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(54) Title: METHODS AND CELLS FOR PRODUCING FATTY ACIDS AND FATTY ACID CHAIN PRODUCTS

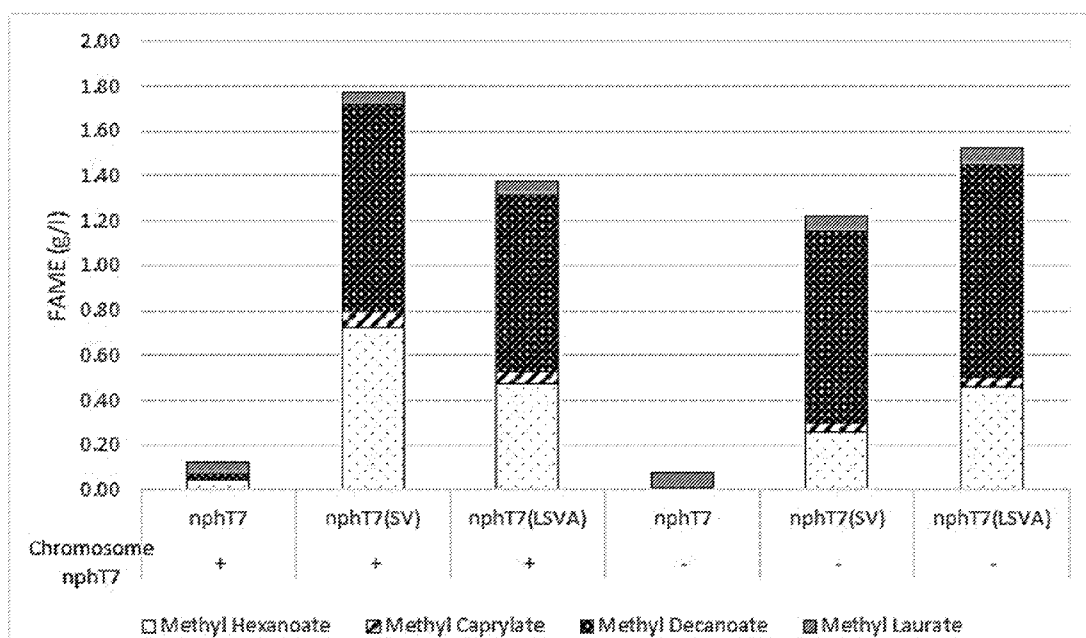


FIG. 3

(57) Abstract: A recombinant cell comprising a heterologous gene encoding a polypeptide with 3-ketoacyl-CoA synthase activity produces fatty acid or fatty acid chain product. Methods for producing fatty acid or fatty acid chain product with the recombinant cell are also disclosed.

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METHODS AND CELLS FOR PRODUCING FATTY ACIDS AND
FATTY ACID CHAIN PRODUCTS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/691,274, filed June 28, 2018, and entitled “METHODS AND CELLS FOR PRODUCING FATTY ACIDS AND FATTY ACID CHAIN PRODUCTS”, which application is hereby incorporated by reference herein in its entirety. This application claims the benefit of U.S. Provisional Application No. 62/693,714, filed July 3, 2018, and entitled “METHODS AND CELLS FOR PRODUCING FATTY ACIDS AND FATTY ACID CHAIN PRODUCTS”, which application is hereby incorporated by reference herein in its entirety.

BACKGROUND

[0002] Fatty acids and fatty acid chain products have a number of industrial uses. For instance, fatty acids and fatty acid chain products can be utilized as solvents for lacquers, paints, varnishes and other compositions; as plasticizers for organic resins, as fragrances and flavorings, as fuels for jet and other internal combustion engines, and as raw materials for making a variety of downstream products. Currently, fatty acids and fatty acid chain products are produced industrially from non-renewable fossil fuels or from plant oils such as palm and coconut oil. However, these conventional methods of producing fatty acids and fatty acid chain products have a number of drawbacks. A particular drawback is poor selectivity of a specific carbon chain length. The products of these conventional methods tend to be a mixture of compounds having a range of carbon chain lengths. Separating these mixtures of compounds can be difficult and can often lead to poor yields with much of the non-renewable fossil fuels or plant oils converted to lower-value byproducts that do not have the desired carbon chain lengths.

[0003] Some biological cells can naturally produce some types of fatty acid chain products. For example, almost all living cells produce triglycerides of fatty acids as well as other fatty acid esters. These triglycerides and other esters play a role in the metabolism, cellular structure, and other biological processes of the cells, and can perform other useful functions such as storing energy. Therefore, some biological cells can potentially be used to produce fatty acid chain products industrially. Among other potential advantages, biological

production of fatty acids and fatty acid products in some cases can rely on annually renewable carbon sources such as sugars, rather than on fossil fuels.

[0004] Biological cells tend to produce fatty acid groups with chain lengths of 12 carbon atoms or greater. Thus, naturally-occurring biological cells are a good source for C12 and higher fatty acids and their chain products. For example, triglycerides produced naturally by such cells can be hydrolyzed to produce C12 or higher fatty acids, which can in turn be converted to other derivatives such as esters or alcohols. On the other hand, few cells naturally produce fatty acids of C6 – C10 chain length in significant quantities.

[0005] Some biological cells can produce fatty acid chain products through a native metabolic pathway that starts with acetyl-CoA and malonyl-ACP (malonyl-acyl-carrier protein). Acetyl-CoA condenses with malonyl-ACP with loss of carbon dioxide and CoA to produce 3-ketobutyryl-ACP. Subsequent enzymatic reactions convert the 3-ketobutyryl-ACP successively to 3-hydroxybutyryl-ACP, then to trans-2-butenoyl-ACP (with loss of water) and finally to butyryl-ACP. The butyryl-ACP can re-enter this reaction cycle in place of acetyl-CoA to produce hexanoyl-ACP. This cycle repeats itself, producing in each iteration a longer carbon atom chain by adding two carbon atoms at a time, until terminated by some other cellular process. However, this native metabolic pathway has low termination rates for C6-C10 chain lengths, resulting in low production of C6-C10 fatty acids and fatty acid chain products.

