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Abstract:

The present disclosure relates generally to production of GPP in a methylotrophic yeast strain containing a modified Erg20 gene and production of CBGA in a methylotrophic yeast strain.

PRODUCTION OF GPP AND CBGA IN A METHYLOTROPHIC YEAST STRAIN**CROSS REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority to United States Provisional Patent Application Numbers US 62/894,146, filed August 30, 2019, and US 62/899,948, filed September 13, 2019, the entire content of both is hereby incorporated by reference.

FIELD

[0002] The present disclosure relates generally to production of GPP and/or cannabigerolic acid (CBGA) in a methylotrophic yeast strain containing a modified Erg20 gene.

BACKGROUND

[0003] Cannabis is a genus of flowering plants that have been consumed by humans since at least 440 BCE. Cannabinoids represent a class of small molecules that interact with the human endocannabinoid system. Plant derived cannabinoids are classified as phytocannabinoids that are found in the cannabis plant as well as a variety of other plant species. Evidence suggests endogenous cannabinoids (endocannabinoids) play a critical role in regulating homeostasis in disease conditions by way of interactions with cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2). Human cannabinoid receptors are G protein-coupled receptors (GPCR) (Mackie, 2006).

[0004] Cannabis has been investigated as a medicinal product for multiple sclerosis spasms, sleep disorders, Tourette syndrome, glaucoma, anxiety, psychosis, depression, appetite stimulation in HIV/AIDS patients, chronic pain, and nausea and vomiting due to chemotherapy treatments. (doi:10.1001/jama.2015.6358)(The Health Effects of Cannabis and Cannabinoids: The Current State of Evidence and Recommendations for Research).

[0005] Despite having a high pharmacological potential for a broad range of therapeutic applications, obtaining highly purified cannabinoids remains a significant challenge that is oftentimes extremely resource intensive and therefore cost prohibitive. Plant extraction can be an especially difficult as cannabinoids are difficult to separate from other plant biomass and result in a contaminated product that is not purified to an extent necessary for most pharmaceutical applications or to meet regulatory standards of purity.

[0006] An inability to cost effectively purify individual cannabinoids from plant extracts not only makes pharmaceutical targets difficult for researchers to study and a challenge for regulators to approve, but in turn leads consumers to self-medicate with plant based cannabis that contains unknown chemical compositions. *Cannabis sativa* strains can vary widely in the level of composition of constituent cannabinoids. Unknown specificities of chemical composition make it nearly impossible to predict the exact pharmacological effects consuming plant based cannabis may provoke and gives little to no specificity to characterize or to target individual biochemical processes. These similar molecules can make isolation difficult. In addition to difficulties in the process of extracting cannabinoids from plants, yields of cannabinoid extract remain extremely low in compared to the overall weight of plant biomass with some sources stating cannabinoid yield extracts as low as 2.5% of total weight (In Med, 2019). Low extraction yields are partially explained by the hydrophobicity of most cannabinoids and add to the cost prohibitive nature of producing purified cannabinoid extracts from plants. Difficulties of extracting plant based cannabinoids are further exacerbated when attempting to purify individual cannabinoids.

[0007] There remains a need to produce the precursors of cannabinoids.

SUMMARY

[0008] In one aspect there is provided a method of producing geranyl pyrophosphate (GPP) in a methylotrophic yeast host cell, comprising:

[0009] - introducing a first polynucleotide encoding an Erg20 (F98W/N128W) polypeptide,

[0010] - culturing the methylotrophic yeast host cell under conditions sufficient for GPP production.

[0011] In one example, said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).

[0012] In one example, the first polynucleotide encoding an Erg20 (F98W / N128W) polypeptide comprises or consists of:

[0013] a) a nucleotide sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2;

[0014] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

[0015] c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

- [0016]** d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [0017]** e) a derivative or variant of a), b), c), or d).
- [0018]** In one example, in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.
- [0019]** In one example there is provided an expression vector comprising an isolated polynucleotide, comprising:
- [0020]** a) a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2,
- [0021]** b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- [0022]** c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- [0023]** d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [0024]** e) a derivative of a), b), c), or d).
- [0025]** In one example, in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.
- [0026]** In one aspect there is provided a methylotrophic yeast host cell comprising an expression vector of claim 5 or 6.
- [0027]** In one aspect there is provided a methylotrophic yeast host cell comprising:
- [0028]** a) a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2,
- [0029]** b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- [0030]** c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- [0031]** d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [0032]** e) a derivative of a), b), c), or d).
- [0033]** In one example, said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffii*).
- [0034]** In one aspect there is provided an isolated polynucleotide comprising or consisting of:
- [0035]** a) a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2,

- [0036]** b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- [0037]** c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- [0038]** d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [0039]** e) a derivative of a), b), c), or d).
- [0040]** In one example, isolated polynucleotide of claim 10, wherein said polynucleotide encodes an Erg20 (F98W / N128W) polypeptide.
- [0041]** In one aspect there is provided a method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising:
- [0042]** - introducing a first polynucleotide encoding an olevitiolic synthase polypeptide,
- [0043]** - introducing a second polynucleotide encoding an olevitiolic acid cyclase polypeptide, and
- [0044]** - introducing a third polynucleotide encoding an aromatic prenyl transferase,
- [0045]** - culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.
- [0046]** In one aspect there is provided a method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising:
- [0047]** - introducing a fourth polynucleotide encoding an olevitiolic synthase polypeptide and encoding olevitiolic acid cyclase polypeptide, and
- [0048]** - introducing a third polynucleotide encoding an aromatic prenyl transferase,
- [0049]** - culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.
- [0050]** In one aspect there is provided a method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising:
- [0051]** - introducing a first polynucleotide encoding an olevitiolic synthase polypeptide, and
- [0052]** - introducing a fifth polynucleotide encoding olevitiolic acid cyclase polypeptide and encoding acormatic prenyl transferase,

[0053] - culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

[0054] In one example, said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).

[0055] In one example, said conditions sufficient for CBGA production comprise methanol induction.

[0056] In one example, said first polynucleotide encoding olevitolic synthase polypeptide comprises or consists of:

[0057] a) a nucleotide sequence as set forth in (SEQ ID NO: 6);

[0058] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

[0059] c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

[0060] d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[0061] e) a derivative of a), b), c), or d).

[0062] In one example, said second polynucleotide encoding olevitolic acid cyclase polypeptide comprises or consists of:

[0063] a) a nucleotide sequence as set forth in (SEQ ID NO: 9);

[0064] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

[0065] c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

[0066] d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[0067] e) a derivative of a), b), c), or d).

[0068] in one example, said third polynucleotide encoding said acornatic prenyl transferase comprises or consists of:

[0069] a) a nucleotide sequence as set forth in (SEQ ID NO: 8);

[0070] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

[0071] c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

[0072] d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[0073] e) a derivative of a), b), c), or d).

[0074] In one example, said fourth polynucleotide encoding olevitiolic synthase polypeptide and encoding olevitiolic acid cyclase polypeptide comprises or consists of:

[0075] a) a nucleotide sequence as set forth in (SEQ ID NO: 3);

[0076] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

[0077] c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

[0078] d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[0079] e) a derivative of a), b), c), or d).

[0080] In one example said fifth polynucleotide encoding olevitiolic acid cyclase polypeptide and encoding aromatic prenyl transferase comprises or consists of:

[0081] a) a nucleotide sequence as set forth in (SEQ ID NO: 10);

[0082] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

[0083] c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

[0084] d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[0085] e) a derivative of a), b), c), or d).

[0086] In one example, in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.

[0087] In one aspect there is provided an expression vector comprising an isolated polynucleotide, comprising:

[0088] a) a nucleotide sequence set forth in SEQ ID NO 3, 6, 8, 9, or 10,

[0089] b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),

[0090] c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),

[0091] d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[0092] e) a derivative of a), b), c), or d).

[0093] In one example, in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.

[0094] In one aspect there is provided a methylotrophic yeast host cell comprising an expression vector of claim 23 or 24.

[0095] In one aspect there is provided methylotrophic yeast host cell comprising:

[0096] a) a nucleotide sequence set forth in SEQ ID NO 3, 6, 8, 9, or 10,

[0097] b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),

[0098] c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),

[0099] d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or

[00100] e) a derivative of a), b), c), or d).

[00101] In one example, said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).

BRIEF DESCRIPTION OF THE FIGURES

[00102] Embodiments of the present disclosure will now be described, by way of example only, with reference to the attached Figures.

[00103] Fig. 1 depicts an Erg20 duplication vector map ;

[00104] Fig. 2 depicts an Erg20 replacement vector map;

[00105] Fig. 3 depicts GPP production.

[00106] Fig. 4 depicts LC-MS/MS analysis. Panel A depicts Farnesyl pyrophosphate injected into water. Panel B is a graph showing Peak Area versus FPP Loaded. Panel C depicts Geranyl pyrophosphates injected into water. Panel D is a graph showing Peak Area versus GPP Loaded. Panel E shows a summary of the data. Panel F shows the ratio of GPP/FPP.

[00107] Fig. 5 depicts LC-MS/MS analysis. Panel A depicts Farnesyl pyrophosphate injected into MeOH grown yeast. Panel B is a graph showing Peak Area versus MeOH FPP Loaded. Panel C depicts Geranyl pyrophosphates injected into MeOH grown in yeast. Panel D is a graph showing Peak Area versus MeOH GPP Loaded. Panel E shows a summary of data. Panel F shows the ratio of GPP/FPP.

[00108] Fig. 6 depicts LC-MS/MS analysis. Panel A depicts Farnesyl pyrophosphate injected into glycerol grown yeast. Panel B is a graph showing Peak Area versus glycerol FPP Loaded. Panel C depicts Geranyl pyrophosphates injected into glycerol grown in yeast. Panel D is a graph showing Peak Area versus glycerol GPP Loaded. Panel E shows a summary of data.

[00109] Fig. 7 depicts Analytical results for CBGA production. LC/MS of GBGA generated from a *P. pastoris* strain that had been modified to contain the biosynthetic

cannabinoid pathway. The sample was separated on a C8 reverse phase column using water and acetonitrile as aqueous and organic phases in a gradient. The detection method was a total ion current, and the CBGA peak appears at the 0.80 min mark.;

[00110] Fig. 8 depicts Quantification of MeOH induced *P. pastoris* CBGA levels. Calibration curve and analysis of LC/MS data showing the quantification of the CBGA produced in *P. pastoris*. Panel A depicts CBGA injected into MeOH grown yeast. Panel B depicts a graph of Peak Area versus MeOH CBGA. Panel C shows a summary of data. Panel D shows a summary of the data.

[00111] Fig. 9 depicts LC-MS/MS Analysis of CBGA production. Panel A depicts Cannabigerolic Acid depicted into glycerol grown in yeast. Panel B depicts a graph of peak area versus glycerol CBGA loaded. Panel C depicts a summary of data.

[00112] Fig. 10 depicts the pGUH CsTKS map.

[00113] Fig. 11 depicts the pJAG dual CsPT4 CsOAC map.

[00114] Fig. 12 depicts the pJUN dual CsPT4 CsOAC map.

[00115] Fig. 13 depicts the pGAH CsTKS map.

DETAILED DESCRIPTION

[00116] Unless otherwise defined, all terms of art, notations and other scientific terminology used herein are intended to have the meanings commonly understood by those of ordinary skill in the art to which the present application pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art.

[00117] Cannabinoids are a group of chemicals known to activate cannabinoid receptors in cells throughout the human body, including the skin. Phytocannabinoids are the cannabinoids derived from cannabis plants. Endocannabinoids are endogenous cannabinoids found in the human body.

[00118] Cannabinoids exert their physiological effects by interacting with cannabinoid receptors present on the surface of cells. To date, two types of cannabinoid receptor have been identified, the CB1 receptor and the CB2 receptor, and are distributed in different tissues and also have different signaling mechanisms. They also differ in their sensitivity to agonists and antagonists.

[00119] It is though that cannabinoids may be used in therapeutics such as chronic pain, multiple sclerosis, cancer-associated nausea and vomiting, weight loss, appetite loss, spasticity, and other conditions.

[00120] The term cannabinoid includes acid cannabinoids and neutral cannabinoids. The term "acidic cannabinoid" refers to a cannabinoid having a carboxylic acid moiety. The carboxylic acid moiety may be present in protonated form (i. e., as -COOH) or in deprotonated form (i. e., as carboxylate -COO⁻). Examples of acidic cannabinoids include, but are not limited to, cannabigerolic acid (CBGA), cannabidiolic acid, and A9-tetrahydrocannabinolic acid. The term "neutral cannabinoid" refers to a cannabinoid that does not contain a carboxylic acid moiety (i. e., does contain a moiety -COOH or -COO⁻). Examples of neutral cannabinoids include, but are not limited to, cannabigerol, cannabidiol, and Δ9 -tetrahydrocannabinol.

[00121] **Production of geranyl geranyl diphosphate**

[00122] In one aspect, the present disclosure provides increased production of geranyl geranyl diphosphate (also referred to as pyrophosphate GPP; C₁₀) in a methylotrophic yeast containing a modified *P. pastoris* Erg20 gene. In one example, the methylotrophic yeast is *P. pastoris*.

[00123] GPP is the diphosphate of the polyprenol compound geraniol, and is a precursor of monoterpenes, and ultimately in downstream processes in the production of cannabinoids, for example, cannabigerolic acid (CBGA).

[00124] The polypeptide Erg20 exhibits both GPP synthase (GPPS) and farnesyl pyrophosphate synthase (FPPS) activities. Erg20 condenses isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAP) to GPP and farnesyl pyrophosphate (FPP). In the methylotrophic yeast *P. pastoris*, a greater amount FPP is produced than GPP in this reaction. However, as noted above, GPP is required for the production of cannabinoids, such as CBGA.

[00125] As described herein, a mutant of the Erg20 *P. Pastoris* gene containing two point mutations (F98W and N128W) was used to increase the amount of GPP produced in *P. Pastoris*. In one example, introduction of the mutant of the Erg20 *P. Pastoris* gene described in to *P. pastoris* increases the ratio of GPP:FPP, relative to the non-mutated Erg20 gene.

[00126] Thus, in one aspect, there is described herein a metabolic engineering strategy in which a mutant of the Erg20 *P. Pastoris* gene containing two point mutations (F98W and N128W) may be used to increase the production of GPP in *P. Pastoris*.

[00127] While not wishing to be bound by theory, it is believed that this mutant Erg20 (F98W/N128W) from *P. Pastoris* encodes a polypeptide that, when produced in *P. Pastoris*, biases the natural production of FPP and GPP in *P. Pastoris* towards GPP.

[00128] In one example, GPP production was achieved by introducing the modified Erg20 gene from *P. pastoris* containing two point mutations (F98W and N128W) (SEQ ID NO: 1 or SEQ ID NO: 2) in to *P. pastoris*.

[00129] In one example, GPP production was achieved by replacing the endogenous Erg20 allele of *P. pastoris* with the modified Erg20 gene from *P. pastoris* containing two point mutations (F98W and N128W) (SEQ ID NO: 1).

