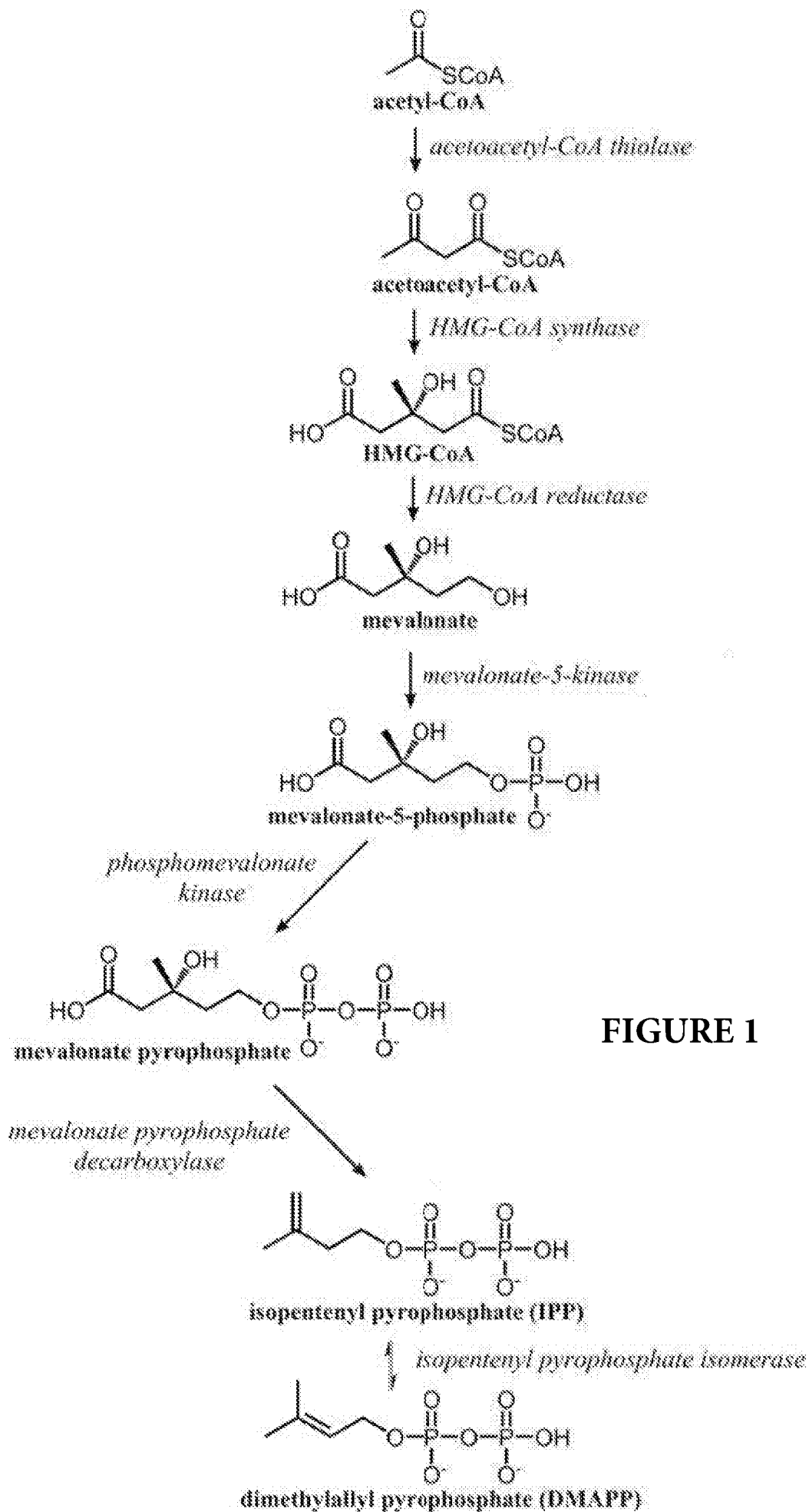
60. The composition of claim 53, wherein the Reb B is released extracellularly into the composition in the range of about 25% to about 100 %, about 30 % to about 100 %, or about 80 % to about 90 % of the total amount of Reb B produced.

61. The composition of any one of claims 52 to 60, wherein the heating does not increase extracellular nitrogen content to more than 45% of total nitrogen content of the composition.

62. The composition of any one of claims 52 to 60, wherein the heating does

not increase extracellular phosphorus content to more than 70% of total phosphorus content of the composition.

63. A method of enriching a composition with steviol glycosides comprising:
heating a composition comprising a host cell engineered to synthesize steviol glycosides to a temperature in the range from 70°C to 95°C for 5 minutes to 2 hours to release the steviol glycosides from the host cells into the composition,
wherein extracellular nitrogen content in the composition does not increase to more than 45% of total nitrogen content of the composition, and/or
wherein extracellular phosphorus content in the composition does not increase to more than 70% of total phosphorous components of the composition.