[0006] Some biological cells can be genetically modified to increase production of C6-C10 chain lengths. These genetic modifications can include insertions of heterologous genes and modification and/or deletions of endogenous genes to increase production of C6-C10 chain lengths. Often, heterologous genes can be inserted to provide enzymes to catalyze specific reactions between chemical intermediates along a pathway that results in production of C6-C10 chain lengths. For example, Okamura et al., in *PNAS* vol. 107, no. 25, pp. 11265-11270 (2010), reported that the enzyme produced by the *nphT7* gene of a soil-isolated *Streptomyces sp.* strain can catalyze a single condensation of acetyl-CoA and malonyl-CoA to produce acetoacetyl-CoA. Additionally, U.S. Application No. 2014/0051136 disclosed that fatty acid chain products can be produced by a cell modified to include the *nphT7* gene and additional heterologous genes. However, the amounts of C6 or longer chain fatty acid chain products produced were limited and selectivity of C6-C10 chain lengths was limited. WO 2015/010103 described mutants of the *nphT7* gene that code for NphT7 enzymes that more efficiently catalyze the condensation of longer-chain acyl-CoA compounds with malonyl-CoA. Cells modified to

include both the wild-type *nphT7* gene and the *nphT7* mutant gene produced greater relative quantities of longer fatty acids, but selectivity of C6-C10 chain lengths remained poor. C6-C10 fatty acid chain products in particular were made in only small amounts.

SUMMARY

[0007] Provided herein are nucleic acid sequences and proteins, as well as biological processes to produce fatty acids at higher yield and with selectivity to C6-C10 fatty acids or fatty acid chain products.

[0008] One aspect provides an expression system comprising at least one expression cassette comprising a promoter operably linked to a heterologous nucleic acid segment encoding a polypeptide with 3-ketoacyl-CoA synthase activity and having at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-15, wherein the polypeptide catalyzes the condensation of acyl-CoA with malonyl-CoA to form 3-ketoacyl-CoA. In one aspect, the polypeptide with 3-ketoacyl-CoA synthase activity has at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-4. In another aspect, the polypeptide catalyzes one or more condensations selected from the group consisting of: a) C2-CoA with malonyl-CoA to form 3-keto-C4-CoA; b) C4-CoA with malonyl-CoA to form 3-keto-C6-CoA; c) C6-CoA with malonyl-CoA to form 3-keto-C8-CoA; and d) C8-CoA with malonyl-CoA to form 3-keto-C10-CoA. In one aspect, an amino acid residue of the polypeptide with 3-ketoacyl-CoA synthase activity that aligns with amino acid residue 147 of SEQ ID NO: 16 is serine and an amino acid residue that aligns with amino acid residue 217 of SEQ ID NO: 16 is valine. In one aspect, the promoter is an inducible promoter, such as an inducible promoter sensitive to lowering phosphate concentration. In one aspect, the promoter is a *PpstSIH* promoter or a *PphoE* promoter.

[0009] One aspect provides a recombinant cell comprising the expression system described herein, wherein the recombinant cell produces a fatty acid and/or fatty acid chain product having a chain length of one or more of C4, C6, C8 and/or C10. In one aspect, the fatty acid chain product comprises one or more of fatty acid ester, fatty alcohol, fatty acid amide and fatty acid amine. In another aspect, the fatty acid ester comprises one or more of fatty acid methyl ester, fatty acid ethyl ester, fatty acid propyl ester, fatty acid butyl ester, fatty acid pentyl ester and fatty acid hexyl ester. In one aspect, the recombinant cell produces a fatty acid ester of C4, C6, C8, and/or C10 chain length. In another aspect, the recombinant cell produces a fatty

acid ester of C8 and/or C10 chain length. In one aspect, the alkoxy group of the fatty acid ester is derived from a C1, C2, C3, or C4 monoalcohol. In another aspect, the alkoxy group is derived from a C1 or C2 monoalcohol. In one aspect, the monoalcohol is methanol.

[0010] In one aspect, the recombinant cell described herein further comprises one or more of: a heterologous nucleic acid segment encoding a polypeptide with enoyl-CoA reductase activity; a heterologous nucleic acid segment encoding a polypeptide with bifunctional 3-hydroxyacyl-CoA dehydrogenase/dehydratase activity; a heterologous nucleic acid segment encoding a polypeptide with ester synthase activity; a heterologous nucleic acid segment encoding a polypeptide with acetyl-CoA carboxylase activity; and a heterologous nucleic acid segment encoding a mutant polypeptide with ketoacyl-CoA synthase activity. In one aspect, the heterologous nucleic acid segment encoding a mutant polypeptide with ketoacyl-CoA synthase activity is an Asch variant selected from the group consisting of Asch(T184I, F236L, V268A, V296A, V317A, S328G), Asch(T184I, V296A, V268A), and Asch(V296A).

[0011] In one aspect, the recombinant cell is a fungal cell, a bacterial cell, or a plant cell. In one aspect, the recombinant cell comprises an *Escherichia coli*. In another aspect, the recombinant cell comprises a yeast cell.

[0012] One aspect provides a cell culture comprising: a) a recombinant cell described herein; and b) one or more fatty acids or fatty acid chain products produced by the recombinant cell.

[0013] One aspect provides a method of producing fatty acids and/or fatty acid chain products comprising growing the recombinant cell described herein in a culture medium, wherein the recombinant cell is grown under conditions in which the heterologous nucleic acid segment encoding the polypeptide with 3-ketoacyl-CoA synthase activity is expressed; and the recombinant cell produces a fatty acid and/or a fatty acid chain product.

[0014] One aspect provides one or more fatty acids or fatty acid chain products produced herein for use in products.

[0015] One aspect provides an isolated 3-ketoacyl-CoA synthase having at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-15 for use in catalyzing the condensation of acyl-CoA with malonyl-CoA to form 3-ketoacyl-CoA. In another aspect, the 3-ketoacyl-CoA synthase has activity for one or more of C2-CoA, C4-CoA and C6-CoA intermediates. In one aspect, the 3-ketoacyl-CoA synthase has activity for C2-CoA and C4-CoA

intermediates. In one aspect, the 3-ketoacyl-CoA synthase has activity for C2-CoA, C4-CoA and C6-CoA intermediates.

