III. SUMMARY

[0006] Provided herein are compositions and methods for the improved conversion of kaurenoic acid to steviol. These compositions and methods are based in part on the production of certain kaurenoic acid hydroxylases (KAHs) that are capable of converting kaurenoic acid to steviol with remarkably high efficiency. Even a modest improvement in strain performance (*e.g.*, ten percent) with new KAHs 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 KAHs described herein are also capable of producing steviol with little or no residual kaurenoic acid. As such, in certain examples, the compositions and methods described herein can reduce the costs of downstream processing to obtain a composition with high yield steviol glycosides such as RebM.

[0008] Provided herein are genetically modified host cells and methods of their use for the production of industrially useful compounds. Provided herein is a genetically modified host cell comprising: a heterologous nucleic acid encoding a *Stevia rebaudiana* kaurenoic acid hydroxylase provided herein. In some examples, the genetically modified host cell further comprises one or more enzymatic pathways capable of producing steviol and/or steviol glycosides. In certain examples, the genetically modified host cell is capable of converting kaurenoic acid to steviol at an efficiency greater than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, or 98%.

[0008a] In one aspect, provided herein is a kaurenoic acid hydroxylase polypeptide with residue numbers according to SEQ ID NO:1, comprising one, two, three, four, five, six, seven, eight, nine, ten, or eleven of the following mutations: I166R, I153L, S158D, G306L, L232D, I333V, I350L, V316L, G447V, and M308L.

[0008b] In another aspect, provided herein is a kaurenoic acid hydroxylase polypeptide with residue numbers according to SEQ ID NO:1, comprising at least one of the following mutations: Y62H, T164R, L76V, Q415L, A60Y, S182C, T167N, Y52H, R266D, T167H, I153L, K100L, G306C, Q120N, L232H, I333V, G351R, L232S, I166S, W447C, I443Y, I166N, N355Y, L232D, S158D, A442G, G306N, V316L, M308L, G447F, G306I, G306V, I350L, G447V, I166R, and G306L.

[0008c] In another aspect, provided herein is a variant kaurenoic acid hydroxylase polypeptide of SEQ ID NO:1 comprising the following mutations I153L, S158D, I166R, L232D, I333V, and I350L.

[0008d] In another aspect, provided herein is a polynucleotide encoding the polypeptide as provided herein.

[0008e] In another aspect, provided herein is a host cell comprising the polypeptide as provided herein or the polynucleotide as provided herein.

[0009] 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 heterologous steviol glycoside as described herein, in a medium with a carbon source under conditions suitable for making said heterologous steviol glycoside compound; and recovering said steviol glycoside from the medium. In some examples, heterologous steviol glycoside is selected from the group consisting of RebD and RebM.

[0009a] In another aspect, provided herein is a method for producing steviol or one or more steviol glycosides comprising the steps: (a) culturing a population of the host cells as provided herein in a medium with a carbon source under conditions suitable for making steviol or one or more steviol glycosides to yield a culture broth; and (b) recovering said steviol or one or more steviol glycosides from the culture broth.

[0010] 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.

[0011] 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.

[0012] Provided herein are methods for producing steviol, the method comprising: contacting kaurenoic acid with a kaurenoic acid hydroxylase described herein, capable of converting kaurenoic acid to steviol, under conditions suitable for forming steviol.

[0013] 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 *Stevia rebaudiana* kaurenoic acid hydroxylase polypeptide provided herein.

[0013a] In another aspect, provided herein is a fermentation composition comprising: (a) a host cell comprising: a nucleic acid encoding a kaurenoic acid hydroxylase polypeptide as provided herein, capable of converting kaurenoic acid to steviol; and (b) steviol glycosides produced from the host cell.

IV. BRIEF DESCRIPTION OF THE FIGURES

[0014] **FIG. 1** provides an enzymatic pathway from the native yeast metabolite farnesyl pyrophosphate (FPP) to Rebaudioside M (RebM). Depicted in the figure are geranylgeranyl pyrophosphate (GGPP), copalyl pyrophosphate (CPP), and several rebaudiosides (Reb).

[0015] **FIG. 2** provides a schematic diagram of a "landing pad" design used to insert individual KAH enzymes for screening for kaurenoic acid to steviol conversion in yeast.

[0016] **FIG. 3** provides Sr.KAH mutants, each containing a single amino acid change, that have activity at least one standard deviation higher than the wild type Sr.KAH allele. The Y-axis represents the ratio of 19-glycoside (Strain 1) or Reb M (Strain 2) produced by Sr.KAH variant to that of wild type Sr.KAH.