[00130] In one example, the modified Erg20 from *P. pastoris* used in replacing the endogenous allele of *P. pastoris* comprises or consists of the following polynucleotide sequence:

[00131] AGTCTCAAGACTGGAGACTCGCTCTTTATAGAAATAGATATAGCCTTT
TACTATTAATTCCATTTTAGTCATGCTGTGTTATATTTGTAGACATGATATAATCACT
GTATATTTTCAACTTTGATGTGCCCTTAAACGTTAACACGCTCAAGCTCCTCTCTACC
TGCCTTGGTGTACAGGTATTCATTGTAGTCTCTAGCTTGAGCCACACGTTCCAAAC
GATGATACCTGGAATAATCACATACAAAGCCTGAGCTTTGGTTCTTCTCCAAACGTTG
AAAATGGCTCTTTCCGATGATCCTTTCAAAGGAGATCCGACGTAAGGGGAGATGGTG
TAGGTAGTGACGTTCTTTTGCTTGGGGGATCCAATGAATCCCCACCAGCCCATGTAA
GCACCTGGATGTCCTGCCATTCTTAATGTAGTTTTAGGGTAAGTAGATGGTAGTAGA
ATTTCCCGTATTATATTCGAGGCCATCTGATTGGTGATAATTCCGTTCTGAAATATTC
GCAGGGAAACTTAGGTTGGTAGCAGTGATACCTGTAACCTTGCAGAGATAATCA
CCAGCTAATAAAGTATGGCCTTGGCCCTTTCGCATAGAGAATGGTCAAGACGTACAG
AACTATGAGGGCCCAACACTGTTTAACTTTAGTTTGGTGCCAACTTATGATTTAAAA
AGCCAGACAATTTAATTCCCCAAACAGACCTCTAATTTTGCCCGAATAATGCCAATT
AACATTCTAGAACTTCGATTAACCTATATTTTCATAAACCATGACCTTTTTCCGCCAA
TATCCTCATATTTAGCCTAAGGAAGACTCTCCTCCTTTTAGCCTACAACGAACCTTGC
CCCATAAATACACCAAGCGCGACCCTCGTTTCATCGTTTAACTCGTGTTTTCTTCTCAC
CTTTCGAAGTAGTAGAAATCAAATGTCCAAAGAAGTAGCAGCTAAGAACAAAGTGAA
GTTCTTGCCTTCTTTCCAGACCTCGTGTTTGAGCTGGAAGTCATGCTGGAAAATTA
CGGCATGCCTGCTGATGCAATCAACTGGTTCAAGAGATCCTTGAACACAACTCC
TGGTGGAAACTCAACAGAGGTATCTCTGTTGTGCGACACCTATGTCATTCTCAAGGG
GTACAAGTCATGGGACGAGTTAAGTGCCGAAGAGTTCAAGAAAGCTGCTATTTTAGG
ATGGTGTGTCGAATTGCTACAAGCCTACTGGTTGGTTGCTGATGATATGATGGACCA

ATCTATTACCAGAAGAGGTCAACCATGTTGGTACAAGGTTGAGAACGTCGGTAACAT
 TGCTATTTGGGACTCTTTCATGTTGGAGGGTGCCATCTACAAGATTTTGAGAAAGTA
 CTTCAAGAAGGAGTCTTACTACGCCGACTTGTTGGACTTGTTGCACGAGGTTACTTT
 CCAAAGTGAATTGGGTCAATTGTTGGACTTGATCACTGCTCCAGAAGACCACGTTGA
 TCTTTCCAAGTTTACCCCTGCTAAACACTCTTTCATTGTCATCTTCAAGACTGCCTAC
 TATTCATTCTACCTTCCTGTTGTTATGGCCATGTACTTGTCTGGTATTACCCATGAAA
 AGGATTTGCAGCAGGCTGAGCACATCTTGATTCCATTGGGTGAGTACTTCCAAATTC
 AAGATGACTACTTGGACTGTTTCGGTAAGCCAGAAGATATTGGTAAGATTGGAAGTG
 ACATCCAGGACAATAAGTGTCTTGGGTGATTAACCAAGCTCTGAGATTGGCCTCTC
 CAGAGCAAAGACAAATTCTTGATGAGAACTACGGAAGAAAAGATGCCGACAAGGAG
 GCAAAGTGCAAGGAAGTTTTTGACCAACTTGACATTGCTGGTAAGTACAAGGCTTAC
 GAGGAAAACATCGGTAAGGAGTTGCAAAGAGAATTGCTGACACTGAGGAGGATCG
 TGGATTCAAGAAGGAAGTTTCCAGGTCTTTTCGACAAGATCTACAAGAGAACCAA
 ATAG (SEQ ID NO: 1)

[00132] In one example, GPP production was achieved by introducing in to *P. pastoris* the modified Erg20 gene from *P. pastoris* containing two point mutations (F98W and N128W) (SEQ ID NO: 2).

[00133] In one example, a mutant of the Erg20 *P. Pastoris* gene containing two point mutations (F98W and N128W) comprises or consists of the following polynucleotide sequence:

[00134] AAGATGTTTTGTTAGTTGACTATAAGTTAATTTTTATATTAATTTGC
 ACTCTGTCCTTCACTAACTTTAGTTTGGTGCCAACTTATGATTAAAAAGCCAGACA
 ATTTTAATTCCCCAACAGACCTCTAATTTGCCCGAATAATGCCAATTAACATTCTAG
 AAACCTCGATTAACTATATATTTTCATAAACCATGACCTTTTTCCGCCAATATCCTCATA
 TTTGAGCCTAAGGAAGACTCTCCTCCTTTTAGCCTACAACGAAGTTGCCCCATAAATA
 CACCAAGCGCGACCCCTCGTTCATCGTTTAACTCGTGTTAGAGACCTATTCCAGACTT
 CAGATCTCCAAAAACAGGACTATATCATAATTTGGCACGTTTGAATCTTCCCCATCCT
 GAAGCGGTATTTGAGATCAACTACTTCAGAGAAAACCCTCATGCTTTTTACACATTGG
 CTGACGAACTTTACCCTGGGAGGGTCTCATTCTTCTCACCTTTGAACTAGTAGAAA
 TCAAATGTCCAAAGAAGTAGCAGCTAAGAACAAAGTGAAGTTCCTTGCGTTCTTTCC
 AGACCTCGTGTTTGAGCTGGAAGTCATGCTGGAAAATTACGGCATGCCTGCTGATG
 CAATCAACTGGTTCAAGAGATCCTTGAAGTACAACACTCCTGGTGGAAAAGTCAACA
 GAGGTATCTCTGTTGTCGACACCTATGTCATTCTCAAGGGGTACAAGTCATGGGACG
 AGTTAAGTGCCGAAGAGTTCAAGAAAGCTGCTATTTTAGGATGGTGTGTCGAATTGC

TACAAGCCTACTGGTTGGTTGCTGATGATATGATGGACCAATCTATTACCAGAAGAG
 GTCAACCATGTTGGTACAAGGTTGAGAACGTCGGTAACATTGCTATTTGGGACTCTT
 TCATGTTGGAGGGTGCCATCTACAAGATTTTGAGAAAGTACTTCAAGAAGGAGTCTT
 ACTACGCCGACTTGTTGGACTTGTTGCACGAGGTTACTTTCCAACTGAATTGGGTC
 AATTGTTGGACTTGATCACTGCTCCAGAAGACCACGTTGATCTTTCCAAGTTTACCCC
 TGCTAAACACTCTTTCATTGTCATCTTCAAGACTGCCTACTATTCACTTACCTTCCTG
 TTGTTATGGCCATGTACTTGTCTGGTATTACCCATGAAAAGGATTTGCAGCAGGCTG
 AGCACATCTTGATTCCATTGGGTGAGTACTTCCAAATTCAAGATGACTACTTGGACTG
 TTTCGGTAAGCCAGAAGATATTGGTAAGATTGGAAGTACATCCAGGACAATAAGTG
 TTCTTGGGTGATTAACCAAGCTCTGAGATTGGCCTCTCCAGAGCAAAGACAAATTCT
 TGATGAGAACTACGGAAGAAAAGATGCCGACAAGGAGGCAAAGTGCAAGGAAGTTT
 TTGACCAACTTGACATTGCTGGTAAGTACAAGGCTTACGAGGAAAACATCGGTAAGG
 AGTTGCAAAGAGAATTGCTGACACTGAGGAGGATCGTGGATTCAAGAAGGAAGTTT
 TCCAGGTCTTTTTGACAAGATCTACAAGAGAACCAAATAG (SEQ ID NO: 2).

[00135] In one example there is provided a method of producing geranyl pyrophosphate (GPP) in a methylotrophic yeast host cell, comprising:

[00136] - introducing a first polynucleotide encoding an Erg20 (F98W / N128W) polypeptide,

[00137] - culturing the methylotrophic yeast host cell under conditions sufficient for GPP production.

[00138] In one example, the host cell is from the *Komagataella* genus.

[00139] In one example, the methylotrophic yeast host cell is *Pichia Pastoris* (*Komagataella phaffii*).

[00140] In one example, the host cell is a cell from *P. pastoris*.

[00141] In one example, an ERG20 duplication vector map is presented in Figure 1.

[00142] In one example, an Erg20 replacement vector map is presented in Figure 2.

[00143] In one example, GPP production in *P. pastoris* is presented in Figure 3.

[00144] In one example, the first polynucleotide encoding an Erg20 (F98W / N128W) polypeptide comprises or consists of:

[00145] a) a nucleotide sequence as set forth in (SEQ ID NO:2);

[00146] b) a nucleic acid having at least 70% identity to the nucleic acid of a),

- [00147]** c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),
- [00148]** d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [00149]** e) a derivative or variant of a), b), c), or d).
- [00150]** In one example, in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.
- [00151]** In one example, there is provided an expression vector comprising an isolated polynucleotide, comprising:
- [00152]** a) a nucleotide sequence set forth in SEQ ID NO 2,
- [00153]** b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- [00154]** c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- [00155]** d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [00156]** e) a derivative of a), b), c), or d).
- [00157]** In one example, the in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.
- [00158]** In one example, there is provided a methylotrophic yeast host cell comprising an expression vector of as described here.
- [00159]** In one example, there is provided a methylotrophic yeast host cell comprising:
- [00160]** a) a nucleotide sequence set forth in SEQ ID NO 2,
- [00161]** b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- [00162]** c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- [00163]** d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- [00164]** e) a derivative of a), b), c), or d).
- [00165]** In one example, said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).

[00166] Fig. 4 depicts LC-MS/MS analysis. Panel A depicts Farnesyl pyrophosphate injected into water. Panel B is a graph showing Peak Area versus FPP Loaded. Panel C depicts Geranyl pyrophosphates injected into water. Panel D is a graph showing Peak Area versus GPP Loaded. Panel E shows a summary of the data. Panel F shows the ratio of GPP/FPP.

[00167] Fig. 5 depicts LC-MS/MS analysis. Panel A depicts Farnesyl pyrophosphate injected into MeOH grown yeast. Panel B is a graph showing Peak Area versus MeOH FPP Loaded. Panel C depicts Geranyl pyrophosphates injected into MeOH grown in yeast. Panel D is a graph showing Peak Area versus MeOH GPP Loaded. Panel E shows a summary of the data. Panel F shows the ratio of GPP/FPP.

[00168] Fig. 6 depicts LC-MS/MS analysis. Panel A depicts Farnesyl pyrophosphate injected into glycerol grown yeast. Panel B is a graph showing Peak Area versus glycerol FPP Loaded. Panel C depicts Geranyl pyrophosphates injected into glycerol grown in yeast. Panel D is a graph showing Peak Area versus glycerol GPP Loaded. Panel E shows a summary of the data.

[00169] Production of cannabigerolic acid

[00170] In one aspect, the present disclosure provides production of cannabigerolic acid (CBGA) in a methylotrophic yeast. In one example, the methylotrophic yeast is *P. pastoris*.

[00171] As described herein, cannabigerolic acid (CBGA) was produced in a methylotrophic yeast.

[00172] The inventors, through genetic engineering, have introduced the pathway required for the conversion of hexanoyl-CoA to olevitolic acid. This pathway begins by a heterologous polyketide synthase (CsTKS) combining one molecule of hexanoyl-CoA with three molecules of malonoyl-CoA to generate 3,5,7-trioxododecanoyl-CoA. 3,5,7-trioxododecanoyl-CoA is cyclized through the activity of an olevitolic acid cyclase (CsOAC) enzyme releasing olevitolic acid and a molecule of coenzyme A. An aromatic prenyltransferase (CsPT4) catalyzes the final step in the reaction pathway combining the olevitolic acid produced in the previous steps with GPP resulting in the production of CBGA.

[00173] In one example, CsTKS, CsOAC, and CsPT4, are from *Cannabis sativa*.

[00174] Thus, in one aspect, there is described herein a metabolic engineering strategy in which a CBGA was produced in *P. Pastoris*.

[00175] In one example, there is provided a method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising: introducing a first polynucleotide encoding olevitiolic synthase polypeptide, introducing a second polynucleotide encoding olevitiolic acid cyclase polypeptide, and introducing a third polynucleotide encoding aromatic prenyl transferase, and culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

[00176] In one example, there is provided a method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising: introducing a fourth polynucleotide encoding olevitiolic synthase polypeptide and encoding olevitiolic acid cyclase polypeptide, and introducing a third polynucleotide encoding aromatic prenyl transferase, culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

[00177] In one example, there is provided a method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising: introducing a first polynucleotide encoding olevitiolic synthase polypeptide, and introducing a fifth polynucleotide encoding olevitiolic acid cyclase polypeptide and encoding acormatic prenyl transferase, culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

[00178] In one example, the host cell is from the *Komagataella* genus.

[00179] In one example, the methylotrophic yeast host cell is *Pichia Pastoris* (*Komagataella phaffii*).

[00180] In one example, the CsPT4/CsOAC sequence is:

[00181] GATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCC
GACATCCACAGGTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGGATACA
CTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCA
CTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCC
AATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCC
CCTGGCGAGGTTTCATGTTTGTGTTATTTCCGAATGCAACAAGCTCCGCATTACACCCG
AACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCCAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCG
TGATCTCATCCAAGATGAACTAAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGT
CAAAAAGAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTTTGGTATTGATTGACGA
ATGCTCAAAAATAATCTCATTAAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCC
GGTGCACCTGTGCCGAAACGCAATGGGGAAACACCCGCTTTTTGGATGATTATGC

ATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGATAGCCTAAC
GTTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACAGAA
GGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTTCATAAT
TGCGACTGGTTCGAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACCTTGAG
AAGATCAAAAAACAACCTAATTATTCGAAACGATGCATCATCATCATCACCATTCCCTCC
GGTAGAGAGAACTTGACTTCCAAGGTATGTCTGCTGGTTCTGACCAAATCGAAGGT
TCCCCACACCACGAGTCTGATAACTCCATTGCTACCAAGATTTTGAACCTTTGGCCATA
CGTGTTGGAAATTGCAAAGACCTTACGTTGTCAAGGGTATGATATCGATTGCTTGTG
GTTTGTTTGGTAGAGAGTTGTTCAACAACAGACATTTGTTCTCTTGGGGTTTGATGTG
GAAGGCTTTTTTTGCCCTTGTTCTTCTTTCTTTCAACTTTTTTGGCGCTATCATGA
ACCAGATTTACGATGTTGATATTGACAGAATCAACAAGCCAGACCTTCCATTGGTTTC
TGGTGAGATGTCCATTGAGACTGCTTGGATTCTGTCAATTATAGTTGCTTTGACTGGT
TTGATTGTCACTATCAAGCTGAAATCTGCTCCACTGTTTGTTTTCATTTACATCTTTGG
TATTTTTGCTGGTTTTGCCTATTCGGTTCCTCCAATTAGATGGAAGCAATATCCATTC
ACCAACTTTCTGATCACAATTCCTCTCACGTTGGTTTGGCTTTCACAAGCTACTCCG
CTACTACCTCTGCTTTGGGTCTTCCATTCTGTGTGGCGTCCAGCCTTTTCTTTCATCAT
TGCTTTCATGACTGTCATGGGTATGACCATTGCTTTTGCCAAGGACATTTCTGATATC
GAAGGTGACGCTAAGTACGGTGTCTCGACCGTTGCTACCAAGTTGGGTGCCAGAAA
TATGACTTTCGTTGTTTCTGGTGTTTTGCTGTTGAACTACCTGGTGAGTATTTCAATT
GGTATTATCTGGCCCCAAGTCTTCAAGTCCAACATTATGATTCTATCTCACGCTATTT
TGGCTTTCGTGTTTGATCTTCCAACCAGAGAGCTGGCTTTGGCCAACCTACGCTTCCG
CTCCATCCAGACAATTCTTTGAATTCATTTGGCTATTGTACTACGCCGAGTACTTCGT
TTATGTTTTCATCTAATGAAATGTATTTAATTTGATATTAAGTAAATGAATGATTATGAC
TTTATGAATTCGCAATGTTTTCTCCTTGATTATTTCTGTATTGTATTGGAATGATTATA
GAATACTCATATATTGAT
TATAGTATTAGCACATAAAACGTTTGTTGTTAACTCACTTCCGTACGCAACCATTCT
ATTTCTAGCTATCTTGATAAGGTGATCTAACATCCAAAGACGAAAGGTTGAATGAAAC
CTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACATAAGTGCCAAACGCAACA
GGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCT
CCTCAACACCCCACTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAG
CTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCTGTC
TATCCTGGCCCCCCTGGCGAGGTTTCATGTTTGTTTATTTCCGAATGCAACAAGCTCC
GCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGT
TTCATGTTCCCAAATGGCCCAAACTGACAGTTTAGAGACCTATTCCAGACTTCAG

ATCTCCAAAAACAGGACTATATCATAATTTGGCACGTTTGAATCTTCCCCATCCTGAA
 GCGGTATTTGAGATCAACTACTTCAGAGAAAACCCTCATGCTTTTTACACATTGGCTG
 ACGAACTTTACCCTGGGAGGGTCTCAAAACGCTGTCTTGGAACCTAATATGACAAAA
 GCGTGATCTCATCCAAGATGAACTAAGTTTGGTTCGTTGAAATGCTAACGGCCAGTT
 GGTCAAAAAGAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTTTGGTATTGATTGA
 CGAATGCTCAAAAATAATCTCATTAAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAAC
 CCCGGTGACCTGTGCCGAAACGCAAATGGGGAAACACCCGCTTTTTTGGATGATTA
 TGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGATAGCC
 TAACGTTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACA
 GAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTTATCATCATTATTAGCTTACTTTTCA
 AATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAACGACTTTTAACGACAACCTT
 GAGAAGATCAAAAAACAATAATTATTCGAAACGATGCATCATCATCATCATAGC
 TCCGGTAGAGAGAACTTGTACTTCCAAGGTATGGCCGTTAAGCATCTGATCGTTTTTA
 AAGTTCAAGGATGAGATCACCGAGGCTCAAAAGGAAGAGTTTTTCAAGACTTACGTC
 AACTTGGTCAACATCATTCCAGCTATGAAGGATGTCTACTGGGGTAAGGACGTCCT
 CAAAAGAACAAGGAGGAGGGATACACCCACATAGTGGAGGTTACTTTGAGTCTGT
 CGAGACTATCCAGGACTACATCATTACCCAGCCACGTTGGATTGCGCGATGTCTA
 CAGATCTTTTTGGGAGAAGTTGCTGATCTTTGACTACACTCCAAGAAAAGGTTTCATAA
 TCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCAGGCTTCATTTTTGATACT
 TTTTTATTTGTAACTATATAGTATAGGATTTTTTTTGTCAATTTGTTTCTTCTCGTACG
 AGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATATCTTGTGGTAGGGGTTTTG
 GGAAAATCATTGAGTTTTGATGTTTTTCTTGGTATTTCCCACTCCTCTTCAGAGTACA
 GAAGATTAAGTGACACGTTTCGTTTGTGCAAGCTTCAACGATGCCAAAAGGGTATAAT
 AAGCGTCATTTGCAGCATTGTGAAGAAAACATGTGGCAAGCCAAGCCTGCGAAGAA
 TGTA (SEQ ID NO:3).