[0016] One aspect provides a recombinant cell described herein for use in producing fatty acid and/or fatty acid chain products having a chain length of one or more of C4, C6, C8 and/or C10.

[0017] One aspect provides a 3-ketoacyl-CoA synthase having at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-4, wherein an amino acid residue of the 3-ketoacyl-CoA synthase that aligns with amino acid residue 147 of SEQ ID NO: 16 is serine and an amino acid residue that aligns with amino acid residue 217 of SEQ ID NO: 16 is valine.

[0018] In one aspect, the recombinant cell produces a fatty acid and/or fatty acid chain product having a chain length of one or more of C6, C8 and/or C10. In another aspect, the recombinant cell produces a fatty acid ester of C6, C8, and/or C10 chain length. In one aspect, the recombinant cell produces a fatty acid ester of C8 and/or C10 chain length. In another aspect, the alkoxy group is derived from a C1, C2, C3, or C4 monoalcohol. In one aspect, the alkoxy group is derived from a C1 or C2 monoalcohol. In some aspects, the monoalcohol is methanol.

BRIEF DESCRIPTION OF THE FIGURES

[0019] FIG. 1 shows graphical views of the sequence alignment. Each line corresponds to a 3-ketoacyl-CoA synthase enzyme as indicated by the designation. The amino acid position of the first residue of each row is indicated. The SV mutations are marked with the boxes. Using the NphT7 numbering, it is I147S and F217V. Sequence alignment was done using Clone Manager alignment software with a Scoring Matrix: Standard Linear and default settings.

[0020] FIGS. 2A-2D show graphical views of the sequence alignment. Each line corresponds to a 3-ketoacyl-CoA synthase enzyme as indicated by the designation. The amino acid position of the first residue of each row is indicated. The SV mutations are marked with the boxes. Using the NphT7 numbering, it is I147S and F217V. Sequence alignment was done using Clone Manager alignment software with a Scoring Matrix: Standard Linear and default settings.

[0021] FIG. 3 shows the FAME production by NphT7 and its mutants SV and LSVA in a C10 FAME production pathway with or without chromosomal expression of wild type *nphT7*.

[0022] FIG. 4 shows FAME production by wild type Nbra, Sten, Sko3, Scin in three different FAME production pathways (C8 FAME, C10 FAME, C8/C10 mix).

[0023] FIG. 5 shows FAME production by wild-type Nbra and its SV mutants vs. NphT7(LSVA) in three different FAME production pathways (C8 FAME, C10 FAME, C8/C10 mix) with or without chromosomal expression of wild type *nphT7*.

[0024] FIG. 6 shows FAME production by wild-type Sten and its SV mutants vs. NphT7(LSVA) in three different FAME production pathways (C8 FAME, C10 FAME, C8/C10 mix) with or without chromosomal expression of wild type *nphT7*.

[0025] FIG. 7 shows FAME production by wild-type Sko3 and its SV mutants vs. NphT7(LSVA) in three different FAME production pathways (C8 FAME, C10 FAME, C8/C10 mix) with or without chromosomal expression of wild type *nphT7*.

[0026] FIG. 8 shows FAME production by wild-type Scin and its SV mutants vs. NphT7(LSVA) in three different FAME production pathways (C8 FAME, C10 FAME, C8/C10 mix) with or without chromosomal expression of wild type *nphT7*.

DETAILED DESCRIPTION

[0027] Although some wild type biological cells can naturally produce some types of fatty acid chain products, these biological cells tend to produce fatty acids and fatty acid chain products with chain lengths of C12 or higher. Unfortunately, few cells naturally produce fatty acids and/or fatty acid chain products of C6-C10 chain length in significant quantities.

[0028] Applicants have shown that recombinant cells can be genetically modified to express heterologous polypeptides for the biosynthesis of C6-C10 fatty acids and/or fatty acid chain products. These recombinant cells can be genetically modified to express identified enzymes having 3-ketoacyl-CoA synthase activity to produce C6-C10 fatty acids and/or fatty acid chain products. In particular, these identified enzymes having 3-ketoacyl-CoA synthase activity can catalyze the stepwise addition of malonyl-CoA to an acyl-CoA substrate that can be further converted to a C6-C10 fatty acid and/or fatty acid chain product. For example, an enzyme having 3-ketoacyl-CoA synthase activity can catalyze the addition of malonyl-CoA to a C2-CoA substrate to lead to a C4-CoA product that can be further converted to its fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C4-CoA substrate to lead to a C6-CoA product that can be further converted to its fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C6-CoA substrate to lead to a C8-CoA product that can be

further converted to its fatty acid and/or fatty acid chain product, and/or the addition of malonyl-CoA to a C8-CoA substrate to lead to a C10-CoA product that can be further converted to its fatty acid and/or fatty acid chain product. In some aspects, an enzyme having 3-ketoacyl-CoA synthase activity can catalyze the addition of malonyl-CoA to a C2-CoA substrate (acetyl-CoA) to lead to a 3-keto-C4-CoA, the addition of malonyl-CoA to a C4-CoA substrate to lead to a 3-keto-C6-CoA, the addition of malonyl-CoA to a C6-CoA substrate to lead to a 3-keto-C8-CoA, and/or the addition of malonyl-CoA to a C8-CoA substrate to lead to a 3-keto-C10-CoA.