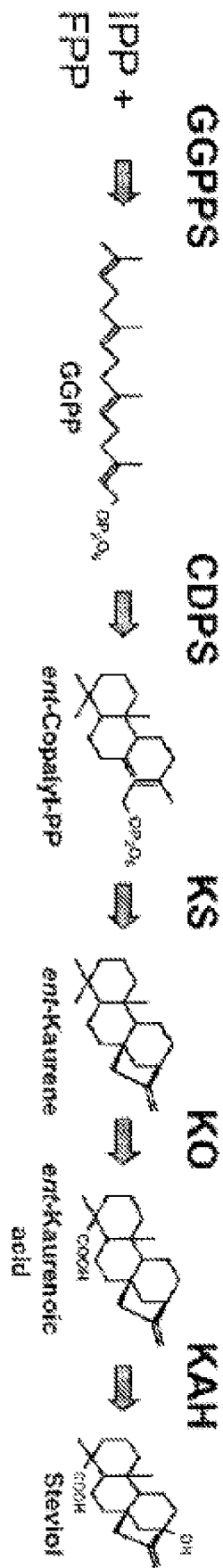


FIGURE 3

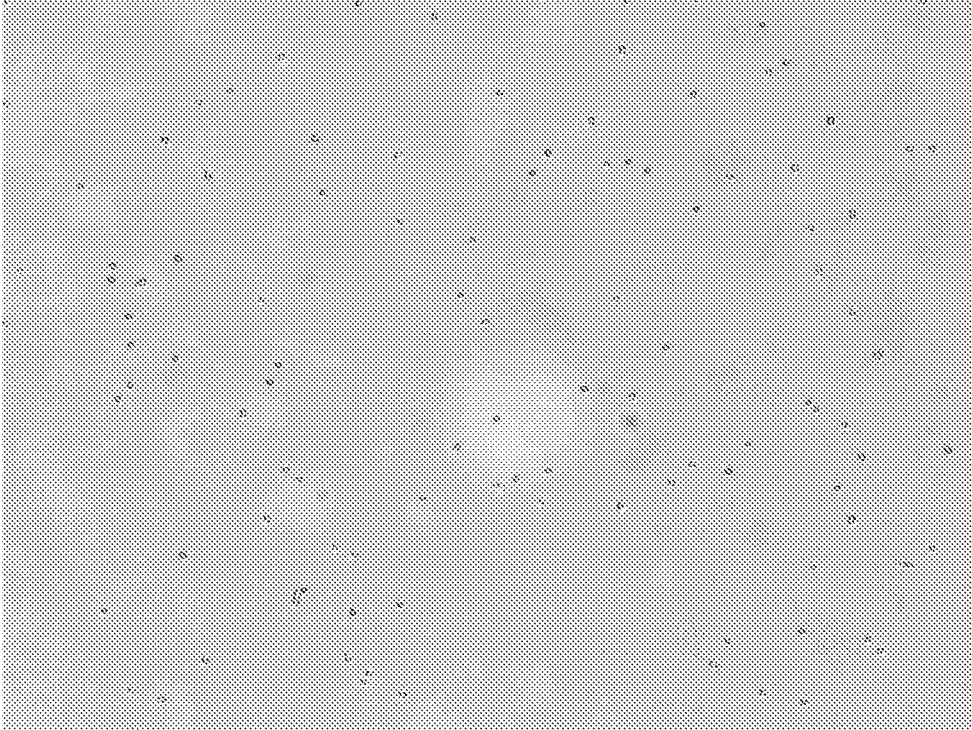


FIGURE 5 A

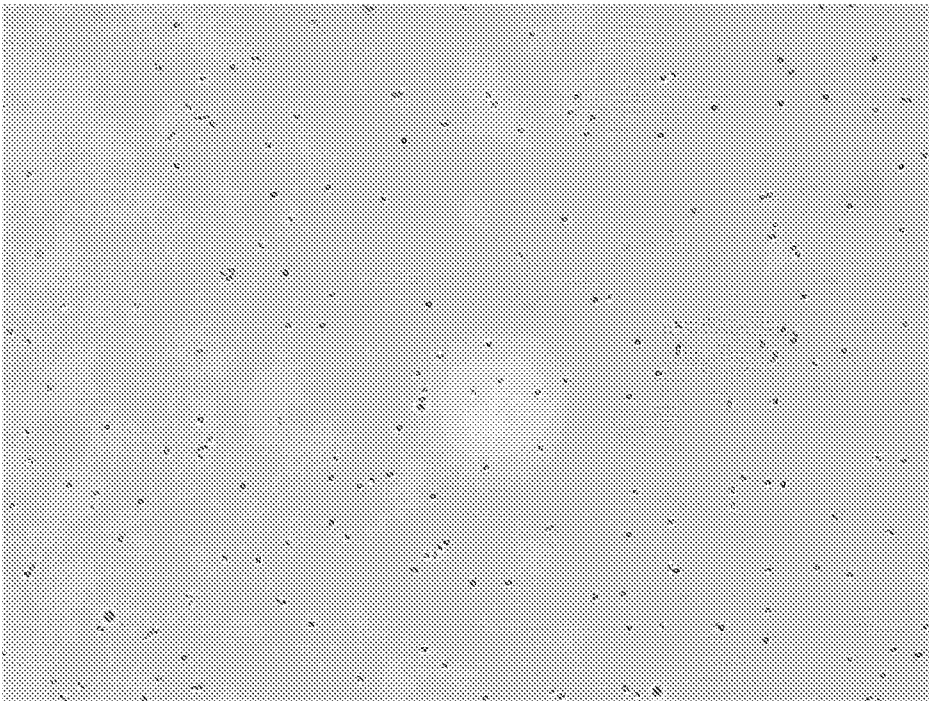


FIGURE 5 B



↓ acetoacetyl-CoA thiolase



↓ HMG-CoA synthase



↓ HMG-CoA reductase



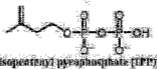
↓ mevalonate-5-kinase



phosphomevalonate
kinase



mevalonate pyrophosphate
decarboxylase



↓ isopentenyl pyrophosphate isomerase