[00182] In one example, the CsPT4 polypeptide sequence is:

[00183] MHHHHHHSSGRENLIFQGM SAGSDQIEGSPHHESDNSIATKILNFGHTC
 WKLQRPYVVKGMISIA CGLFGREL FNNRH LFSWGLMWKAFFALVPILSFNFFAAIMNQIY
 DVDIDRINKPDLPLVSGEMSIETAWILSIIVALTGLIVTIKLKSAPLFVFIYIFGIFAGFAYSVP
 PIRWKQYPFTNFLITISSHVGLAFTSYSATTSALGLPFVWRPAFSFIIFMTVMGMTIAFAK
 DISDIEGDAKYGVSTVATKLGARNMTFVSGVLLLNYSISIGIWPQVFKSNIMILSHAIL
 AFCLIFQTRELALANYASAPSRQFFEFIWLLYYAEYFVYVFI.(SEQ ID NO:4)

[00184] In one example, the CsOAC polypeptide sequence is:

[00185] MHHHHHHSSGRENLYFQGMVVKHLIVLKFDEITEAQKEEFFKTYVNLV
 NIIPAMKDVYWGKDVQKNKEEGYTHIVEVTFESVETIQDYIIHPAHVGFQDVYRSFWEK
 LLIFDYTPRKGS (SEQ ID NO: 5)

[00186] In one example, the CsTKS polynucleotide sequence is :

[00187] ATGCATCATCATCATCATCATTCCTCCGGTAGAGAGAACTTGACTTC
 CAAGGTATGAACCATTTGAGAGCTGAAGGTCCTGCTTCCGTTTTGGCTATTGGAAC
 GCCAATCCAGAGAACATCCTTTTGCAAGATGAGTTTCCAGACTACTATTTCCGTGTCA
 CCAAGTCTGAACACATGACTCAATTGAAGGAGAAGTTCAGAAAGATTTGTGATAAGT
 CAATGATCAGAAAGAGAACTGTTTTTTGAACGAGGAACATTTGAAACAAAACCCAA
 GATTAGTTGAGCATGAAATGCAAACATTGGACGCTAGACAGGATATGTTGGTTGTTG
 AGTTTCCAAAGTTGGGTAAGGATGCTTGTGCCAAGGCCATCAAGGAATGGGGTCAG
 CCAAAGTCCAAGATTACTCACTTGATCTTTACAAGCGCTTCTACCACTGACATGCCA
 GGAGCCGACTACCACTGCGCCAAGTTGTTGGGACTGTCTCCTTCTGTTAAGAGAGTT
 ATGATGTACCAATTGGGATGTTACGGTGGTGGTACCGTTTTGAGAATTGCCAAGGAC
 ATTGCTGAGAACAACAAGGGTGCCAGAGTTTTGGCTGTTTGTGTGATATCATGGCT
 TGTGTGTTGAGAGGACCAAGCGAATCAGATTTGGAATTGTTGGTTGGCCAAGCCATT
 TTTGGAGATGGTGCTGCTGCTGTTATTGTTGGTGCCGAACCAGACGAATCTGTTGGA
 GAGAGACCAATCTTCGAATTAGTTTCGACTGGTCAGACAATTTTGCCCAACTCTGAA
 GGAACAATTGGTGGTCACATCAGAGAAGCTGGTTTGATCTTCGACTTGACACAAGGAT
 GTACCAATGCTGATTTCCAACAACATTGAGAAGTGTTTGATTGAGGCTTTTACCCCTA
 TTGGTATTTCTGATTGGAACCTCATCTTCTGGATCACTCATCCAGGTGGTAAGGCCA
 TTTTGGACAAAGTTGAGGAGAAGTTGCACTTGAAGTCTGATAAGTTTGTGACTCCA
 GACACGTGCTCTCTGAGCACGGTAATATGTCATCTTCCACTGTTCTGTTTCGTCATGG
 ATGAGTTGAGAAAGAGATCTTTGGAGGAAGGTAAGTCTACTACAGGCGATGGATTTG
 AATGGGGTGTTTTGTTTCGGTTTTCGGGCCAGGTTTGACCGTCGAGAGAGTTGTTGTTA
 GATCTGTTCCAATTAAGTACGGATCTTAA.(SEQ ID NO: 6).

[00188] In one example, the CsTKS polypeptide sequence is:

[00189] MHHHHHHSSGRENLYFQGMNHLRAEGPASVLAIGTANPENILLQDEFPD
 YYFRVTKSEHMTQLKEKFRKICDKSMIRKRNCFLNEEHLKQNPRLVEHEMQTLARQD
 MLVVEVPKLGKDACAIAKEWGQPKSKITHLIFTSASTTDMPGADYHCAKLLGLSPSVKR
 VMMYQLGCGYGGTVLRIAKDIAENNKARVLAVCCDIMACLFRGPSESDLELLVGQAIF
 GDGAAAVIVGAEPDESVERPIFELVSTGQTILPNSEGTIGGHIREAGLIFDLHKDVPMLIS
 NNIEKCLIEAFTPIGISDWNISIFWITHPGGKAILDKVEEKLHLKSDKFVDSRHVLSEHGNM

SSSTVLFVMDLRKRSLEEGKSTTGDGFEWGVLFVFGPGLTVERVWVRSVPIKYGS
(SEQ ID NO:7).

[00190] In one example, the CsPT4 polynucleotide sequence is:

[00191] ATGCATCATCATCATCACCATTCCTCCGGTAGAGAGAACTTGACTTC
CAAGGTATGTCTGCTGGTTCTGACCAAATCGAAGGTTCCCCACACCACGAGTCTGAT
AACTCCATTGCTACCAAGATTTTGAACCTTGGCCATACGTGTTGGAAATTGCAAAGAC
CTTACGTTGTCAAGGGTATGATATCGATTGCTTGTGGTTTGTGGTAGAGAGTTGTT
CAACAACAGACATTTGTTCTCTTGGGGTTTGATGTGGAAGGCTTTTTTGGCCCTTGTT
CCTATTCTTTCTTTCAACTTTTTTGGCGCTATCATGAACCAGATTTACGATGTTGATAT
TGACAGAATCAACAAGCCAGACCTTCCATTGGTTTCTGGTGAGATGTCCATTGAGAC
TGCTTGGATTCTGTCAATTATAGTTGCTTTGACTGGTTTGATTGTCACTATCAAGCTG
AAATCTGCTCCACTGTTTGTTCATTTACATCTTTGGTATTTTGTCTGGTTTGCCTA
TTCGGTTCCTCCAATTAGATGGAAGCAATATCCATTCACCAACTTTCTGATCACAATT
TCCTCTCACGTTGGTTTGGCTTTCACAAGCTACTCCGCTACTACCTCTGCTTTGGGT
CTTCCATTCTGTGTGGCGTCCAGCCTTTTCTTTCATCATTGCTTTCATGACTGTCATGG
GTATGACCATTGCTTTTGCCAAGGACATTTCTGATATCGAAGGTGACGCTAAGTACG
GTGTCTCGACCGTTGCTACCAAGTTGGGTGCCAGAAATATGACTTTCGTTGTTTCTG
GTGTTTTGCTGTTGAACTACCTGGTGAGTATTTCAATTGGTATTATCTGGCCCCAAGT
CTTCAAGTCCAACATTATGATTCTATCTCACGCTATTTTGGCTTTCTGTTTGATCTTCC
AAACCAGAGAGCTGGCTTTGGCCAACTACGCTTCCGCTCCATCCAGACAATTCTTTG
AATTCATTTGGCTATTGTACTACGCCGAGTACTTCGTTTATGTTTTCATCTAA (SEQ ID
NO: 8).

[00192] In one example, the CsOAC polynucleotide sequence is:

ATGCATCATCATCATCATAGCTCCGGTAGAGAGAACTTGACTTCCAAGGTATG
GCCGTTAAGCATCTGATCGTTTTAAAGTTCAAGGATGAGATCACCGAGGCTCAAAG
GAAGAGTTTTTCAAGACTTACGTCAACTTGGTCAACATCATTCCAGCTATGAAGGATG
TCTACTGGGGTAAGGACGTCACTCAAAAGAACAAGGAGGAGGGATACCCACATA
GTGGAGGTTACTTTGAGTCTGTCTGAGACTATCCAGGACTACATCATTACCCAGCC
CACGTTGGATTGGCGATGTCTACAGATCTTTTTGGGAGAAGTTGCTGATCTTTGAC
TACACTCCAAGAAAAGGTTTCATAA (SEQ ID NO: 9)

[00193] In one example, the ScPT4/CsOAC polynucleotide is:

[00194] GATCTAACATCCAAAGACGAAAGGTTGAATGAAACCTTTTTGCCATCC
GACATCCACAGGTCCATTCTCACACATAAGTGCCAAACGCAACAGGAGGGGATACA
CTAGCAGCAGACCGTTGCAAACGCAGGACCTCCACTCCTCTTCTCCTCAACACCCA

CTTTTGCCATCGAAAAACCAGCCCAGTTATTGGGCTTGATTGGAGCTCGCTCATTCC
AATTCCTTCTATTAGGCTACTAACACCATGACTTTATTAGCCTGTCTATCCTGGCCCC
CCTGGCGAGGTTTCATGTTTGTATTTCCGAATGCAACAAGCTCCGCATTACACCCG
AACATCACTCCAGATGAGGGCTTTCTGAGTGTGGGGTCAAATAGTTTCATGTTCCCC
AAATGGCCCCAAACTGACAGTTTAAACGCTGTCTTGGAACCTAATATGACAAAAGCG
TGATCTCATCCAAGATGAACTAAGTTTGGTTCGTTGAAATGCTAACGGCCAGTTGGT
CAAAAAGAACTTCCAAAAGTCGGCATACCGTTTGTCTTGTTTGGTATTGATTGACGA
ATGCTCAAAAATAATCTCATTAAATGCTTAGCGCAGTCTCTCTATCGCTTCTGAACCCC
GGTGACCTGTGCCGAAACGCAAAATGGGGAAACACCCGCTTTTTGGATGATTATGC
ATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGGGAATACTGCTGATAGCCTAAC
GTTTCATGATCAAAATTTAACTGTTCTAACCCCTACTTGACAGCAATATATAAACAGAA
GGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATCATTATTAGCTTACTTTTCATAAT
TGCGACTGGTTCCAATTGACAAGCTTTTGATTTAACGACTTTTAACGACAACCTTGAG
AAGATCAAAAAACAACCTAATTATTCGAAACGATGCATCATCATCATCACCATTCTCC
GGTAGAGAGAAGCTTGACTTCCAAGGTATGTCTGCTGGTTCTGACCAAATCGAAGGT
TCCCCACACCACGAGTCTGATAACTCCATTGCTACCAAGATTTTGAACCTTTGGCCATA
CGTGTTGGAAATTGCAAAGACCTTACGTTGTCAAGGGTATGATATCGATTGCTTGTG
GTTTGTGTTGGTAGAGAGTTGTTCAACAACAGACATTTGTTCTCTTGGGGTTTGATGTG
GAAGGCTTTTTTTGCCCTTGTTCCCTATTCTTTCTTTCAACTTTTTTGCCGCTATCATGA
ACCAGATTTACGATGTTGATATTGACAGAATCAACAAGCCAGACCTTCCATTGGTTTC
TGGTGAGATGTCCATTGAGACTGCTTGGATTCTGTCAATTATAGTTGCTTTGACTGGT
TTGATTGTCACTATCAAGCTGAAATCTGCTCCACTGTTTGTGTTTCATTTACATCTTTGG
TATTTTGTCTGGTTTTGCCTATTTCGTTTCTCCAATTAGATGGAAGCAATATCCATTC
ACCAACTTTCTGATCACAATTCCTCTCACGTTGGTTTGGCTTTCACAAGCTACTCCG
CTACTACCTCTGCTTTGGGTCTTCCATTTCGTGTGGCGTCCAGCCTTTTCTTTCATCAT
TGCTTTCATGACTGTCATGGGTATGACCATTGCTTTTGCCAAGGACATTTCTGATATC
GAAGGTGACGCTAAGTACGGTGTCTCGACCGTTGCTACCAAGTTGGGTGCCAGAAA
TATGACTTTCGTTGTTTCTGGTGTGTTTGTGTTGAACTACCTGGTGAGTATTTCAATT
GGTATTATCTGGCCCCAAGTCTTCAAGTCCAACATTATGATTCTATCTCACGCTATTT
TGGCTTTCGTGTTTGATCTTCCAAACCAGAGAGCTGGCTTTGGCCAACCTACGCTTCCG
CTCCATCCAGACAATTCTTTGAATTCATTTGGCTATTGTACTACGCCGAGTACTTCGT
TTATGTTTTTCATCTAATGAAATGTATTTAATTTGATATTAAGTAAATGAATGATTATGAC
TTTATGAATTCGCAATGTTTTCTCCTTGATTATTTCTGTATTGTATTGGAATGATTATA
GAATACTCATATATTGATTATAGTATTAGCACATAAAACGTTTGTTGTTAACTCACTT

CCGTACGCAACCATTCTATTTCTAGCTATCTTGATAAGGTGATCTAACATCCAAAGA
CGAAAGGTTGAATGAAACCTTTTTGCCATCCGACATCCACAGGTCCATTCTCACACA
TAAGTGCCAAACGCAACAGGAGGGGATACACTAGCAGCAGACCGTTGCAAACGCAG
GACCTCCACTCCTCTTCTCCTCAACACCCACTTTTGCCATCGAAAAACCAGCCCAGT
TATTGGGCTTGATTGGAGCTCGCTCATTCCAATTCCTTCTATTAGGCTACTAACACCA
TGACTTTATTAGCCTGTCTATCCTGGCCCCCCTGGCGAGGTTTCATGTTTGTTTATTTT
CGAATGCAACAAGCTCCGCATTACACCCGAACATCACTCCAGATGAGGGCTTTCTGA
GTGTGGGGTCAAATAGTTTCATGTTCCCCAAATGGCCCAAACTGACAGTTTAGAGA
CCTATTCCAGACTTCAGATCTCCAAAACAGGACTATATCATAATTTGGCACGTTTGA
ATCTTCCCCATCCTGAAGCGGTATTTGAGATCAACTACTTCAGAGAAAACCCTCATG
CTTTTACACATTGGCTGACGAACTTTACCCTGGGAGGGTCTCAAACGCTGTCTTG
GAACCTAATATGACAAAAGCGTGATCTCATCCAAGATGAACTAAGTTTGTTTCGTTGA
AATGCTAACGGCCAGTTGGTCAAAAAGAACTTCCAAAAGTCGGCATAACGTTTGTC
TTGTTTGGTATTGATTGACGAATGCTCAAAAATAATCTCATTAAATGCTTAGCGCAGTC
TCTCTATCGCTTCTGAACCCCGGTGCACCTGTGCCGAAACGCAAATGGGGAAACAC
CCGCTTTTTTGGATGATTATGCATTGTCTCCACATTGTATGCTTCCAAGATTCTGGTGG
GAATACTGCTGATAGCCTAACGTTTCATGATCAAAATTTAACTGTTCTAACCCTACTT
GACAGCAATATATAAACAGAAGGAAGCTGCCCTGTCTTAAACCTTTTTTTTTATCATC
ATTATTAGCTTACTTTTCATAATTGCGACTGGTTCCAATTGACAAGCTTTTGATTTTAAC
GACTTTTAACGACAACCTTGAGAAGATCAAAAACAATAATTATTCGAAACGATGCAT
CATCATCATCATAGCTCCGGTAGAGAGAACTTGTAATTCCAAGGTATGGCCGTT
AAGCATCTGATCGTTTTAAAGTTCAAGGATGAGATCACCGAGGCTCAAAGGAAGAG
TTTTTCAAGACTTACGTCAACTTGGTCAACATCATTCCAGCTATGAAGGATGTCTACT
GGGGTAAGGACGTCACTCAAAGAACAAGGAGGAGGGATACACCCACATAGTGGAG
GTTACTTTTCGAGTCTGTGAGACTATCCAGGACTACATCATTACCCAGCCCACGTT
GGATTGGCGATGTCTACAGATCTTTTTGGGAGAAGTTGCTGATCTTTGACTACACT
CCAAGAAAAGGTTTATAATCAAGAGGATGTCAGAATGCCATTTGCCTGAGAGATGCA
GGCTTCATTTTTGATACTTTTTTATTTGTAACCTATATAGTATAGGATTTTTTTTGTGAT
TTTGTCTTCTCGTACGAGCTTGCTCCTGATCAGCCTATCTCGCAGCTGATGAATAT
CTTGTTGGTAGGGGTTTTGGGAAAATCATTGAGTTTGATGTTTTTCTGGTATTTCCCA
CTCCTCTTCAGAGTACAGAAGATTAAGTGACACGTTTCGTTTGTGCAAGCTTCAACGA
TGCCAAAAGGGTATAATAAGCGTCATTTGCAGCATTGTGAAGAAAATATGTGGCAA
GCCAAGCCTGCGAAGAATGTAGTCGAGAATTGAGCTTGC (SEQ ID NO: 10).