[0029] In addition to enzymes having 3-ketoacyl-CoA synthase activity, the recombinant cells can be genetically modified to express other heterologous polypeptides for the biosynthesis of C6-C10 fatty acids and/or fatty acid chain products. For example, the recombinant cells can be genetically modified to express one or more heterologous polypeptides capable of converting a 3-keto-CX-CoA substrate (e.g., 3-keto-C4-CoA, 3-keto-C6-CoA, 3-keto-C8-CoA, and/or 3-keto-C10-CoA) to a 3-OH CX-CoA substrate (e.g., 3-OH-C4-CoA, 3-OH-C6-CoA, 3-OH-C8-CoA, and/or 3-OH-C10-CoA) (where X represents the carbon chain length). The recombinant cells can be genetically modified to express one or more heterologous polypeptides capable of converting a 3-OH-CX-CoA substrate (e.g., 3-OH-C4-CoA, 3-OH-C6-CoA, 3-OH-C8-CoA, and/or 3-OH-C10-CoA) to a trans-enoyl-CX-CoA substrate (e.g., enoyl-C4-CoA, enoyl-C6-CoA, enoyl-C8-CoA, and/or enoyl-C10-CoA) (where X represents the carbon chain length). The recombinant cells can be genetically modified to express one or more heterologous polypeptides capable of converting a trans-enoyl-CX-CoA substrate (e.g., enoyl-C4-CoA, enoyl-C6-CoA, enoyl-C8-CoA, and/or enoyl-C10-CoA) to a CX-CoA substrate (e.g., C4-CoA, C6-CoA, C8-CoA, and/or C10-CoA) (where X represents the carbon chain length). Additionally, the recombinant cells can be genetically modified to express one or more heterologous polypeptides capable of converting a CX-CoA substrate (e.g., C4-CoA, C6-CoA, C8-CoA, and/or C10-CoA) to a fatty acid ester.

[0030] As used herein, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. For example, reference to “a nucleic acid” means one or more nucleic acids.

[0031] Methods available to those skilled in the art can be used to construct genetic expression constructs and recombinant cells as described. These methods include, but are not limited to, *in vitro* recombinant DNA techniques, synthetic DNA techniques, *in vivo* recombination techniques, and polymerase chain reaction (PCR) techniques. For example,

techniques as described in Green & Sambrook, 2012, MOLECULAR CLONING: A LABORATORY MANUAL, Fourth Edition, Cold Spring Harbor Laboratory, New York; Ausubel et al., 1989, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates and Wiley Interscience, New York, and PCR Protocols: A Guide to Methods and Applications (Innis et al., 1990, Academic Press, San Diego, CA) can be used to construct the genetic expression constructs and the recombinant cells described in this application.

[0032] The terms “polynucleotide,” “nucleotide,” “oligonucleotide,” and “nucleic acid” can be used interchangeably to refer to nucleic acid comprising DNA, RNA, derivatives thereof, or combinations thereof.

[0033] Amino acid residues in all amino acid sequences described herein are ordered in the N-terminus to C-terminus direction. “Upstream” means in the direction toward the N-terminus, and “downstream” means toward the C-terminus direction. The “start” of an amino acid sequence is the first amino acid residue in the N-terminus direction. The first amino acid residue (amino acid residue 1) for any sequence or sub-sequence described herein is the amino acid residue at its N-terminus.

[0034] A “sub-sequence” is a sequence of amino acid residues contained within a larger amino acid sequence.

[0035] Polypeptides refer to polymeric chains of amino acids connected by peptide bonds. In some aspects, polypeptides can fold into unique three dimensional structures that allow the polypeptides to function as enzymes that catalyze distinct biochemical reactions. For example, a 3-ketoacyl-CoA synthase polypeptide catalyzes the condensation reaction of an acyl-CoA with malonyl-CoA to form a 3-ketoacyl-CoA.

[0036] “Identity” is used herein to indicate the extent to which two (nucleotide or amino acid) sequences have the same residues at the same positions in side-by-side alignment of their sequences. The identity is expressed herein as a % identity as determined using BLAST (National Center for Biological Information (NCBI) Basic Local Alignment Search Tool) version 2.2.31 software (National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland, USA), using default parameters unless indicated otherwise in this paragraph. Identity between amino acid sequences is determined using protein BLAST with the following parameters: Max target sequences: 100; Short queries: Automatically adjust parameters for short input sequences; Expect threshold: 10; Word size: 6; Max matches in a

query range: 0; Matrix: BLOSUM62; Gap Costs: (Existence: 11, Extension: 1); Compositional adjustments: Conditional compositional score matrix adjustment; Filter: none selected; Mask: none selected. Nucleic acid % sequence identity between nucleic acid sequences is determined using standard nucleotide BLAST with the following default parameters: Max target sequences: 100; Short queries: Automatically adjust parameters for short input sequences; Expect threshold: 10; Word size: 28; Max matches in a query range: 0; Match/Mismatch Scores: 1, -2; Gap costs: Linear; Filter: Low complexity regions; Mask: Mask for lookup table only. A sequence having a % identity score of XX% (for example, 80% or 95%) to a reference sequence as determined in this manner is considered to be XX% identical to or, equivalently, have XX% sequence identity to, the reference sequence.

[0037] An amino acid residue of a sequence or sub-sequence under investigation "aligns" to an amino acid residue of a reference sequence or sub-sequence when:

- i) in the case of an entire sequence, the sequences are aligned using the BLAST version 2.2.31 software in the manner described above, and the amino acid residue of the sequence under investigation occupies the same position in the alignment as does the amino acid residue of the reference sequence;
- ii) in the case of a sub-sequence, the sequence containing the sub-sequence under investigation is aligned with the reference sequence, and the amino acid residue of the sub-sequence under investigation occupies the same position in the alignment as does the amino acid residue of the reference sub-sequence.

[0038] The enzymes described herein can be expressed from expression cassettes. Such an expression cassette (also referred to as a transgene) can include a promoter operably linked to a nucleic acid segment that encodes any of the enzymes described herein.

[0039] The terms "expression vector" and "expression cassette" refer to a recombinant nucleic acid molecule containing a desired nucleic acid coding segment and appropriate nucleic acid sequences for the expression of the operably linked nucleic acid coding segment in a particular host cell/recombinant cells or organism. Nucleic acid sequences for expression of proteins in prokaryotes usually include a promoter, an operator (optional), and a ribosome-binding site, often along with other sequences. Eukaryotic cells can utilize promoters, enhancers, and termination and polyadenylation signals.

[0040] Nucleic acid sequences for expression of proteins can be heterologous to the nucleic acid coding segment. A "heterologous nucleic acid," "heterologous gene" and

"heterologous protein" indicate a nucleic acid, gene or protein, respectively, which has come from a source other than its native source. The terms "heterologous" and "exogenous" are sometimes used interchangeably with "recombinant." Such heterologous nucleic acid, heterologous genes, and/or heterologous proteins may have been artificially supplied to the biological system or host/recombinant cell.