[0017] **FIG. 4** provides combinations of Sr.KAH protein mutations leading to improvement of *in vivo* activity from 4.3x to 6.3 x in a yeast strain; Sr.KAH activity is measured in the Strain 3 background. The Y-axis is the relative fold increase of mutant Sr.KAH alleles over wild type Sr.KAH; wild type Sr.KAH activity is normalized to one.

[0018] **FIG. 5** provides improved KAH mutants leading to a reduction in the substrate kaurenoic acid compared to wild type Sr.KAH demonstrating that Sr.KAH alleles with improved activity are converting more substrate to steviol.

[0019] **FIG. 6** provides *in vivo* KAH activity of the degenerate codon library mutants measured in a Tier 1 screen versus the Sr.KAH mutant #3 allele using the titer of total steviol glycosides (μM) as described in Example 7.

WHAT IS CLAIMED:

1. A kaurenoic acid hydroxylase polypeptide with residue numbers according to SEQ ID NO:1, comprising one, two, three, four, five, six, seven, eight, nine, ten, or eleven of the following mutations: I166R, I153L, S158D, G306L, L232D, I333V, I350L, V316L, G447V, and M308L.

2. The kaurenoic acid hydroxylase polypeptide of claim 1 further comprising a heterologous amino terminal domain.

3. A kaurenoic acid hydroxylase polypeptide with residue numbers according to SEQ ID NO:1, comprising at least one of the following mutations: Y62H, T164R, L76V, Q415L, A60Y, S182C, T167N, Y52H, R266D, T167H, I153L, K100L, G306C, Q120N, L232H, I333V, G351R, L232S, I166S, W447C, I443Y, I166N, N355Y, L232D, S158D, A442G, G306N, V316L, M308L, G447F, G306I, G306V, I350L, G447V, I166R, and G306L.

4. The kaurenoic acid hydroxylase polypeptide of any one of claims 1 to 3, further comprising at least one mutation at kaurenoic acid hydroxylase positions E492C, M427A, G306D, V333C, D191L, I40S, L445I, S114A, D191Y, D191F, L497R, G306L, N29G, T167G, T164S, Q415H, T89A, L13D, R258T, and L13V, wherein the residue numbers are according to SEQ ID NO: 1.

5. A variant kaurenoic acid hydroxylase polypeptide of SEQ ID NO:1 comprising the following mutations I153L, S158D, I166R, L232D, I333V, and I350L.

6. The variant kaurenoic acid hydroxylase polypeptide of claim 5 further comprising one or more mutations selected from E492C, M427A, G306D, V333C, D191L, I40S, L445I, S114A, D191Y, D191F, L497R, G306L, N29G, T167G, T164S, Q415H, T89A, L13D, R258T, and L13V.

7. The variant kaurenoic acid hydroxylase polypeptide of claim 6 further comprising the mutation L13D.

8. The variant kaurenoic acid hydroxylase polypeptide of claim 6 further comprising the mutation R258F.

9. The variant kaurenoic acid hydroxylase polypeptide of claim 6 further comprising the mutation L13V.

10. A polynucleotide encoding the polypeptide of any of the preceding claims.

11. A host cell comprising the polypeptide of any of claims 1-9 or the polynucleotide of claim 10.

12. The host cell of claim 11 that is capable of producing steviol.

13. The host cell of claim 12 that is capable of producing one or more steviol glycosides.

14. The host cell of any one of claims 11-13 that is capable of producing RebA, RebB, RebD, RebE, or RebM.

15. The host cell of any one of claims 11-14further comprising one or more nucleic acids encoding one or more enzymes of a pathway for making steviol.

16. The host cell of any one of claims 11-15 further comprising one or more nucleic acids encoding one or more enzymes of a pathway for making a steviol glycoside.

17. The host cell of any one of claims 11-16 wherein the one or more nucleic acids are heterologous.

18. The host cell of any one of claims 11-17, 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.

19. A method for producing steviol or one or more steviol glycosides comprising the steps:

(a) culturing a population of the host cells of any of claims 11 to 18 in a medium with a carbon source under conditions suitable for making steviol or one or more steviol glycosides to yield a culture broth; and

(b) recovering said steviol or one or more steviol glycosides from the culture broth.

20. A fermentation composition comprising:

(a) a host cell comprising:

i. a nucleic acid encoding a kaurenoic acid hydroxylase polypeptide of any of claims 1 to 9, capable of converting kaurenoic acid to steviol; and

(b) steviol glycosides produced from the host cell.