[00195] Fig. 8 depicts Quantification of MeOH induced *P. pastoris* CBGA levels. Calibration curve and analysis of LC/MS data showing the quantification of the CBGA produced in *P. pastoris*. Panel A depicts CBGA injected into MeOH grown yeast. Panel B should a graph of Peak Area versus MeOH CBGA. Panel C shows a summary of data. Panel D shows a summary of the data.

[00196] Fig. 9 depicts

[00197] Fig. 10 depicts the pGUH CsTKS map.

[00198] Fig. 11 depicts the pJAG dual CsPT4 CsOAC map.

[00199] Fig. 12 depicts the pJUN dual CsPT4 CsOAC map.

[00200] Fig. 13 depicts the pGAH CsTKS map.

[00201] In one example, CBGA production was introducing CsTKS, CsOAC, and CsPT4, in to the methylotropic yeast, *P. pastoris*.

[00202] As used herein, the term "polynucleotide(s)", "nucleic acid molecule(s)" or "nucleic acid sequence(s)" are interchangeable and it generally refers to any polyribonucleotide or poly- deoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA or any combination thereof. As used herein, the term "nucleic acid(s)" also includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "nucleic acids". The term "nucleic acids" as it is used herein embraces such chemically, enzymatically or metabolically modified forms of nucleic acids, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including for example, simple and complex cells. In some examples, one or more nucleotides are modified at the base, sugar or backbone to make the nucleic acid more stable or less likely to be cleared e.g. phosphorothioate backbone, pegylated backbone.

[00203] A polynucleotide may consist of an entire gene, or any portion thereof.

[00204] A gene is a polynucleotide that encodes (which may also be referred to as "coding for") a functional polypeptide or RNA molecule.

[00205] In some examples, polynucleotides as described herein may also encompass polynucleotide sequences that differ from the disclosed sequences (which may also be know as the reference sequence) but which, as a result of the degeneracy of genetic code, encode a polypeptide which is the same as that encoded by a polynucleotide of the present disclosure.

[00206] In some examples, the polynucleotides described herein are isolated and purified, as those terms are commonly used in the art.

[00207] The term "isolated" refers to sequences that are removed from their natural cellular or other naturally occurring biological environment or from the environment of the experiment. An isolated molecule may be obtained by any method or combination of methods including biochemical, recombinant, and synthetic techniques. The polypeptide sequences may be prepared by at least one purification step.

[00208] The nucleotide sequences of described herein, may be modified. In some examples, the polynucleotides contains one or more chemical modifications. The modifications may be various distinct modifications. In some embodiments, the regions may contain one, two, or more (optionally different) nucleoside or nucleotide modifications.

[00209] Polypeptides described herein may be produced by inserting a polynucleotide that encodes the polypeptide into an expression vector and expressing the polypeptide in an appropriate host. Any of a variety of expression vectors known to those of ordinary skill in the art may be employed. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a polypeptide encoding a recombinant polypeptide.

[00210] The polynucleotide(s) described herein may be used in a vector.

[00211] The term "vector" as used herein refers to any nucleic acid molecule for the cloning of and/or transfer of a nucleic acid into a cell. A vector may be a replicon to which another nucleotide sequence may be attached to allow for replication of the attached nucleotide sequence.

[00212] A "replicon" can be any genetic element (for example a plasmid, phage, cosmid, chromosome, viral genome) that functions as an autonomous unit of nucleic acid replication *in vivo*, and for example, is capable of replication under its own control. The term "vector" includes both viral and non viral (e.g., plasmid) nucleic acid molecules for introducing a nucleic acid into a cell *in vitro*, *ex vivo*, and/or *in vivo*. A large number of vectors known in the art may be used to manipulate nucleic acids, incorporate response elements and promoters into genes, and the like. For example, the insertion of the nucleic acid fragments corresponding to response elements and promoters into a suitable vector can be accomplished by ligating the appropriate nucleic acid fragments into a chosen vector that has complementary cohesive termini. Alternatively, the ends of the nucleic acid molecules may be enzymatically modified or any site may be produced by ligating nucleotide sequences (linkers) to the nucleic acid termini. Such vectors may be engineered to contain sequences encoding selectable markers that provide for the

selection of cells that contain the vector and/or have incorporated the nucleic acid of the vector into the cellular genome. Such markers allow identification and/or selection of host cells that incorporate and express the proteins encoded by the marker. A "recombinant" vector refers to a viral or non-viral vector that comprises one or more heterologous nucleotide sequences.

[00213] As used herein, the term "isolated" refers to material, for example a polynucleotide, a polypeptide, or a cell, that is substantially or essentially free from components that normally accompany it in its native state.

[00214] As used herein, the term "variant" refers to a polynucleotide or polypeptide sequences different from the specifically identified sequences, wherein one or more nucleotides or amino acid residues is deleted, substituted, or added. Variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variant polynucleotide sequences preferably exhibit at least 70%; more preferably at least 80%; more preferably yet at least 90%; and most preferably at least 95% identity to a sequence of the present invention. Variant polypeptide sequences preferably exhibit at least 70%; more preferably at least 80%; more preferably yet at least 90%; and most preferably at least 95% identity to a sequences described herein.

[00215] In some examples, variant polynucleotides of the polynucleotides described herein hybridize to the polynucleotide sequences recited in SEQ ID NO: 2, or complements, reverse sequences, or reverse complements of those sequences under high stringent conditions (also referred to as high stringency).

[00216] In one example, as used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65 C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65 C and two washes of 30 minutes each in 0.2XSSC, 0.1% SDS at 65 C.

[00217] The term "polypeptide" refers to amino acid chains of any length, including full length sequences in which amino acid residues are linked by covalent peptide bonds. Polypeptides may be isolated natural products, or may be produced partially or wholly using recombinant or synthetic techniques. Thus, the term "polypeptide" may also refer to "protein".

[00218] The vector comprising a polynucleotide which encodes a fusion polypeptide may be introduced in to a cell.

[00219] The term "introducing" as used herein in the context of a cell or organism refers to presenting the nucleic acid molecule to the organism and/or cell in such a

manner that the nucleic acid molecule gains access to the interior of a cell. Where more than one nucleic acid molecule is to be introduced these nucleic acid molecules can be assembled as part of a single polynucleotide or nucleic acid construct, or as separate polynucleotide or nucleic acid constructs, and can be located on the same or different nucleic acid constructs. Accordingly, these polynucleotides can be introduced into cells in a single transformation event or in separate transformation events.

[00220] Thus, the term "transformation" as used herein refers to the introduction of a nucleic acid into a cell. Transformation of a cell may be stable or transient.

[00221] The term "transient transformation" as used herein in the context of a polynucleotide refers to a polynucleotide that may be introduced into the cell and does not integrate into the genome of the cell.

[00222] The term "stably introducing" or "stably introduced" as used herein in the context of a polynucleotide introduced into a cell refers to a polynucleotide that may be stably incorporated into the genome of the cell, and thus the cell is stably transformed with the polynucleotide.

[00223] As used herein, the terms "contacting" refers to a process by which, for example, a compound may be delivered to a cell. The compound may be administered in a number of ways, including, but not limited to, direct introduction into a cell (i.e., intracellularly) and/or extracellular introduction into a cavity, interstitial space, or into the circulation of the organism.

[00224] A "cell" or "host cell" refers to an individual cell or cell culture that can be or has been a recipient of any recombinant vector(s), isolated polynucleotide, or polypeptide. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected in vivo or in vitro with a recombinant vector or a polynucleotide of the invention. A host cell which comprises a recombinant vector of the invention is a recombinant host cell.

[00225] The term "about" or "approximately" means within an acceptable range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, e.g., the limitations of the measurement system. For example, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can

mean within an order of magnitude, preferably within 5 fold, and more preferably within 2 fold, of a value. Unless otherwise stated, the term 'about' means within an acceptable error range for the particular value, such as $\pm 1-20\%$, preferably $\pm 1-10\%$ and more preferably $\pm 1-5\%$.

[00226] Where a range of values is provided, it is understood that each intervening value, between the upper and lower limit of that range and any other stated or intervening value in that stated range is encompassed within the invention. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the invention, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either both of those included limits are also included in the invention.

[00227] Method of the invention are conveniently practiced by providing the compounds and/or compositions used in such method in the form of a kit. Such kit preferably contains the composition. Such a kit preferably contains instructions for the use thereof.

[00228] To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in anyway.

[00229] EXAMPLES

[00230] Metabolic engineering

[00231] Metabolic engineering is a technique that targets specific pathways in the organism to adapt, enhance, or add a novel metabolite. This can be used as an alternate to the wild type biosynthesis of a higher order, and slower growth, organism such as a plant or animal, and produce the identical natural metabolite in a different host organism. The metabolites can be flavors, agricultural products, cosmetics, pharmaceuticals, and other high value small molecules or proteins. (Bhantna 2015). Early products such as Beta-Galactoside alpha- 2,6 sialyltransferase as a glycosylation enzyme in Chinese Hamster Ovary cells for the production of erythropoietin, or D-Amino Acid Oxidase in *A. Chrysogenum* have been commercialized since the early 1990's. This technique shows promise for production of many high value molecules. The low cost, fast doubling times, and relative genetic simplicity make microorganisms the ideal target for metabolic engineering.

[00232] The anabolic metabolites are generated by first identifying the intrinsic processes that are present in the microorganism. Once a suitable common precursor is found, the metabolic pathway for the desired metabolite is then introduced into the organism. While the process of introducing the genes into the organism is important to the overall production of the metabolite, the actual techniques used will vary and should all result in the same product. The genes for the enzymes that will generate these metabolites will be inserted and expressed either constitutively or via an induction methodology.

[00233] Induction methods will grow the organism in its natural state, and then switch over to an alternate carbon source, that will refocus the resources of the microbe to generate the desired product. At this point the culture has grown close to its desired concentration, and then can move from a growth state, to a metabolic production state. Other than induction, the desired metabolite production can be optimized by diverting resources to the precursors for the introduced pathway from other less desirable products. This technique needs to be sure that vital systems remain intact.

[00234] Other foci of metabolic engineering include decreasing the cells catabolism, increasing the flux of carbon through the intended target pathway, optimizing, replacing, or adding better enzymes in the rate limiting steps of the process, decreasing the competitive pathways, or increasing the biomass of the production strain

[00235] Problems with Cannabinoid Biosynthesis in Alternative Microorganisms:

[00236] Cannabinoid biosynthesis in microorganisms commonly utilized in broader biotechnology applications fail to sufficiently meet the needs required to mass produce purified cannabinoid products at scale. *Escherichia coli* (*E. Coli*) is unable to perform the post translational modifications required for the glycosylation steps of cannabinoid biosynthesis. Evidence suggests that *saccharomyces cerevisiae* (*S. Cerevisiae*) as an organism can overcome the limitations presented with *E. coli* in an attempt to biosynthesize cannabinoids, but that yields from *S. Cerevisiae* fail to reach levels practical for commercialization. Biosynthesis of small molecule isolates assayed by Lou and others have only shown cannabinoid yields in mg quantities with little evidence to suggest significantly higher yields are attainable (Lou et al., 2019).

[00237] Problems with Cell Free Cannabinoid Biosynthesis:

[00238] Existing literature demonstrates that CBD/THC cannabinoid production yields of 1 g/L have been synthesized with biotechnologies that perfuse sugar, oxygen

and nutrients via stainless steel fermentation tanks (Valliere et al., 2019). Despite early evidence suggesting reasonable cannabinoid yields could be attainable through cell free biosynthesis platforms, challenges still remain in that individual proteins would still be required to be synthesized and extracted for each enzyme required in the process. Work performed by Valliere et al. Incorporates numerous individual enzymes that would each require biosynthesis and extraction. Therefore, the process of cell free biosynthesis proposed by Valliere and others does not solve the immediate need for a more cost effective method of cannabinoid biosynthesis.

[00239] Description of the Technology

[00240] Cannabinoid production in *P. pastoris* overcomes the disadvantages of previous systems of cannabinoid production for commercial use falling into one or more of the following categories 1) extensive requirements for environmental resources, 2) limited production of cannabinoids in plant systems, 3) requirements for post-translational modifications not available in *E. coli* expression systems, 4) low solubility of intermediates in cannabinoid synthesis pathway, 5) low expression of starting materials in *P. pastoris*, 6) production of non-native cannabinoid and related molecules not found naturally in cannabis, and/or 7) lower biomass yield in other biosynthetic strains.

[00241] *Pichia pastoris* is synonymous with *Komagataella phaffii*, *Komagataella pastoris*, and *Komagataella pseudopastoris* for many uses and in the case of this patent.

[00242] In one example, described herein is a robust, scalable platform for the production of GPP.

[00243] In one example, described herein is a robust, scalable platform for the production of CBGA..

[00244] EXAMPLE 1

[00245] In one example, described herein is a robust, scalable platform for the production GPP.

[00246] Creation of a stable methylotrophic yeast strain containing a modified Erg20 gene

[00247] Two stable transformations of the methylotrophic yeast *P. pastoris* were created through genetic recombination to generate either an insertion of the modified Erg20 gene or a replacement of the endogenous Erg20 gene.

[00248] In one embodiment of this technology a methylotrophic yeast strain (Bg10, Biogrammatix, Carlsbad, CA) was transformed with an Erg20 variant gene (SEQ ID NO: 1) in an integrating vector with homology arms adjacent to the sites of the mutations

which direct the mutation to the endogenous Erg20 location resulting in replacement. 5ug of Erg20 replacement gene in a vector containing a gene for hygromycin B resistance was linearized via restriction enzyme digestion, transformed into cells lacking hygromycin B resistance through electroporation of the cell membrane, and grown on YPD agar containing hygromycin B at inhibitory concentrations to facilitate selection. Methylophilic yeast cells that lacked successful incorporation of the integration plasmid, which included the hygromycin B resistance gene, died. Several colonies, with successful vector insertion, were picked and grown in BMGY media (10g/L yeast extract, 20g/L peptone, 0.1M potassium phosphate buffer pH 6.0, 1.34% w/v yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 1% glycerol) at 28-30°C. All colonies were screened for successful integration by PCR. Clones that were validated via PCR were stored at -80°C in glycerol stocks.

[00249] In another embodiment of this technology a methylophilic yeast strain (Bg10, Biogrammatix, Carlsbad, CA) was transformed with an Erg20 variant gene in an integrating vector with a complete endogenous promoter and homology arms that allow for the incorporation of an additional copy of the Erg20 variant gene alongside the endogenous copy (SEQ ID NO:2). 5ug of Erg20 duplication gene in a vector containing a gene for hygromycin B resistance was linearized via restriction enzyme digestion, transformed into cells lacking hygromycin B resistance through electroporation of the cell membrane, and grown on YPD agar containing hygromycin B at inhibitory concentrations to facilitate selection. Methylophilic yeast cells that lacked successful incorporation of the integration plasmid, which included the hygromycin B resistance gene, died. Several colonies, with successful vector insertion, were picked and grown in BMGY media (10g/L yeast extract, 20g/L peptone, 0.1M potassium phosphate buffer pH 6.0, 1.34% w/v yeast nitrogen base, $4 \times 10^{-5}\%$ biotin, 1% glycerol) at 28-30°C. All colonies were screened for successful integration by PCR. Clones that were validated via PCR were stored at -80°C in glycerol stocks.

[00250] Production of Erg20 in methylophilic yeast

[00251] To initiate the Erg20 variant expression a fresh colony was generated from glycerol stock of Erg20 replacement or Erg20 duplication through streaking on an agar plate to generate single colonies. A single colony was then picked and grown in 5mL of BMGY media overnight at 30°C. The overnight culture was then pelleted by centrifugation, washed with 10mL phosphate buffered saline and re-pelleted. The cell pellet was then resuspended in 50mL of BMGY and grown to an optical density (OD) of approximately 15.

[00252] Production of Erg20 variant production of GPP and FPP was analyzed. Cells were spun down, washed with PBS, and then the cell pellet was stored at -20°C . To each cell pellet, 0.5 mL of 2-propanol:100mM NH_4HCO_3 , pH 7.4 (1:1 v/v), was added, cells were then sonicated, and 300 μL of the resulting cell homogenate was used for further preparation. Subsequently, 1.0 mL of methanol was added for deproteinization, and then mixture was cooled (-20°C) for 10 min. The samples were then centrifuged for 10 min at $14,000\times g$ at 4°C . After centrifugation, supernatants were transferred to glass tubes and dried under a stream of nitrogen at 40°C . The residues were then dissolved in 100 μL acetonitrile: water (1:1, v/v) and 5 μL of this solution was injected into the LC–MS/MS system to confirm and quantitate the production of GPP and the ratio of GPP:FPP.