[0041] Promoters provide for expression of mRNA. In some cases, the promoter can be a native promoter. However, the promoter can in some cases be heterologous to the nucleic acid segment. In other words, such a heterologous promoter may not be naturally linked to such nucleic acid segment. Instead, some expression cassettes and expression vectors have been recombinantly engineered to include a nucleic acid coding segment (such as one encoding an enzyme described herein) operably linked to a heterologous promoter. A nucleic acid coding segment is operably linked to the promoter, for example, when it is located downstream from the promoter.

[0042] A variety of promoters can be included in the expression cassettes and/or expression vectors. In some cases, an endogenous promoter, or an endogenous promoter that expresses the native enzyme, can be employed. Promoter regions are typically found in the flanking DNA upstream from the coding sequence in both prokaryotic and eukaryotic cells. A promoter sequence provides for regulation of transcription of the downstream gene sequence and typically includes from about 50 to about 2,000 nucleotide base pairs. Promoter sequences can also contain regulatory sequences such as enhancer sequences that can influence the level of gene expression. Some isolated promoter sequences can provide for gene expression of heterologous DNAs, that is a DNA different from the native or homologous DNA.

[0043] Promoters can be strong or weak, or inducible. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a low level of gene expression. An inducible promoter is a promoter that provides for the turning on and off gene expression in response to an exogenously added agent, or to an environmental or developmental stimulus. Promoters can also provide for cell specific, tissue specific or developmental regulation. A strong promoter for heterologous DNAs can be advantageous because it provides for a sufficient level of gene expression for easy detection and selection of transformed cells and provides for a high level of gene expression when desired. In some cases, the promoter within such expression cassettes / vectors can be functional in a fungal cell.

[0044] An enzyme-encoding nucleic acid segment can be combined with a promoter by standard methods to yield an expression cassette or transgene, for example, as described in Sambrook et al. (MOLECULAR CLONING: A LABORATORY MANUAL. Second Edition (Cold Spring Harbor, NY: Cold Spring Harbor Press (1989); MOLECULAR CLONING: A LABORATORY MANUAL. Third Edition (Cold Spring Harbor, NY: Cold Spring Harbor Press (2000)). Briefly, a plasmid containing a promoter can be constructed. Typically, these plasmids are constructed to have multiple cloning sites having specificity for different restriction enzymes downstream from the promoter. The enzyme encoding nucleic acids can be subcloned downstream from the promoter region using restriction enzymes and positioned to ensure that the nucleic acid segment is inserted in proper orientation with respect to the promoter so that the coding segment can be expressed as sense or antisense RNA. Once the enzyme nucleic acid is operably linked to a promoter, the expression cassette so formed can be subcloned into a plasmid or other vector.

[0045] A “recombinant cell” is a cell whose genetic material has been altered by a human using genetic engineering techniques including molecular cloning. Recombinant cells can comprise cells whose genetic material has been altered by the addition of genetic material from a cell of a different organism by genetic engineering. Recombinant cells can also comprise cells whose genetic material has been altered by the deletion of genetic material by genetic engineering. Recombinant cells can also comprise cells altered by the addition of extra copies of genetic material that is normally native to the recombinant cell by genetic engineering.

[0046] For purposes of this application, genetic material such as genes, promoters and terminators is “heterologous” if it is (i) non-native to the wild-type cell before it was converted to a recombinant cell and/or (ii) is native to the cell, but is present at a location different than where that genetic material is present in the wild-type cell and/or (iii) is under the regulatory control of a non-native promoter and/or non-native terminator. Extra copies of native genetic material are considered as “heterologous” for purposes of this application, even if such extra copies are present at the same locus as that genetic material is present in the wild-type cell.

[0047] A gene (such as a gene coding for a 3-ketoacyl-CoA synthase) is “heterologous” if it is not natively expressed by the native cell before it is converted to a recombinant cell, if it is native to the wild-type cell, but is present at a location different than where that genetic material is present in the wild-type cell, if it is under the regulatory control of a non-native promoter and/or non-native terminator, and/or if extra copies are present at the same locus where

that genetic material is normally present in the wild-type cell. A heterologous gene can produce a heterologous polypeptide.

[0048] A “3-ketoacyl-CoA synthase” is an enzyme that catalyzes the condensation reaction of an acyl-CoA with malonyl-CoA to form a 3-ketoacyl-CoA (which is 2 carbon longer in chain length than the acyl-CoA primer), CoA-SH, and CO₂. One method for evaluating the ability of an enzyme to catalyze this reaction is by measuring the release of free CoA-SH using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), malonyl-CoA as the donor substrate, and a C2-C8 acyl-CoA as the primer substrate, in the presence of the enzyme. The formation of the corresponding 3-ketoacyl-CoA from any of these primer substrates indicates the enzyme is a 3-ketoacyl-CoA synthase.

[0049] The ability of an enzyme to catalyze this condensation reaction can also be evaluated by using an acyl-CoA as the primer substrate and malonyl-CoA as the donor substrate in the presence of 5 mM Mg⁺⁺ salt by measuring the increase in absorbance at 303 nm as a function of the increase in the formation of a Mg⁺⁺-complex with the 3-ketoacyl-CoA product. 3-Ketoacyl-CoA synthase will produce acetoacetyl-CoA from acetyl-CoA primer, 3-ketohexanoyl-CoA from butyryl-CoA primer, 3-ketooctanoyl-CoA from hexanoyl-CoA primer, and 3-ketodecanoyl-CoA from octanoyl-CoA primer.

