Metabolic Engineering of E. Coli for the Biosynthesis of Cannabinoid Products

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application Number 62/554,494, the contents of which are hereby incorporated by reference in the entirety and for all purposes.

SEQUENCE LISTING

[0001.1] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 17, 2018, is named NMD-003_PCT_SL.txt and is 109,691 bytes in size.

BACKGROUND OF THE INVENTION

[0002] The glandular trichomes of the plant *Cannabis sativa* accumulate a variety of terpenophenolic chemical compounds (Cannabinoids). These plant derived natural products are capable of interacting directly to the cannabinoid receptors (CBl and CB2) found throughout the animal and human body. CB-1 receptors are primarily found in the nervous system and CB-2 receptors are predominantly found in the immune system, or immune-derived cells.

[0003] Cannabinoids, and derivatives thereof, have several properties with therapeutic potential. Activation or blocking of CB-1 and/or CB-2 receptors with a cannabinoid can regulate downstream signalling and metabolic pathways and subsequently influence synaptic transmission, including transmission of pain and other sensory signals in the periphery, immune response, and inflammation. Thus, there is an interest in use of natural or synthetic cannabinoids for therapeutic purposes. However, low extraction yields, and high separation costs have rendered the use of naturally-derived cannabinoids uneconomical. Similarly, fully synthetic methods of cannabinoid production are hampered by the complexity of these compounds.

[0004] Heterologous systems for production of cannabinoids known in the art rely on eukaryotic host organisms for production and secretion of cannabinoid synthase enzymes, which are then used to produce a cannabinoid product in an in vitro enzyme-catalyzed reaction. For example, U.S. Patent Nos. 9,587,212; 9,512,391; 9,394,512; 9,526,715; 9,359,625 each describe methods and compositions and bioreactors for making cannabinoids using a recombinant *Pichia pastoris* that secretes THCA synthase or CBDA synthase. Unfortunately, however, this system requires additional means to generate a suitable substrate for the secreted enzyme. Thus, there is a long felt and unmet need to develop a cost-effective heterologous system for the production of cannabinoids in vivo.

SUMMARY OF THE DISCLOSURE

[0005] Described herein are methods, compositions, and host cells for production of cannabinoids, and terpenoids.

[0005a] In one aspect, the present invention provides an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a bifunctional single-chain enzyme having two active sites, wherein a first active site exhibits 2-C-methyl-D-erythritol 4-phosphate

cytidylyltransferase (ispD) activity and a second active site exhibits 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (ispF) activity (bifunctional ispDF enzyme), wherein the bifunctional ispDF enzyme comprises an amino acid sequence at least 75% identical to SEQ ID No. 1. SEQ ID No. 2, or SEQ ID No. 3.

[0005b] In another aspect, the present invention provides a plasmid comprising an expression cassette as described herein.

[0005c] In another aspect, the present invention provides a host cell comprising: a. an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a bifunctional ispDF enzyme, wherein the bifunctional ispDF enzyme comprises an amino acid sequence at least 75% identical to SEQ ID No. 1. SEQ ID No. 2, or SEQ ID No. 3; and b. an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a terpenoid synthase.

[0006] In one aspect, the present invention provides an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a bifunctional ispDF enzyme. In some embodiments, the expression cassette increases the flux through the MEP pathway in a host cell in which the expression cassette is present. The increased flux through the MEP pathway can increase the

CLAIMS

What is claimed is:

1. An expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a bifunctional single-chain enzyme having two active sites, wherein a first active site exhibits 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (ispD) activity and a second active site exhibits 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (ispF) activity (bifunctional ispDF enzyme),
wherein the bifunctional ispDF enzyme comprises an amino acid sequence at least 75% identical to SEQ ID No. 1. SEQ ID No. 2, or SEQ ID No. 3.