[00253] EXAMPLE 2

[00254] In one example, described herein is a robust, scalable platform for the production GPP.

[00255] Biosynthetic production of cannabigerolic acid (CBGA) and related cannabinoids in methylotrophic yeast

[00256] This technology embodies a system of converting glucose or other readily available sugars such as, but not limited to dextrose, to cannabinoids in the methylotrophic yeast *Pichia pastoris*. Cannabinoids act on the endogenous endocannabinoid system in humans resulting in changes in psychological behavior through modulation of neurotransmitters and brain chemistry. Members of this family are synthesized naturally in humans as well as in plants and have been shown to have a variety of medicinal and therapeutic value making them key targets of the pharmaceutical industry.

[00257] Current cannabinoid production is limited to organic synthesis, limited production in some microorganisms, and extraction from natural sources. Current methods of cannabinoid production have significant drawbacks in their requirement for expensive chemical precursors, biologically incompatible solvents, limited yields of desired products, stereochemical racemization of products, reduced potency compared to naturally produced cannabinoids, and limited yields. Isolation of cannabinoids from natural sources also suffer due to significant requirements for arable land, large amounts of water, long growth times, mixed cannabinoid production, and low yields as an overall percentage of plant mass. Another limitation to the plant production of cannabinoids is the prevalence of similar compounds.

[00258] Disclosed herein are strategies for the production of chemically pure cannabinoid molecules using the methylotrophic yeast *P. pastoris* (*Komagataella phaffi*). Through genetic engineering of this microorganism along with directed evolution and generation of novel proteins with specific enzymatic function we can achieve the production of the desired cannabinoid compound from a variety of sugars (glucose, galactose, fructose, sucrose, and naturally occurring mixtures containing high sugar contents).

[00259] Through genetic engineering the inventors have reconstituted a pathway for the production of cannabigerolic (CBGA) acid into the methylotrophic yeast *P. pastoris*. Production of CBGA is the crucial intermediate in the synthesis of pharmaceutically active cannabinoid molecules (including but not limited to THCA, CBDA, CBCA, etc.). While synthesized naturally in the acid containing forms, most cannabinoid molecules are only active in the decarboxylated forms which are achieved through heating of the molecules following their isolation.

[00260] The biosynthetic route for the production of CBGA in *P. pastoris* requires the production of hexanoyl-CoA from acetyl-CoA precursor molecules through a series of enzymatic activities. In the natural plant this process is achieved through the conversion of an available pool of hexanoic acid through the use of an acyl-activating enzyme (AAE). Most yeasts, including methylotrophic yeasts like *P. pastoris*, lack an endogenous pool of short and medium chain fatty acids requiring the introduction of a heterologous system for the production of the required hexanoyl-CoA precursor molecule (our system addresses this issue below). Hexanoyl-CoA is then combined with three molecules of malonoyl-CoA to form olivetolic acid (OA) through a prenylation event catalyzed by a prenyl transferase enzyme followed by a cyclization event catalyzed by an olivetolic acid cyclase. OA is then combined with a molecule of geranyl pyrophosphate (GPP) through the activity of an aromatic prenyl transferase to produce CBGA. Subsequent transformation of CBGA into pharmaceutically relevant cannabinoids is accomplished by one of a number of synthase proteins specific to the final cannabinoid product.

[00261] Embodiments of this technology include a heterologous pathway for the production of CBGA. The inventors, through genetic engineering, have introduced the pathway required for the conversion of hexanoyl-CoA to olivetolic acid. This pathway begins by a heterologous polyketide synthase (CsTKS) combining one molecule of hexanoyl-CoA with three molecules of malonoyl-CoA to generate 3,5,7-trioxododecanoyl-CoA. 3,5,7-trioxododecanoyl-CoA is cyclized through the activity of an olivetolic acid

cyclase (CsOAC) enzyme releasing olevitolic acid and a molecule of coenzyme A. An aromatic prenyltransferase (CsPT4) catalyzes the final step in the reaction pathway combining the olevitolic acid produced in the previous steps with GPP resulting in the production of CBGA.

[00262] Fig. 7 depicts Analytical results for CBGA production. LC/MS of GBGA generated from a *P. pastoris* strain that had been modified to contain the biosynthetic cannabinoid pathway. The sample was separated on a C8 reverse phase column using water and acetonitrile as aqueous and organic phases in a gradient. The detection method was a total ion current, and the CBGA peak appears at the 0.80 min mark.;

[00263] Fig 5 depicts Quantification of MeOH induced *P. pastoris* CBGA levels. Calibration curve and analysis of LC/MS data showing the quantification of the CBGA produced in *P. pastoris*. Panel A depicts CBGA injected into MeOH grown yeast. Panel B should a graph of Peak Area versus MeOH CBGA. Panel C shows a summary of data. produced in *P. pastoris*.

[00264] In one example, the AOX1 promoter, which responds to methanol induction, may be used.

[00265] **Materials**

[00266] **Culture Medium:**

[00267] Prior to all transformation procedures, *P. pastoris* strains are cultured in YPD medium (1% yeast extract, 2% peptone, and 2% dextrose). Growth of *P. pastoris* in liquid media and on agar media plates is at 30°C unless otherwise specified.

[00268] **BMGY Medium:**

[00269] 1% yeast extract

[00270] 2% peptone

[00271] 100mM potassium phosphate, pH 6.0

[00272] 1.34% yeast nitrogen base (YNB)

[00273] 4 x 10⁻⁵% biotin

[00274] 1% glycerol

[00275] **BMMY Medium:**

[00276] 1% yeast extract

[00277] 2% peptone

[00278] 100mM potassium phosphate, pH 6.0

[00279] 1.34% yeast nitrogen base (YNB)

[00280] 4 x 10⁻⁵% biotin

- [00281] 0.5–1% methanol
- [00282] **Electroporation:**
- [00283] 1. H₂O (1 L).
- [00284] 2. 1 M sorbitol (100 mL).
- [00285] 3. YND agar plates: 0.67% YNB, 2% glucose, and 2% agar (0.5 L/~25 plates).
- [00286] 4. 1M DTT (2.5 mL).
- [00287] 5. YPD medium (100 mL) with 20 mL 1 M HEPES buffer (pH 8.0).
- [00288] 6. Electroporation instrument (e.g., BTX Electro Cell Manipulator 600, BTX, San Diego, CA; Bio-Rad Gene Pulser, Bio-Rad, Hercules, CA; Electroporator II, Invitrogen, San Diego, CA).
- [00289] 7. Sterile electroporation cuvettes.
- [00290] All solutions should be autoclaved except the DTT and HEPES solutions, which should be filter sterilized.
- [00291] **Rapid Heat-Shock/Electroporation:**
- [00292] 1. BEDS solution (9 mL): 10 mM bicine-NaOH (pH 8.3), 3% ethylene glycol, and 5% dimethyl sulfoxide (DMSO).
- [00293] 2. 1 M sorbitol supplemented with 1 mL 1.0M DTT.
- [00294] **Methods**
- [00295] This procedure is a modified version of that described by Becker and Guarente. Electroporation was conducted at 1150V, 600 ohms, and 25uF with 4-5ms pulses.
- [00296] **Preparation of Competent Cells:**
- [00297] 1. Inoculate a 10 mL YPD culture with a single fresh *P. pastoris* colony of the strain to be transformed from an agar plate and grow overnight with shaking.
- [00298] 2. In the morning, use the overnight culture to inoculate a 500-mL YPD culture in a 2.8-L Fernback culture flask to a starting OD₆₀₀ of 0.1 and grow to an OD₆₀₀ of 1.0 (see Note 1).
- [00299] 3. Harvest the culture by centrifugation at 2000g at 4°C, and suspend the cells in 100 mL of YPD medium plus HEPES.
- [00300] 4. Add 2.5 mL of 1M DTT and gently mix.
- [00301] 5. Incubate at 30°C for 15 min.

[00302] 6. Bring to 500 mL with cold water. Wash by centrifugation at 4°C once in 250 mL of cold water, once in 20 mL of cold 1M sorbitol and resuspend in 0.5 mL of cold 1M sorbitol. (Final volume including cells will be 1.0 to 1.5 mL.)

[00303] 7. For highest frequencies, transform the cells directly without freezing as described below.

[00304] 8. To freeze competent cells, distribute in 40 µL aliquots to sterile 1.5-mL microcentrifuge tubes, and place the tubes in a -80°C freezer until use.

[00305] Rapid Heat-Shock/Electroporation:

[00306] 1. Grow 5 mL culture of *P. pastoris* cells in YPD overnight with shaking.

[00307] 2. The next morning, dilute the overnight culture to an OD₆₀₀ of 0.15 to 0.20 in a volume of 50 mL YPD medium in a flask large enough to provide good aeration.

[00308] 3. Grow to an OD₆₀₀ of 0.8 to 1.0 with shaking (4–5 h).

[00309] 4. Centrifuge cells at 500g for 5 min at room temperature then decant supernatant.

[00310] 5. Suspend cells in 9 mL of ice-cold BEDS solution supplemented with DTT.

[00311] 6. Incubate the cell suspension for 5 min at with shaking at 30°C.

[00312] 7. Centrifuge cells at 500g for 5 min at room temperature and resuspend in 1 mL of BEDS (without DTT).

[00313] 8. Immediately perform electroporation as described in below, or freeze cells in small aliquots at -80°C.

[00314] Electroporation:

[00315] 1. Mix up to 10 µg of DNA sample in no more than 5 µL total volume of water or TE buffer to a tube containing 40 µL of frozen or fresh competent cells and transfer to a 2-mm gap electroporation cuvette held on ice (see Notes 2 and 3).

[00316] 2. Pulse cells according to the parameters suggested for yeast by the manufacturer of the specific electroporation instrument being used (1150V, 600 ohms, and 25µF with 4-5ms pulses, see Note 4).

[00317] 3. Immediately add 1 mL of cold 1M sorbitol and transfer the cuvette contents to a sterile 1.5-mL microcentrifuge tube.

[00318] 4. Spread selected aliquots onto agar plates containing selective medium and incubate for 2 to 4 days (see Note 5).

[00319] UPP Promoter Growth:

- [00320]** 1. Select a single colony from agar plate (if available) or streak a previously stored glycerol stock onto an agar plate containing the appropriate selection media to generate a single colony to select.
- [00321]** 2. Add selected colony to 5mL of BMGY growth media and incubate while shaking overnight.
- [00322]** 3. Spin down overnight culture at 2,000g for 5 minutes.
- [00323]** 4. Remove spent BMGY media and resuspend cells in phosphate buffered saline (PBS).
- [00324]** 5. Spin down culture, remove PBS, resuspend cells in 50mL of fresh BMGY media.
- [00325]** 6. Grow culture until OD₆₀₀ of approximately 15 has been reached.
- [00326]** 7. Spin down culture at 2,000g for 5 minutes.
- [00327]** 8. Remove spent BMGY media and store cells at -80°C for LC-MS/MS analysis.
- [00328]** **AOX1 Promoter Growth:**
- [00329]** 1. Select a single colony from agar plate (if available) or streak a previously stored glycerol stock onto an agar plate containing the appropriate selection media to generate a single colony to select.
- [00330]** 2. Add selected colony to 5mL of BMGY growth media and incubate while shaking overnight.
- [00331]** 3. Spin down overnight culture at 2,000g for 5 minutes.
- [00332]** 4. Remove spent BMGY media and resuspend cells in phosphate buffered saline (PBS).
- [00333]** 5. Spin down culture, remove PBS, resuspend cells in 50mL of fresh BMGY media.
- [00334]** 6. Grow culture until OD₆₀₀ of approximately 15 has been reached.
- [00335]** 7. Spin down culture at 2,000g for 5 minutes.
- [00336]** 8. Remove spent BMGY media and resuspend cells in phosphate buffered saline (PBS).
- [00337]** 9. Spin down culture, remove PBS, resuspend cells in 50mL of fresh BMMY media.
- [00338]** 10. After 24hrs incubation at 30°C, add additional methanol to a final concentration of 0.5-1%

- [00339] 11. Supplement methanol at 12hr time points until a total incubation time of 60-72hrs has been reached.
- [00340] 12. Spin down culture at 2,000g for 5 minutes.
- [00341] 13. Remove spent BMMY media and store cells at -80°C for LC-MS/MS analysis.
- [00342] LC-MS/MS Analysis GPP/FPP:
- [00343] 1. Add 0.5mL of 1:1 (v/v) 2-propanol:100mM NH_4HCO_3 , pH 7.4.
- [00344] 2. Sonicate cell mixture to disrupt cell membranes.
- [00345] 3. Add 1.0mL methanol and cool mixture to -20°C for 10 minutes.
- [00346] 4. Centrifuge samples for 10 minutes at 14,000g at 4°C.
- [00347] 5. Transfer supernatant to glass tubes.
- [00348] 6. Dry under a stream of nitrogen at 40°C.
- [00349] 7. Dissolve residue in 100uL 1:1 (v/v) acetonitrile:water.
- [00350] 8. Inject 5uL of the solution into Zorbax 50 x 2.1mm column at 20°C on an Agilent HPLC 1100, API 4000 Qtrap Mass Spectrometer.
- [00351] LC-MS/MS Analysis CBGA:
- [00352] 1. Lyse cells using any mechanical technique.
- [00353] 2. Add 1 mL of 4M KCl, pH2.0 to each 1 mL of cell lysate.
- [00354] 3. Add 1-2mL of ethyl acetate for each 1 mL of cell lysate.
- [00355] 4. Rigorously mix for 1 min.
- [00356] 5. Centrifuge the mixture for 5 minutes at 1000g at 4°C.
- [00357] 6. Remove the top ethyl acetate layer and transfer to glass tubes.
- [00358] 7. Dry under a stream of nitrogen at 40°C.
- [00359] 8. Dissolve residue in 100uL 1:1 (v/v) acetonitrile:water.
- [00360] 9. Inject 5uL of the solution into Zorbax 50 x 2.1mm column at 20°C on an Agilent HPLC 1100, API 4000 Qtrap Mass Spectrometer.
- [00361] Notes:
- [00362] 1. One OD_{600} unit of *P. pastoris* culture equals approx. 5×10^7 cells.
- [00363] 2. If using Invitrogen electroporation cuvettes, the volume of cells should be 80 μL . If using BRL 1.5-mm gap chambers, the volume of cells should be 20 μL .
- [00364] 3. Cell competence decreases very rapidly after cells thaw even if the cells are held on ice. Therefore, adding DNA to frozen samples is critical. To transform large numbers of samples, it is convenient to process them in groups of about six at a time.

[00365] 4. In general, procedures described for *S. cerevisiae* also work well for *P. pastoris*. Thus, if your instrument is not listed, use the protocol recommended for electroporation of *S. cerevisiae* with your instrument. See technical literature provided by manufacturers for information on the use of specific electroporation devices.

[00366] 5. *P. pastoris* cells are flocculent (i.e., tend to grow in multi-cell clumps). As a result, transformant colonies are frequently composed of more than one transformed strain. To avoid the problem of mixed colonies, pick and re-streak them for single colonies on selective medium at least once before proceeding with further analysis. When looking for gene replacement transformants by replica plate screening for a recessive phenotype (e.g., AOX1 gene replacements with a recessive methanol-utilization slow or Mut^s phenotype), colonies should be recovered from the transformation plates, suspended in water, and re-plated on selective medium before screening.

[00367] Transformation and plasmid expansion

[00368] Equipment:

[00369] Shaking incubator at 37°C

[00370] Stationary incubator at 37°C

[00371] Water bath at 42°C

[00372] Ice bucket filled with ice

[00373] Microcentrifuge tubes

[00374] Sterile spreading device

[00375] Materials:

[00376] LB agar plate (10g/L peptone, 5g/L yeast extract, 5g/l NaCl, 12g/L agar)

[00377] SOC media (20g/L tryptone, 5g/L yeast extract, 0.5g/L NaCl)

[00378] Competent *E. coli* cells (DH5- α)

[00379] Ampicillin

[00380] DNA plasmid for transformation

[00381] Method:

[00382] 1. Take competent cells out of -80°C and thaw on ice (approximately 20-30 mins).

[00383] 2. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature and then (optional) incubate in 37°C incubator.

[00384] 3. Mix 1 - 5µl of DNA (usually 10pg - 100ng) into 20-50µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.

[00385] 4. Incubate the competent cell/DNA mixture on ice for 20-30 mins.

[00386] 5. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 secs (45 seconds is usually ideal, but this varies depending on the competent cells you are using).

[00387] 6. Put the tubes back on ice for 2 min.

[00388] 7. Add 250-1,000µl SOC media (without antibiotic) to the bacteria and grow in 37°C shaking incubator for 45 min.

[00389] 8. Plate some 50uL of the transformation onto a 10 cm LB agar plate containing the appropriate antibiotic.