[0050] EC numbers are established by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) (*Enzyme Nomenclature* 1992 (Academic Press, San Diego, California, ISBN 0-12-227164-5 (hardback), 0-12-227165-3 (paperback)) with Supplement 1 (1993), Supplement 2 (1994), Supplement 3 (1995), Supplement 4 (1997) and Supplement 5 (in *Eur. J. Biochem.* 1994, 223, 1-5; *Eur. J. Biochem.* 1995, 232, 1-6; *Eur. J. Biochem.* 1996, 237, 1-5; *Eur. J. Biochem.* 1997, 250; 1-6, and *Eur. J. Biochem.* 1999, 264, 610-650). The EC numbers referenced herein are derived from the KEGG Ligand database, maintained by the Kyoto Encyclopedia of Genes and Genomics, sponsored in part by the University of Tokyo. Unless otherwise indicated, the EC numbers are as provided in the database as of April 2009.

[0051] A “CoA” or “CoA-SH”, as described here refers to Coenzyme A. A CoA substrate can refer to a straight chain carbon chain covalently linked to Coenzyme A. For example, C4-CoA refers to a C4 chain covalently linked to Coenzyme A via a thioester bond between the thiol of the Coenzyme A and a terminal carbon of the C4 chain.

[0052] A “fatty acid,” as described here, is a straight-chain or branched-chain monoalkanoic acid having at least four carbon atoms.

[0053] A “fatty acid chain product” is a compound having a straight chain alkyl group formed in a series of one or more reactions at the site of the terminal carboxyl group of a fatty acid (or a thioester bond of a corresponding -CoA compound). A fatty acid chain product can comprise a straight carbon chain with a different end group such as, for example, an ester, an alcohol group, an amino group, an aldehyde group, a ketone, a methyl group, or an alkenyl group. Fatty acid chain product can include fatty acid ester, fatty alcohol, fatty acid amide, fatty acid imide, and fatty acid amine.

[0054] A “fatty acid ester” is an ester compound corresponding to the reaction product of a fatty acid or a fatty acyl-CoA and an alcohol (with loss of a water or a CoA-SH). A fatty acid ester can also correspond to the reaction product of a fatty acyl CoA and an alcohol. Fatty acid ester can include fatty acid methyl ester, fatty acid ethyl ester, fatty acid propyl ester, fatty acid butyl ester, fatty acid pentyl ester, and fatty acid hexyl ester. Fatty acid methyl ester (FAME) is an ester compound corresponding to the reaction product of a fatty acid or a fatty acyl-CoA and methanol.

[0055] Chain lengths of fatty acids and fatty acid chain products are sometimes indicated herein by the shorthand “CX,” wherein X is a number designating the number of carbon atoms. The number of carbon atoms designated in each case represents the carbon length of the straight-chain compound (after removal of CoA or ACP coenzymes) formed by the recombinant cell through one or more iterations of the reaction cycle: acyl-CoA (or acyl-ACP) + malonyl-CoA to form a 3-ketoacyl compound; reduction of the 3-ketoacyl compounds to form a 3-hydroxyacyl compound; dehydration of the 3-hydroxyacyl-CoA to form a 3-enoylacyl compound; and reduction of the 3-enoylacyl compound to the corresponding acyl compound.

[0056] Each iteration of this reaction cycle adds two carbon atoms to the starting acyl-CoA or acyl-ACP. The number of carbon atoms does not include additional carbon atoms that may be added during the formation of any fatty acid chain products of the fatty acid, such as, for example, carbons included in an ester group following an esterification. Thus, hexanoic acid methyl ester is considered as a “C6” fatty acid ester compound, the carbon of the methyl ester group not being counted.

[0057] The “CX” designation can also apply to fatty acid chain products. For example, C4 FAME refers to methyl butanoate, C6 FAME refers to methyl hexanoate, C8 FAME refers to methyl octanoate, C10 FAME refers to methyl decanoate, and C12 FAME refers to methyl

dodecanoate. The “CX” designation can also be used for fatty acid intermediates. For example, C2-CoA refers to acetyl-CoA, C4-CoA refers to butyryl-CoA, C6-CoA refers to hexanoyl-CoA, C8-CoA refers to octanoyl-CoA, C10-CoA refers to decanoyl-CoA, and C12-CoA refers to dodecanoyl-CoA.

[0058] The term “engineered biosynthetic pathway” refers to a biosynthetic pathway that is at least partially genetically engineered into a recombinant cell and comprises one or more enzymes to carry out a sequence of steps to produce a desired product, such as a fatty acid or fatty acid chain product. In some aspects, one or more of the enzymes of the engineered biosynthetic pathway do not naturally occur in the unmodified cell. These enzymes can be introduced into the cell by inserting a heterologous enzyme by genetic engineering. In some aspects, one or more of the enzymes of the engineered biosynthetic pathway do not naturally occur in sufficient copy number in an unmodified cell and additional copies of the enzymes are inserted by genetic engineering. In some cases, the engineered biosynthetic pathway also comprises genetic modifications to the cell to reduce or eliminate competing metabolic pathways and/or to reduce interfering activities, such as degradation of desired products or necessary intermediates.

[0059] The term “endogenous” gene refers to a gene that is naturally found in a particular cell. The term “overexpress” is used to refer to the expression of a heterologous gene in a recombinant cell at levels higher than the level of gene expression in a wild type cell. In some aspects, an endogenous gene is deleted. The terms “deletion,” “deleted,” “knockout,” and “knocked out” can be used interchangeably to refer to an endogenous gene that has been engineered to no longer be expressed in a recombinant cell. In some aspects, a deleted/knocked out gene is an endogenous gene that is deleted to increase production of a desired product, such as a fatty acid or fatty acid chain product.

[0060] The terms “variant” and “mutant” protein are used to describe a protein sequence that has been modified at one or more amino acids, compared to the wild type sequence of a particular protein. Mutations to the amino acid residues encoded by the wild-type genes are designated herein by the shorthand designation for the wild-type enzyme, followed in parenthesis by a 3-, 4- or 5 character code consisting of a first letter designating the amino acid residue in the native enzyme, a 1-, 2- or 3-digit number indicating the position of that amino acid residue in the native enzyme, and a final letter designating the amino acid residue in that position in the mutated enzyme. For example the designation NphT7(I147S, F217V) indicates

that an isoleucine (I) at amino acid residue position 147 in the wild type NphT7 enzyme has been replaced with a serine (S) and that a phenylalanine at amino acid residue position 217 in the wild type NphT7 enzyme has been replaced with a valine (V). Mutations to the amino acid residues encoded by the wild-type genes can also be designated herein by the shorthand designation for the wild-type enzyme, followed in parenthesis by the final letter or letters designating the amino acid residue in that was mutated. For example the designation NphT7(SV) indicates that two amino acids have been mutated to a serine (S) and valine (V) respectively. The single-letter designations are IUPAC amino acid abbreviations as reported, for example, at *Eur. J. Biochem.* 138:9-37(1984).