2. The expression cassette of claim 1, wherein the bifunctional ispDF enzyme differs by at least one amino acid from the following ispDF enzymes H. pylori HP1020, H. pylori J99 jhp0404, H. pylori HPAG1 HPAG1_0427, H. hepaticus HH1582, H. acinonychis st. Sheeba Hac_1124, W. succinogenes DSM 1740 WS1940, S. denitrificans DSM 1251 Suden_1487, C. jejuni subsp. jejuni NCTC 11168 Cj1607, C. jejuni RM1221 CJE1779, C. jejuni subsp. jejuni 81-176 CJJ81176_1594, and C. fetus subsp. fetus 82-40 CFF8240_0409.

3. The expression cassette of claim 1, wherein the promoter operably linked to the nucleic acid encoding the bifunctional ispDF enzyme is a constitutive promoter.

4. The expression cassette of claim 1, wherein the promoter operably linked to the nucleic acid encoding the bifunctional ispDF enzyme is an inducible promoter.

5. The expression cassette of claim 1, wherein the nucleic acid encoding the bifunctional ispDF enzyme is codon optimized.

6. The expression cassette of claim 1, wherein the expression cassette further comprises a nucleic acid encoding one or more, two or more, or all of the enzymes selected from the group consisting of DOXP synthase (dxs), isopentenyl/dimethylallyl diphosphate isomerase (idi), and 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (ispE).

7. The expression cassette of claim 1, wherein the expression cassette further comprises a nucleic acid encoding dxs and idi.

8. The expression cassette of claim 1, wherein the expression cassette further comprises a nucleic acid encoding dxs, idi, and ispE.

9. A plasmid comprising an expression cassette according to any one of claims 1-8.

10. The plasmid of claim 9, wherein the plasmid further comprises an expression cassette comprising a nucleic acid encoding an isoprene synthase (ispS).

11. The plasmid of claim 9, wherein the plasmid further comprises an expression cassette comprising a nucleic acid encoding a geranyl pyrophosphate synthase (GPP synthase).

12. The plasmid of claim 9, wherein the GPP synthase is a GPP synthase derived from a eukaryote.

13. The plasmid of claim 12, wherein the GPP synthase is a plant-derived GPP synthase.

14. The plasmid of claim 12, wherein the nucleic acid encoding the GPP synthase is codon optimized.

15. The plasmid of claim 11, wherein the expression cassette comprising the nucleic acid encoding GPP synthase further comprises a nucleic acid encoding one or more components of a lycopene synthesis pathway selected from phytoene synthase (crtE), phytoene dehydrogenase (crtI), and prephytoene pyrophosphate synthase crtB) or a monoterpene synthase.

16. The plasmid of claim 11, wherein the expression cassette comprising the nucleic acid encoding GPP synthase further comprises a nucleic acid encoding carene synthase, myrcene synthase, or limonene synthase.

17. The plasmid of claim 11, wherein the expression cassette comprising the nucleic acid encoding GPP synthase further comprises a nucleic acid encoding a cannabinoid synthase.

18. The plasmid of claim 17, wherein the cannabinoid synthase is selected from the group consisting of a Cannabis cannabigerolic acid (CBGA) synthase, tetrahydrocannabinolic acid (THCA) synthase, cannabidiolic acid (CBDA) synthase, and cannabichromenic acid (CBCA) synthase.

19. A host cell comprising any one of the expression cassettes of claims 1-8 and/or any one of the plasmids of claims 9-18.

20. A host cell comprising:

a. an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a bifunctional ispDF enzyme, wherein the bifunctional ispDF enzyme comprises an amino acid sequence at least 75% identical to SEQ ID No. 1. SEQ ID No. 2, or SEQ ID No. 3; and

b. an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a terpenoid synthase.

21. The host cell of claim 20, wherein the terpenoid synthase is an isoprene synthase.

22. The host cell of claim 20, wherein the terpenoid synthase is a component of a lycopene synthesis pathway.

23. The host cell of claim 20, wherein the terpenoid synthase is a cannabinoid synthase.

24. The host cell of claim 23, wherein the cannabinoid synthase is selected from the group consisting of a CBGA synthase, THCA synthase, CBDA synthase, and CBCA synthase.

25. The host cell of claim 24, wherein the cannabinoid synthase is a truncated cannabinoid synthase selected from the group consisting of a THCA synthase, CBDA synthase, and CBCA synthase, wherein the truncation is a deletion of all or part of a signal peptide.