[00390] 9. Incubate plates at 37°C overnight.

[00391] The embodiments described herein are intended to be examples only. Alterations, modifications and variations can be effected to the particular embodiments by those of skill in the art. The scope of the claims should not be limited by the particular embodiments set forth herein, but should be construed in a manner consistent with the specification as a whole.

[00392] All publications, patents and patent applications mentioned in this Specification are indicative of the level of skill those skilled in the art to which this invention pertains and are herein incorporated by reference to the same extent as if each individual publication patent, or patent application was specifically and individually indicated to be incorporated by reference.

[00393] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modification as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of producing geranyl pyrophosphate (GPP) in a methylotrophic yeast host cell, comprising:
 - introducing a first polynucleotide encoding an Erg20 (F98W/N128W) polypeptide,
 - culturing the methylotrophic yeast host cell under conditions sufficient for GPP production.
2. The method of claim 1, wherein said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).
3. The method of claim 1 or 2, wherein the first polynucleotide encoding an Erg20 (F98W / N128W) polypeptide comprises or consists of:
 - a) a nucleotide sequence as set forth in SEQ ID NO:1 or SEQ ID NO:2;
 - b) a nucleic acid having at least 70% identity to the nucleic acid of a),
 - c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),
 - d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
 - e) a derivative or variant of a), b), c), or d).
4. The method of claim 3, wherein in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.
5. An expression vector comprising an isolated polynucleotide, comprising:
 - a) a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2,
 - b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
 - c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
 - d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
 - e) a derivative of a), b), c), or d).

6. The expression vector of claim 5, wherein in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.
7. A methylotrophic yeast host cell comprising an expression vector of claim 5 or 6.
8. A methylotrophic yeast host cell comprising:
 - a) a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2,
 - b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
 - c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
 - d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
 - e) a derivative of a), b), c), or d).
9. The methylotrophic yeast host cell claim 8, wherein said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).
10. An isolated polynucleotide comprising or consisting of:
 - a) a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:2,
 - b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
 - c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
 - d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
 - e) a derivative of a), b), c), or d).
11. The isolated polynucleotide of claim 10, wherein said polynucleotide encodes an Erg20 (F98W / N128W) polypeptide.
12. A method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising:
 - introducing a first polynucleotide encoding an olevitiolic synthase polypeptide,

- introducing a second polynucleotide encoding an olevitiolic acid cyclase polypeptide, and
- introducing a third polynucleotide encoding an aromatic prenyl transferase,
- culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

13. A method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising:

- introducing a fourth polynucleotide encoding an olevitiolic synthase polypeptide and encoding olevitiolic acid cyclase polypeptide, and
- introducing a third polynucleotide encoding an aromatic prenyl transferase,
- culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

14. A method of producing cannabigerolic acid (CBGA), in a methylotrophic yeast host cell, comprising:

- introducing a first polynucleotide encoding an olevitiolic synthase polypeptide, and
- introducing a fifth polynucleotide encoding olevitiolic acid cyclase polypeptide and encoding acormatic prenyl transferase,
- culturing the methylotrophic yeast host cell under conditions sufficient for CBGA production.

15. The method of any one of claims 12 to 14, wherein said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).

16. The method of any one of claims 12 to 14, wherein said conditions sufficient for CBGA production comprise methanol induction.

17. The method of any one of claims 12 to 16, wherein said first polynucleotide encoding olevitiolic synthase polypeptide comprises or consists of:

- a) a nucleotide sequence as set forth in (SEQ ID NO: 6);
- b) a nucleic acid having at least 70% identity to the nucleic acid of a),
- c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),

- d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

18. The method of any one of claims 12 to 16, wherein said second polynucleotide encoding olevitolic acid cyclase polypeptide comprises or consists of:

- a) a nucleotide sequence as set forth in (SEQ ID NO: 9);
- b) a nucleic acid having at least 70% identity to the nucleic acid of a),
- c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),
- d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

19. The method of any one of claims 12 to 16, wherein said third polynucleotide encoding said acornatic prenyl transferase comprises or consists of:

- a) a nucleotide sequence as set forth in (SEQ ID NO: 8);
- b) a nucleic acid having at least 70% identity to the nucleic acid of a),
- c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),
- d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

20. The method of any one of claims 12 to 16, wherein said fourth polynucleotide encoding olevitolic synthase polypeptide and encoding olevitolic acid cyclase polypeptide comprises or consists of:

- a) a nucleotide sequence as set forth in (SEQ ID NO: 3);
- b) a nucleic acid having at least 70% identity to the nucleic acid of a),
- c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),
- d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

21. The method of any one of claims 12 to 16, wherein fifth polynucleotide encoding oleivitiolic acid cyclase polypeptide and encoding aromatic prenyl transferase comprises or consists of:

- a) a nucleotide sequence as set forth in (SEQ ID NO: 10);
- b) a nucleic acid having at least 70% identity to the nucleic acid of a),
- c) a nucleic acid that hybridizes with the complementary strand of the nucleic acid of a),
- d) a nucleic acid that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

22. The method of any one of claims 17 to 21, wherein in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.

23. An expression vector comprising an isolated polynucleotide, comprising:

- a) a nucleotide sequence set forth in SEQ ID NO 3, 6, 8, 9, or 10,
- b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

24. The expression vector of claim 23, wherein in step (c) said polynucleotide hybridizes with the complementary strand of the nucleic acid of a) under conditions of high stringency.

25. A methylotrophic yeast host cell comprising an expression vector of claim 23 or 24.

26. A methylotrophic yeast host cell comprising:

- a) a nucleotide sequence set forth in SEQ ID NO 3, 6, 8, 9, or 10,

- b) a nucleotide sequence having at least 70% identity to the nucleotide sequence of a),
- c) a nucleotide sequence that hybridizes with the complementary strand of the nucleic acid of a),
- d) a nucleotide sequence that differs from a) by one or more nucleotides that are substituted, deleted, and/or inserted; or
- e) a derivative of a), b), c), or d).

27. The methylotrophic yeast host cell of any one of claims 25 or 26, wherein said methylotrophic yeast host cell is from *Pichia Pastoris* (*Komagataella phaffi*).

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ERG20 Duplication vector map

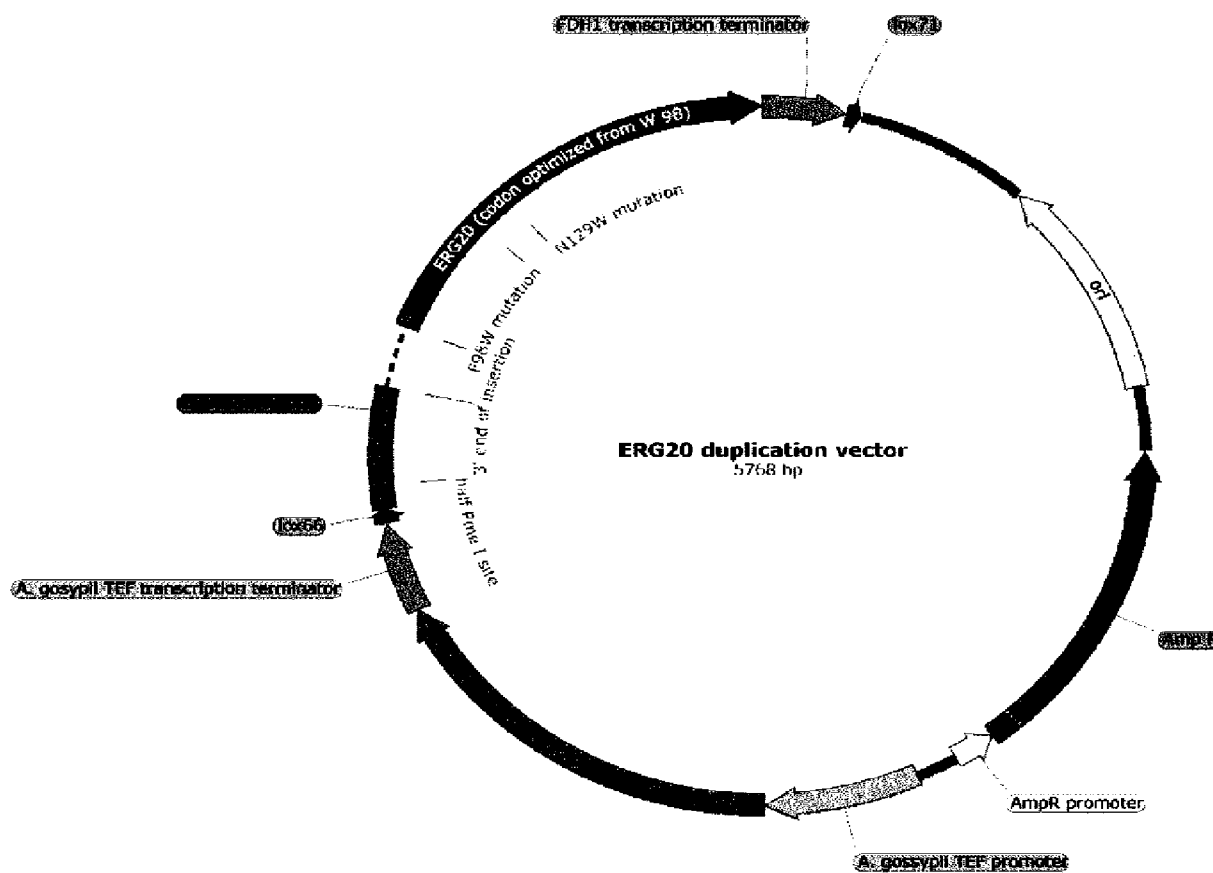


FIGURE 1

Erg20 replacement vector map

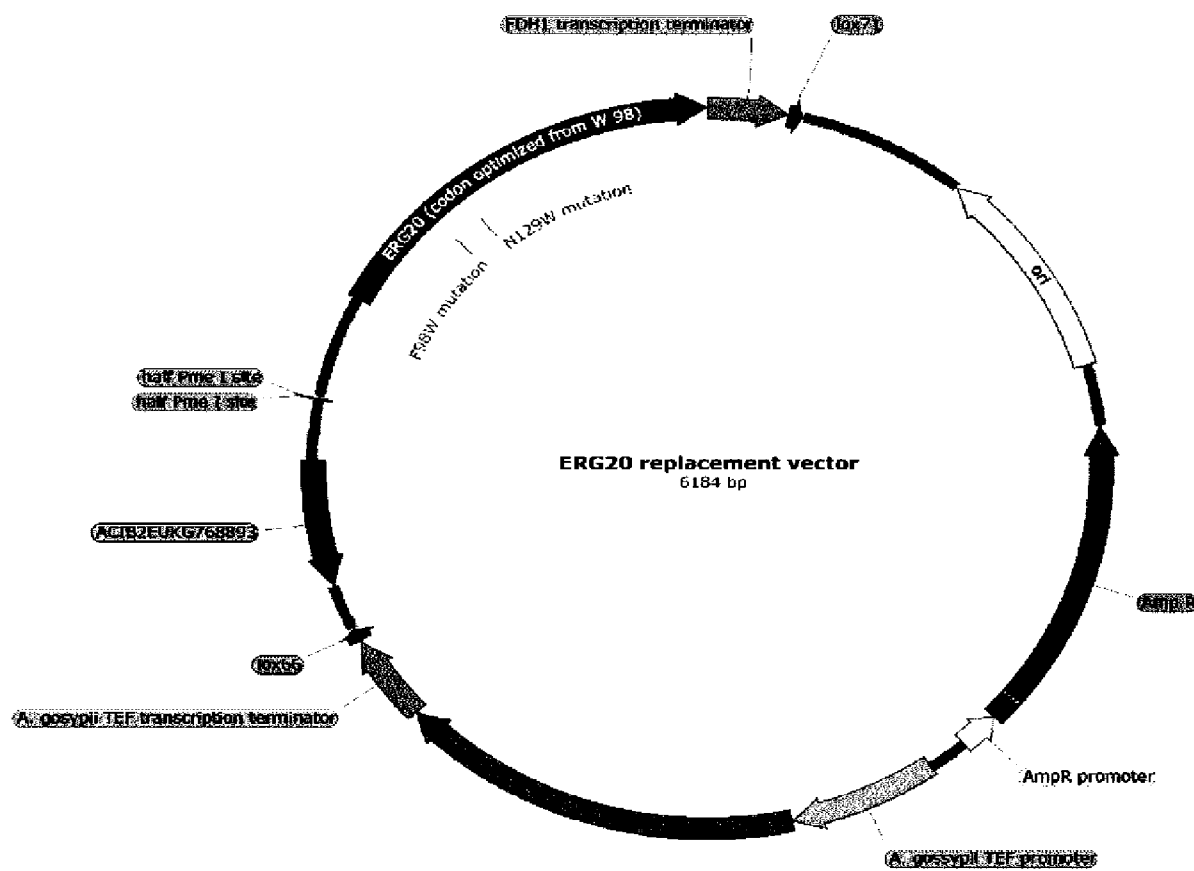
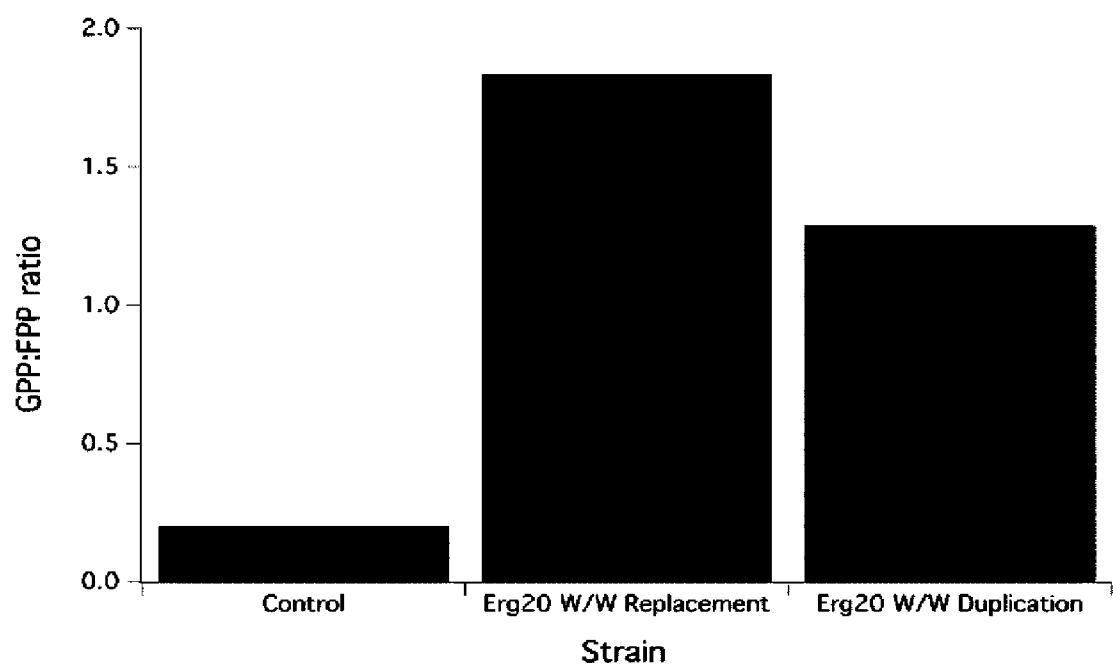


FIGURE 2

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**FIGURE 3**

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FPP = Farnesyl pyrophosphate injected into water

Sample ID	FPP Loaded (ng)	Peak Area (FPP)
Blank	0	0
FPP-1	0.0025	0
FPP-2	0.025	0
FPP-3	0.25	4100
FPP-4	2.5	314000
FPP-5	25	7280000