[0061] In some aspects, the recombinant cell is a prokaryotic cell. In some aspects, the recombinant cell is a eukaryotic cell.

[0062] In some aspects, the recombinant cell is a microorganism, and may be a single-celled microorganism.

[0063] The recombinant cell may be a plant cell, including a cell from a plant within any of the *Chlorophyta*, *Charophyta*, *Marchantiophyta*, *Anthocerotophyta*, *Bryophyta*, *Lycopodiophyta*, *Pteridophyta*, *Cycadophyta*, *Ginkgophyta*, *Pinophyta*, *Gnetophyta* or *Magnoliophyta* plants. Such a plant cell may be, for example, a cell from a plant within any of the genera *Arabidopsis*, *Beta*, *Glycine*, *Jatropha*, *Miscanthus*, *Panicum*, *Phalaris*, *Populus*, *Saccharum*, *Salix*, *Simmondsia*, and *Zea*.

[0064] The recombinant cell may be a fungal, microalgae, algae or red algae (heterokont) cell. The recombinant cell may be a yeast cell. A yeast or fungal cell may be an oleaginous yeast or fungus, and/or may be a Crabtree negative yeast or fungus.

[0065] The term “oleaginous fungi” refers to yeasts or filamentous fungi, which accumulate at least 10%, 12.5%, 15%, 17.5%, preferably at least 20% or even at least 25% (w/w) of their biomass as lipid. They may even accumulate at least 30%, 40%, 50%, 60%, 70%, 80% (w/w) or more of their biomass as lipids. The biomass is usually measured as cell dry weight (CDW).

[0066] A “Crabtree-positive” organism is one that is capable of producing ethanol in the presence of oxygen, whereas a “Crabtree-negative” organism is not. A yeast cell having a Crabtree-negative phenotype is any yeast cell that does not exhibit the Crabtree effect. The term “Crabtree-negative” refers to both naturally occurring and genetically modified organisms. Briefly, the Crabtree effect is defined as the inhibition of oxygen consumption by a

microorganism when cultured under aerobic conditions due to the presence of a high concentration of glucose (e.g., 10 g-glucose L⁻¹). In other words, a yeast cell having a Crabtree-positive phenotype continues to ferment irrespective of oxygen availability due to the presence of glucose, while a yeast cell having a Crabtree-negative phenotype does not exhibit glucose mediated inhibition of oxygen consumption. Crabtree-positive yeast produce an excess of alcohol rather than biomass production.

[0067] Examples of suitable yeast cells include, *Pichia*, *Candida*, *Klebsiella*, *Hansenula*, *Kluyveromyces*, *Trichosporon*, *Brettanomyces*, *Pachysolen*, *Issatchenkia*, *Yamadazyma*, *Saccharomyces*, *Schizosaccharomyces*, *Zygosaccharomyces*, *Debaryomyces*, *Cryptoococcus*, *Rhodotorula*, *Rhodospiridium*, *Lipomyces* and *Yarrowia*. Examples of specific yeast cells include *C. sonorensis*, *K. marxianus*, *K. thermotolerans*, *C. methanesorbosa*, *Saccharomyces bulderi* (*S. bulderi*), *I. orientalis*, *C. lambica*, *C. sorboxylosa*, *C. zemplinina*, *C. geochares*, *P. membranifaciens*, *Z. kombuchaensis*, *C. sorbosivorans*, *C. vanderwaltii*, *C. sorbophila*, *Z. bisporus*, *Z. lentus*, *Saccharomyces bayanus* (*S. bayanus*), *D. castellii*, *C. boidinii*, *C. etchellsii*, *K. lactis*, *P. jadinii*, *P. anomala*, *Saccharomyces cerevisiae* (*S. cerevisiae*) *Pichia galeiformis*, *Pichia* sp. YB-4149 (NRRL designation), *Candida ethanolica*, *P. deserticola*, *P. membranifaciens*, *P. fermentans*, *Rhodospiridium toruloide*, *Lipomyces starkeyii*, *L. lipoferus*, *Candida revkaufi*, *C. pulcherrima*, *C. tropicalis*, *C. utilis*, *Trichosporon pullas*, *T. cutaneum*, *Rhodotorula glutinous*, *R. garminis*, *Yarrowia lipolytica* and *Saccharomycopsis crataegensis* (*S. crataegensis*). Suitable strains of *K. marxianus* and *C. sonorensis* include those described in WO 00/71738 A1, WO 02/42471 A2, WO 03/049525 A2, WO 03/102152 A2 and WO 03/102201A2. Suitable strains of *I. orientalis* are ATCC strain 32196 and ATCC strain PTA-6648.

[0068] In some aspects, the recombinant cell is a bacteria cell. The bacteria may be a gram-positive or gram-negative bacteria. It may be a cell within any of the *Chlamydiae*, green nonsulfur, actinobacteria, planctomycetes, spirochaetes, fusobacteria, cyanobacteria, thermophilic sulphate-reducer, acidobacteria or proteobacteria classifications of bacteria (Ciccarelli *et al.*, *Science* 311 (5765): 1283-7 (2006)).