26. The host cell of claim 20, comprising an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding a GPP synthase.

27. The host cell of claim 20, wherein the expression cassette of a) or b) further comprises a nucleic acid encoding a GPP synthase.

28. The host cell of claim 20, wherein the host cell exhibits higher flux through the MEP pathway as compared to a control cell that does not comprise at least one of the one or more expression cassette(s).

29. The host cell of claim 20, wherein the host cell does not comprise: a heterologous nucleic acid encoding DOXP reductase (ispC), 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (ispE), HMB-PP synthase (ispG), or HMB-PP reductase (ispH); a combination thereof; or all thereof.

30. The host cell of any one of claims 20-29, wherein the cell is a prokaryote.

31. The host cell of any one of claims 20-30, wherein the expression cassette of a) and/or b) is integrated into the genome of the host cell.

32. The host cell of any one of claims 20-31, wherein the host cell comprises a nucleic acid encoding a cannabinoid synthase operably linked to a promoter and the promoter operably linked to the nucleic acid encoding the cannabinoid synthase is a constitutive promoter.

33. The host cell of claims 20-31, wherein the host cell comprises a nucleic acid encoding a cannabinoid synthase operably linked to a promoter and the promoter operably linked to the nucleic acid encoding the cannabinoid synthase is an inducible promoter.

35. The host cell of any one of claims 20-33, wherein the expression cassette of a) and the expression cassette of b) are present in a single plasmid or inserted into a genome of the host cell at a single locus.

35. The host cell of any one of claims 20-33, wherein the expression cassette of a) and the expression cassette of b) are in different plasmids or inserted into a genome of the host cell at different loci.

36. The host cell of any one of claims 20-35, wherein the host cell further comprises olivetolic acid (OA).

37. The host cell of claim 36, wherein the olivetolic acid is exogenous to the host cell.

38. The host cell of any one of claims 20-37, wherein the host cell comprises an expression cassette comprising a heterologous promoter operably linked to a nucleic acid encoding one or more glycosylation pathway genes.

39. The host cell of any one of claims 20-38, wherein the host cell comprises a deletion in 1, 2, 3, 4, 5, 6, or all of the genes selected from the group consisting of acetate kinase-phosphate acetyltransferase (ackA-pta), pyruvate oxidase (poxB), D-lactate dehydrogenase (ldhA), quinone-dependent D-lactate dehydrogenase (dld), alcohol dehydrogenase enzyme (adhE), phosphoenolpyruvate synthase (pps), and acetate CoA-transferase subunit alpha and subunit beta (atoDA).

40. A method of obtaining a target metabolic product, the method comprising culturing a host cell according to any one of claims 20-39 in a suitable culture medium under conditions suitable to induce expression in one or more host cell expression cassettes, and then harvesting the cultured cells or spent medium, thereby obtaining the target metabolic product.

41. The method of claim 40, wherein the metabolic product is a cannabinoid.

42. The method of claim 41, wherein the cannabinoid is tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), cannabichromenic acid (CBCA), cannabinol (CBN), tetrahydrocannabinol (THC), Δ2-cannabidiol (CBD), or cannabichromene (CBC), or a mixture of one or more thereof.

43. The method of claim 40, wherein the metabolic product is a terpenoid or is isoprene.

44. The method of any one of claims 40-43, wherein the method comprises harvesting and lysing the cultured cells, thereby producing cell lysate.

45. The method of claim 44, wherein the method comprises purifying the target metabolic product from the cell lysate, thereby producing a purified target metabolic product.

46. The method of any one of claims 40-43, wherein the method comprises purifying the target metabolic product from the spent culture medium, thereby producing a purified

target metabolic product.

47. The method of claim 45 or 46, wherein the purified target metabolic product is a cannabinoid and the method comprises formulating the cannabinoid in a pharmaceutical composition.

48. The method of claim 45 or 46, wherein the purified target metabolic product is a cannabinoid and the method comprises forming a salt, prodrug, or solvate of the purified cannabinoid.