FIGURE 4A

FPP Peak Area = 293626 x Mass Loaded - 93014

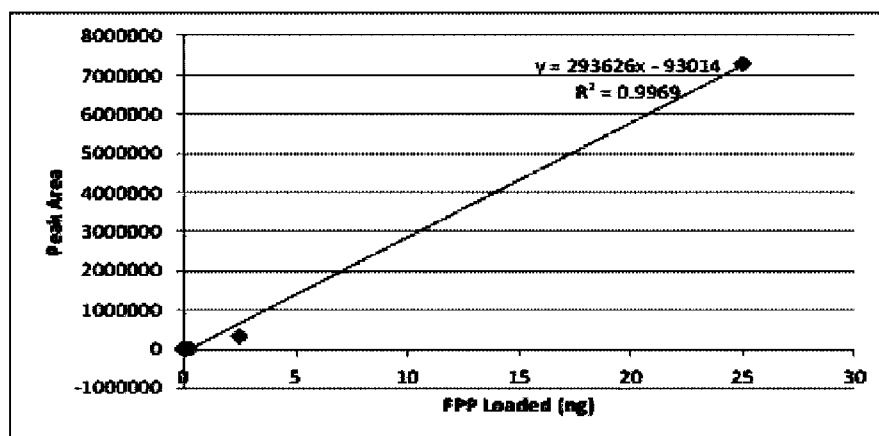
R² = 0.9969

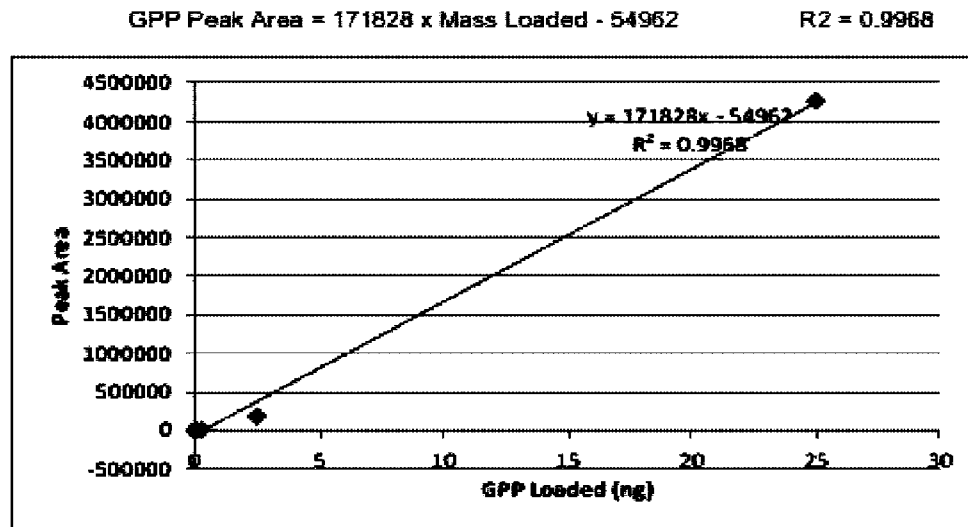
FIGURE 4B

GPP = Geranyl pyrophosphate injected into water

Sample ID	GPP Loaded (ng)	Peak Area (GPP)
Blank	0	0
GPP-1	0.0025	0
GPP-2	0.025	0
GPP-3	0.25	3190
GPP-4	2.5	180000
GPP-5	25	4260000

FIGURE 4C

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Sample ID	FPP Peak Area	Amount of FPP Loaded (ng)	Total FPP in Sample (ng)	Amount of Yeast	FPP ppm in Sample
ER-8	136250	0.781	31.232	1g	0.031
ED-10-1	97625	0.649	25.970	1g	0.026
ED-11-1	81875	0.596	23.825	1g	0.024
Sample ID	GPP Peak Area	Amount of GPP Loaded (ng)	Total GPP in Sample (ng)	Amount of Yeast	GPP ppm in Sample
ER-8	163750	1.273	50.914	1g	0.051
ED-10-1	107125	0.943	37.732	1g	0.038
ED-11-1	81750	0.796	31.825	1g	0.032

FIGURE 4E

Sample ID	GPP/FPP Ratio
ER-8	1.630
ED-10-1	1.453
ED-11-1	1.336

FIGURE 4F

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FPP = Farnesyl pyrophosphate injected into MeOH grown yeast

Sample ID	MeOH FPP Loaded (ng)	Peak Area (MeOH FPP)
Blank	0	72000
MeOH FPP-1	0.25	84300
MeOH FPP-2	0.5	71000
MeOH FPP-3	1	107000
MeOH FPP-4	2.5	119000
MeOH FPP-5	5	225000

FIGURE 5A

MeOH FPP Peak Area = 29525 x Mass Loaded + 67533

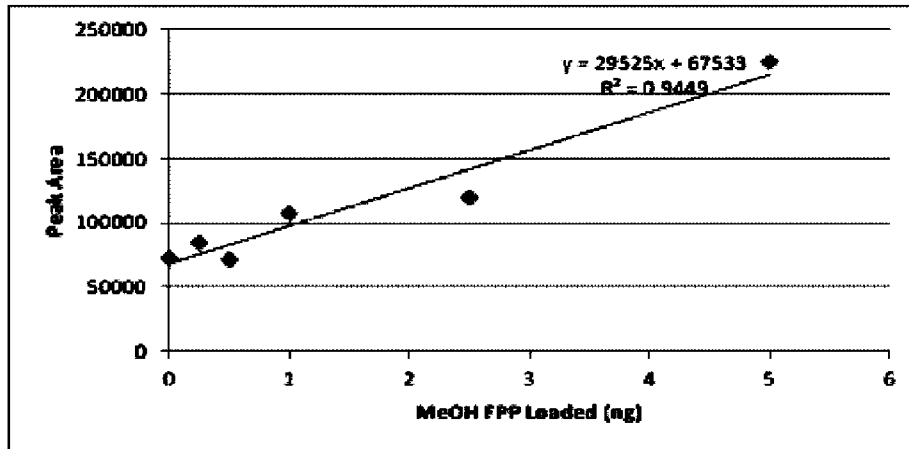
R² = 0.9449

FIGURE 5B

GPP = Geranyl pyrophosphate injected into MeOH grown yeast

Sample ID	MeOH GPP Loaded (ng)	Peak Area (MeOH GPP)
Blank	0	126000
MeOH GPP-1	0.25	147000
MeOH GPP-2	0.5	119000
MeOH GPP-3	1	148000
MeOH GPP-4	2.5	154000
MeOH GPP-5	5	211000

FIGURE 5C

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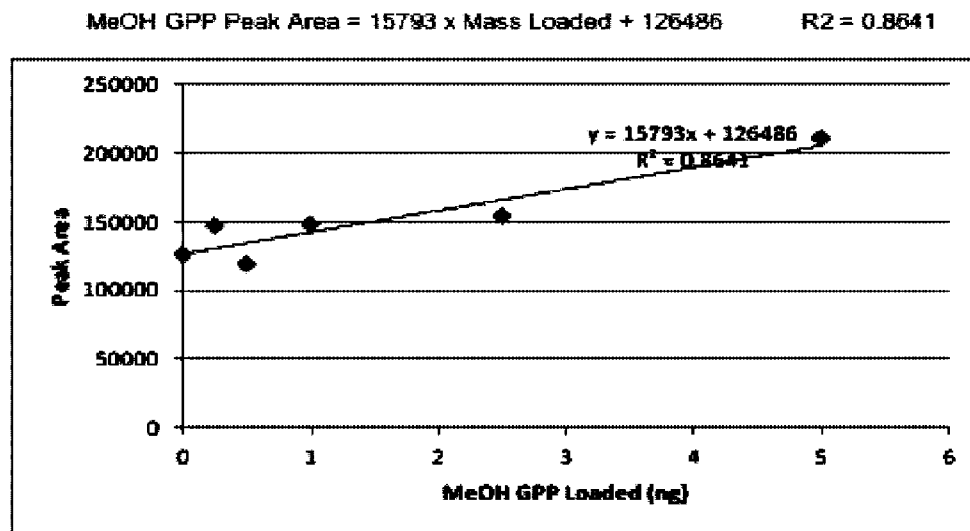


FIGURE 5D

Sample ID	FPP Peak Area	Amount of FPP Loaded (ng)	Total FPP in Sample (ng)	Amount of Yeast	FPP ppm in Sample
ER-8	272500	8.942	277.686	1g	0.278
ED-10-1	195250	4.326	173.029	1g	0.173
ED-11-1	163750	3.258	130.353	1g	0.130
Sample ID	GPP Peak Area	Amount of GPP Loaded (ng)	Total GPP in Sample (ng)	Amount of Yeast	GPP ppm in Sample
ER-8	327500	12.728	509.122	1g	0.509
ED-10-1	214250	5.557	222.286	1g	0.222
ED-11-1	163500	2.344	93.748	1g	0.094

FIGURE 5E

Sample ID	GPP/FPP Ratio
ER-8	1.833
ED-10-1	1.285
ED-11-1	0.719

FIGURE 5F

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FPP = Farnesyl pyrophosphate injected into glycerol grown yeast

Sample ID	Gly FPP Loaded (ng)	Peak Area (Gly FPP)
Blank	0	63400
Gly FPP-1	0.25	54500
Gly FPP-2	0.5	44200
Gly FPP-3	1	64300
Gly FPP-4	2.5	84500
Gly FPP-5	3.75	122000
Gly FPP-6	5	152000

FIGURE 6A

Gly FPP Peak Area = 19684 x Mass Loaded + 47002

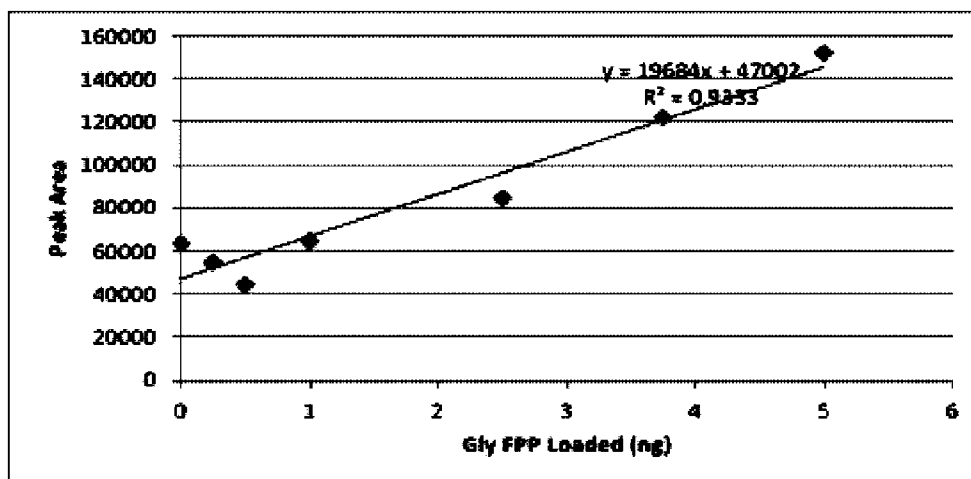
R² = 0.9338

FIGURE 6B

GPP = Geranyl pyrophosphate injected into glycerol grown yeast

Sample ID	Gly GPP Loaded (ng)	Peak Area (Gly GPP)
Blank	0	75200
Gly GPP-1	0.25	64500
Gly GPP-2	0.5	53200
Gly GPP-3	1	66000
Gly GPP-4	2.5	84500
Gly GPP-5	3.75	107000
Gly GPP-6	5	127000

FIGURE 6C

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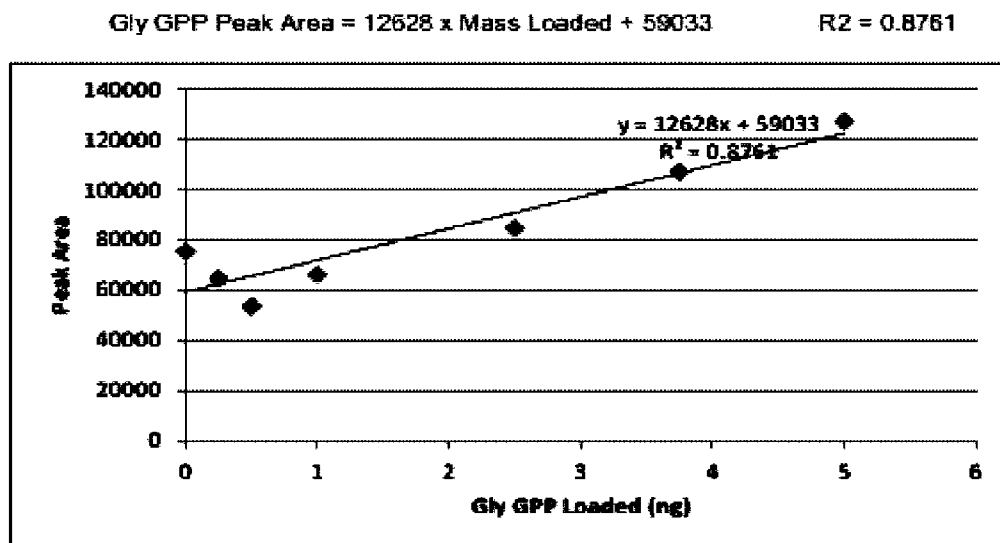


FIGURE 6D

Sample ID	FPP Peak Area	Amount of FPP Loaded (ng)	Total FPP in Sample (ng)	Amount of Yeast	FPP ppm in Sample
ER-8	272500	11.456	458.236	1g	0.458
ED-10-1	195250	7.531	301.258	1g	0.301
ED-11-1	163750	5.931	237.244	1g	0.237
Sample ID	GPP Peak Area	Amount of GPP Loaded (ng)	Total GPP in Sample (ng)	Amount of Yeast	GPP ppm in Sample
ER-8	327500	21.260	850.386	1g	0.850
ED-10-1	214250	12.291	491.660	1g	0.492
ED-11-1	163500	8.273	330.906	1g	0.331

FIGURE 6E

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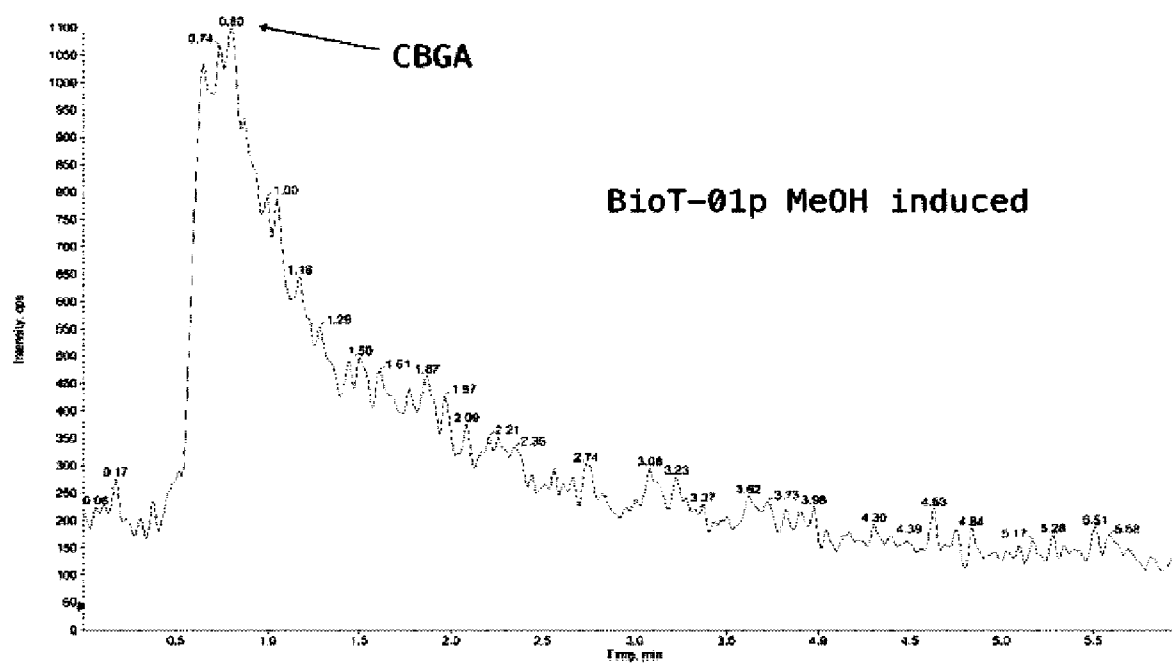


FIGURE 7

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MeOH CBGA = Cannabigerolic Acid injected into MeOH grown yeast

Sample ID	MeOH CBGA Loaded (ng)	Peak Area (MeOH CBGA)
Blank	0	0
MeOH CBGA-1	0.25	11000
MeOH CBGA-2	0.5	25300
MeOH CBGA-3	1	43000
MeOH CBGA-4	2.5	103000

FIGURE 8A

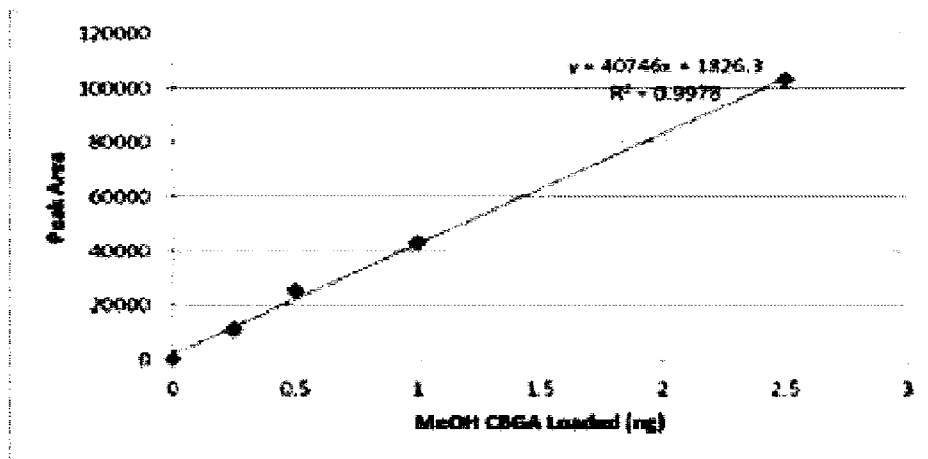
MeOH CBGA Peak Area = 40746 × Mass Loaded + 1826.3 R² = 0.9978

FIGURE 8B

Sample ID	CBGA Peak Area	Amount of CBGA Loaded (ng)	Total CBGA in Sample (ng)	Amount of Yeast	CBGA ppm in Sample
2A 72hr pMi	21800	0.490	39.216	0.5 g	0.078
3A 72hr pMi	19300	0.429	34.308	0.5 g	0.069

FIGURE 8C

Sample ID	CBGA Peak Area	Amount of CBGA Loaded (ng)	Total CBGA in Sample (ng)	Amount of Yeast	CBGA % in Sample
2A 72hr pMi	21800	0.490	39.216	0.5 g	0.00784
3A 72hr pMi	19300	0.429	34.308	0.5 g	0.00686
B1 pUPP	3580	0.043	3.443	0.5 g	0.00069
B5 pUPP	9080	0.178	14.242	0.5 g	0.00285
B14 pUPP	5770	0.097	7.743	0.5 g	0.00155

FIGURE 8D

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Glycerol CBGA = Cannabigerolic Acid injected into glycerol grown yeast

Sample ID	Glycerol CBGA Loaded (ng)	Peak Area (Glycerol CBGA)
Blank	0	0
Glycerol CBGA-1	0.25	15300
Glycerol CBGA-2	0.5	33600
Glycerol CBGA-3	1	50700
Glycerol CBGA-4	2.5	137000

FIGURE 9A

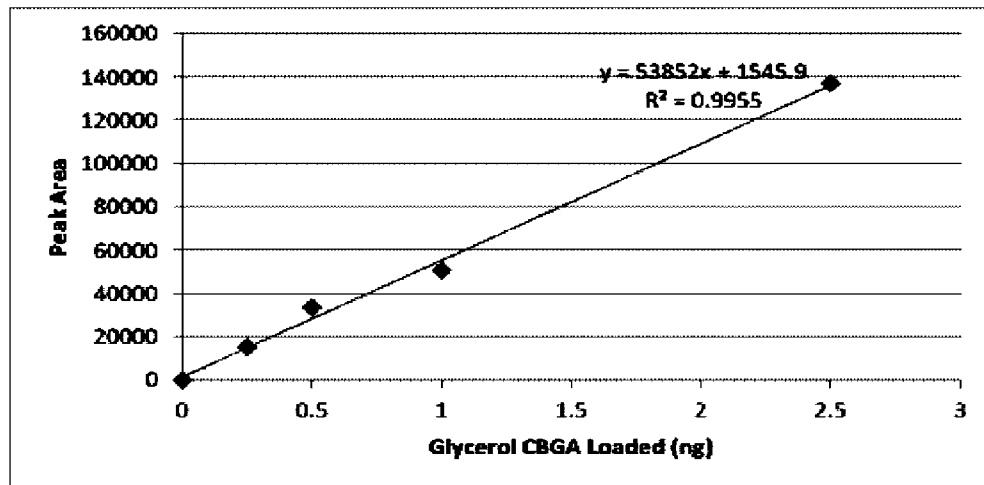
Glycerol CBGA Peak Area = 53852 x Mass Loaded + 1545.9 $R^2 = 0.9955$ 

FIGURE 9B

Sample ID	CBGA Peak Area	Amount of CBGA Loaded (ng)	Total CBGA in Sample (ng)	Amount of Yeast	CBGA % In Sample
2A 72hr pMi	21800	0.376	30.089	0.5 g	0.00602
3A 72hr pMi	19300	0.330	26.375	0.5 g	0.00528
B1 pUPP	3580	0.038	3.022	0.5 g	0.00060
B5 pUPP	9080	0.140	11.192	0.5 g	0.00224
B14 pUPP	5770	0.078	6.275	0.5 g	0.00126

FIGURE 9C

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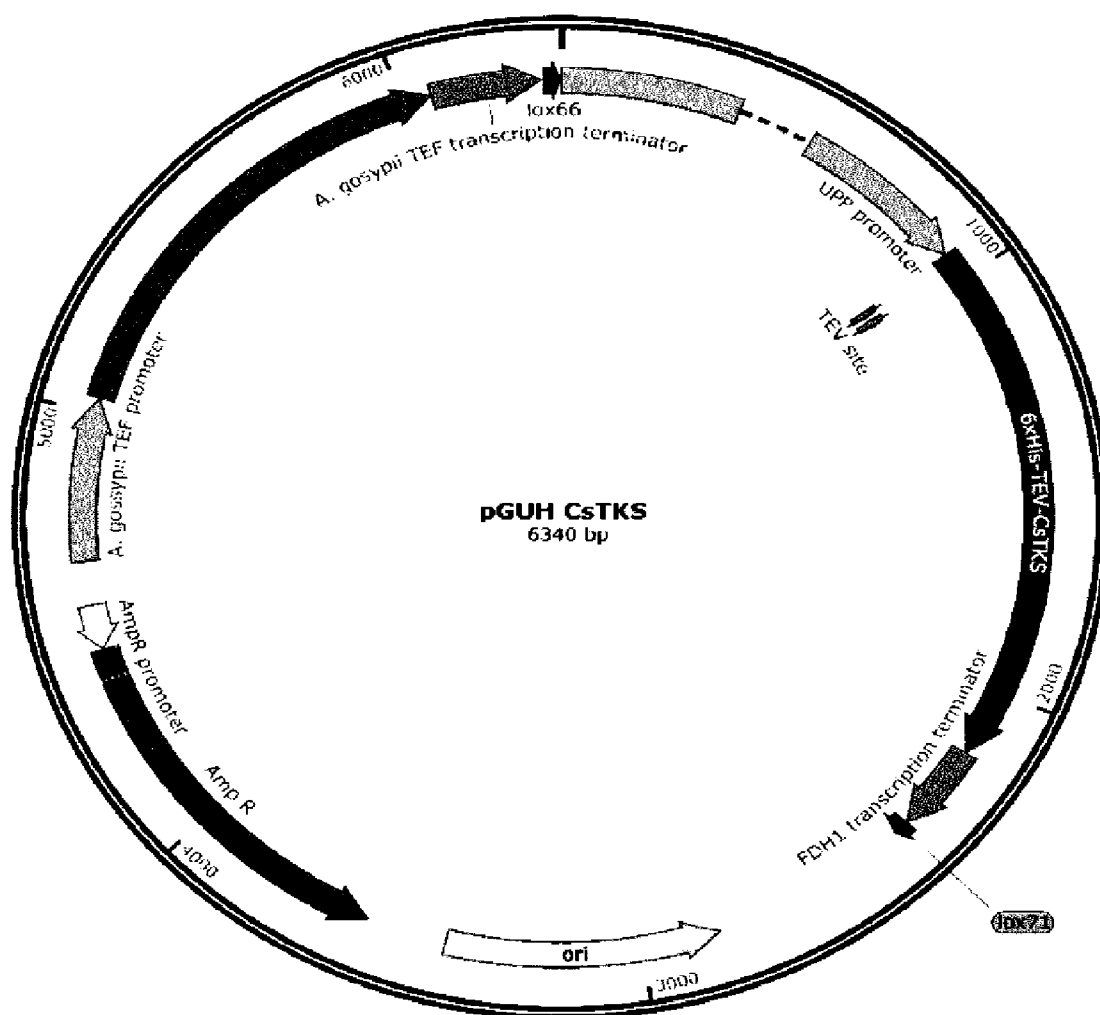


FIGURE 10

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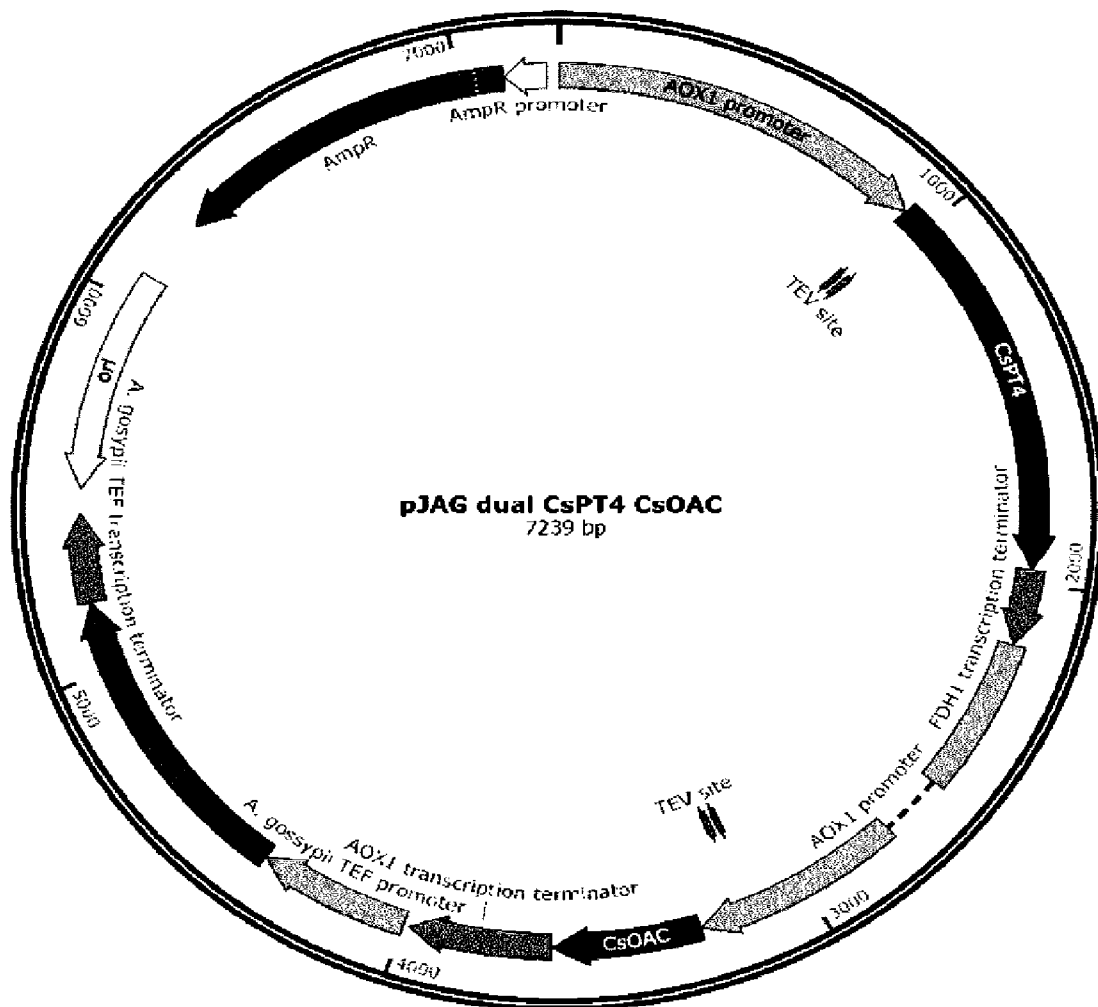


FIGURE 11

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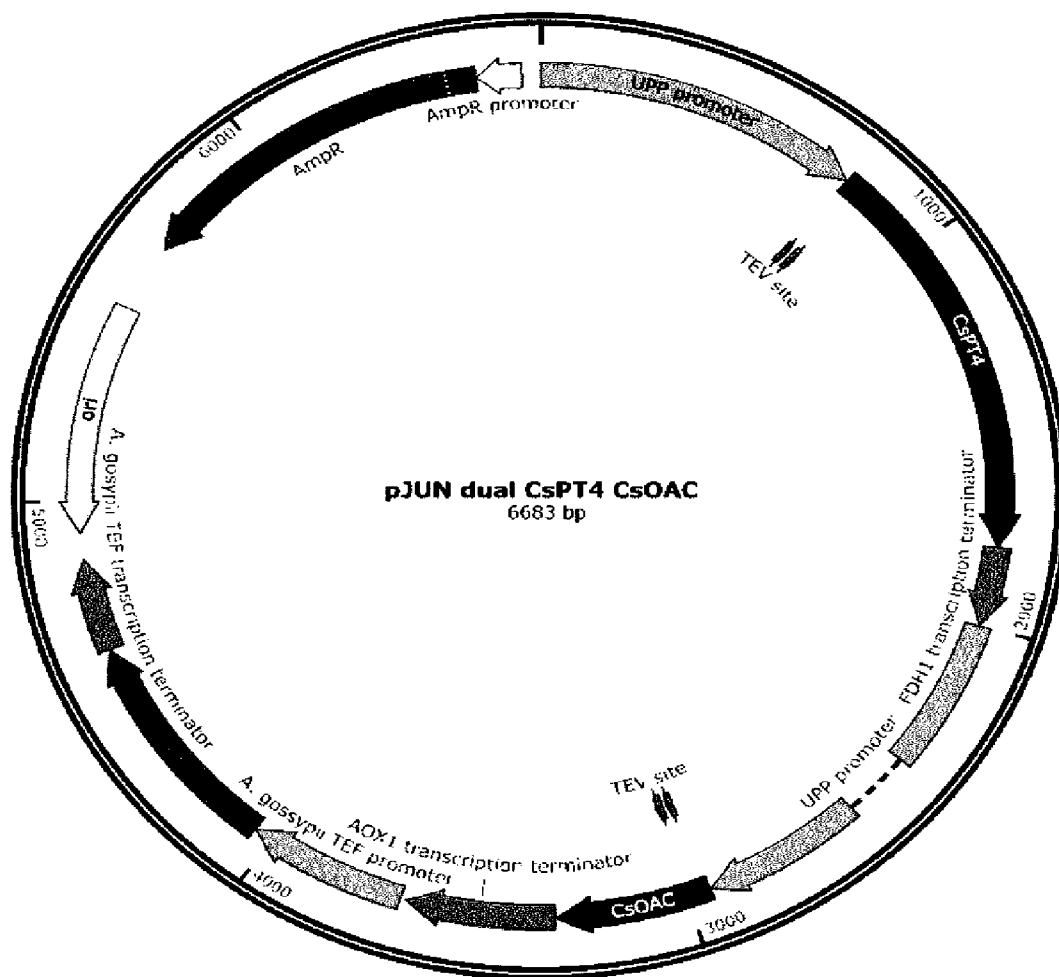


FIGURE 12

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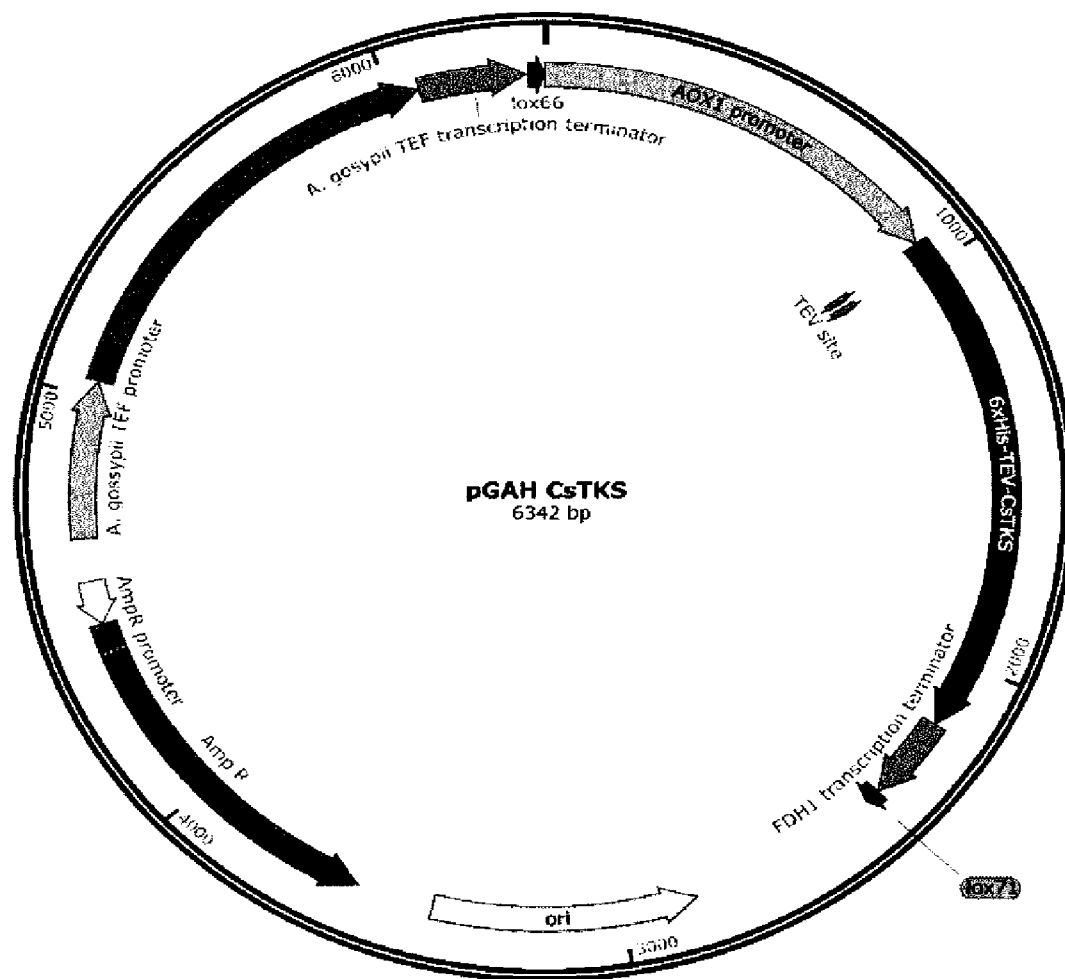


FIGURE 13