[0069] Examples of suitable bacterial cells include, for example, those within any of the genera *Clostridium*, *Zymomonas*, *Escherichia*, *Salmonella*, *Rhodococcus*, *Pseudomonas*, *Streptomyces*, *Bacillus*, *Lactobacillus*, *Enterococcus*, *Alcaligenes*, *Klebsiella*, *Paenibacillus*, *Arthrobacter*, *Corynebacterium*, *Bacteriophage*, *Brevibacterium*, *Acanthoceras*, *Acanthococcus*,

Acarvochloris, Achnanthes, Achnanthidium, Actinastrum, Actinochloris, Actinocyclus, Actinotaenium, Amphichrsis, Amphidinium, Amphikrikos, Amphipleura, Amphiprora, Amphithrix, Amphora, Anabaena, Anabaenopsis, Aneumnastus, Ankistrodesmus, Ankyra, Anomoeoneis, Apatococcus, Aphanizomenon, Aphanocapsa, Aphanochaete, Aphanothece, Apiocystis, Apistonema, Arthrodesmus, Artherospira, Ascochloris, Asterionella, Asterococcus, Audouinella, Aularoseira, Bacillaria, Balbiania, Bambiuseria, Bangia, Basichlamys, Batrarihospermum, Binurlearia, Bitrichia, Blidingia, Botrydiopsis, Botrydium, Botryococcus, Botryosphaerella, Brachiomonas, Brachysira, Brachytrichia, Brebissonia, Bulbochaete, Bumilleria, Buinilleriopsis, Caloneis, Calothrix, Campylodiscus, Capsosiphon, Carteria, Catena, Cavinula, Cenrtractus, Centroniella, Ceratium, Chaetoceros, Chaetochloris, Chaetomorpha, Chaetonella, Chaetomemna, Chaetopeltis, Chaetophora, Chaetosphaeridium, Chamaesiphon, Chara, Characiochloris, Characiopsis, Characium, Charales, Chilomonas, Chlainomonas, Chlamydolepharis, Chlamydocapsa, Chlamydomonas, Chlamydomonopsis, Chlamydomnixa, Chlamydonephris, Chlorangiella, Chlorangiopsis, Chlorella, Chlorobotrys, Chlorobrachis, Chlorochytrium, Chlorocun, Chlorogloea, Chlorogloeopsis, Chlorogonium, Chlorolobion, Chloromonas, Chlorophysema, Chlorophyta, Cholorosaccus, Cholorosarcina, Choricystis, Chromophyton, Chromulina, Chroococciopsis, Chroococcus, Chroodactylon, Chroomonas, Chroothece, Chrysamoeba, Chrysopsis, Chrysidiastrium, Chrysocapsa, Chrysocapsella, Chrysochaete, Chrysochromulina, Chrysococcus, Chrysocrinus, Chrysolepidomonas, Chrysolykos, Chrysonebula, Chrysophyta, Chrysopyxis, Chrysosaccus, Chrysosphaerella, Chrysotephanosphaera, Cladophora, Cladidium, Closteriopsis, Closterium, Coccomyxa, Cocconeis, Coelastrella, Coelastrum, Coelosphaerium, Coenochloris, Coenococcus, Coenocystis, Colacium, Coleochaete, Collodictyon, Compsogonopsis, Compsopogon, Conjugatophyta, Conoechaete, Coronastrum, Cosmarium, Cosmonioneis, Cosmocladium, Crateriportula, Craticula, Crinalium, Crucigenia, Crucigeniella, Cryptaulax, Cryptomonas, Cryptophyta, Ctenophora, Cyanodictyon, Cyanonephron, Cyanophora, Cyanophyta, Cyanothece, Cyanothomonas, Cyclonexis, Cyclostephanos, Cyclotella, Cylirocapsa, Cylirocystis, Cylirospermum, Cylirotheca, Cymatopleura, Cymbella, Cymbeilonitzschia, Cystodinium Dactylococcopsis, Debarya, Denticula, Dermatochrysis, Dermorarpa, Dermocarpella, Desmatractum, Desmidium, Desmococcus, Desmonema, Desmosiphon, Diacanthos, Diacronema, Diadesmis, Diatoma, Diatomella, Dicellula, Dichothrix, Dichtotomococcus, Dicranochaete, Dictyochloris, Dictyococcus, Dictyosphaerium,

Didymocystis, Didymogenes, Didymosphenia, Dilabifilum, Dimorphococcus, Dinobryon, Dinococcus, Diplochloris, Diploneis, Diplostauron, Distrionella, Docidium, Draparnaldia, Dunaliella, Dysmorphococcus, Ecballocystis, Elakatothrix, Ellerbeckia, Encyonema, Enteromorpha, Entocladia, Entomoeis, Entophysalis, Ephichrysis, Epipyxis, Epithemia, Eremosphaera, Euastropsis, Euatstrum, Eucapsis, Eucocconeis, Eudorina, Euglena, Euglenophyta, Eunotia, Eustigmatophyta, Eutreptia, Fallcia, Ficherella, Fragilaria, Fragilariforma, Franceia, Frustulia, Curcilla, Geminella, Genicularia, Glaucocystis, Glaucophyta, Glenodiniopsis, Glenodinium, Gloeomonas, Gloeoplax, Gloeotheca, Gloeotila, Gloeotrichia, Gloiodictyon, Golenkinia, Golenkiniopsis, Gomontia, Gomphocymbella, Gomphonema, Gomphosphaeria, Gonatozygon, Gongrosia, Gongrosira, Goniochloris, Gonium, Gonyostomum, Granulochloris, Granulocystopsis, Groenbladia, Gymnodiunium, Gymnozyga, Gyrosigma, Haematococcus, Hafniomonas, Hallassia, Hammatoidea, Hannaea, Hantzchia, Hapalosiphon, Haplotaenium, Haptophyta, Haslea, Hemidinium, Hemitonia, Heribaudiella, Heteromastix, Heterothrix, Hibberdia, Hildenbrandia, Hillea, Holopedium, Homoeothrix, Hormanthonema, Hormotila, Hyalobrachion, Hyalocardium, Hyalodiscus, Hyalogonium, Hyalotheca, Hydrium, Hydrococcus, Hydrocoleum, Hydrocoryne, Hydrodictyon, Hydrosera, Hydrurus, Hyella, Hymenomonas, Isthmochloron, Johannesbaptistia, Juranyiella, Karayevia, Kathablepharis, Katodinium, Kaphyrion, Keratococcus, Kirchneriella, Klebsormidium, Kolbesia, Koliella, Komarekia, Korshikoviella, Kraskella, Lagerheimia, Lagynion, Lamprothamnium, Lemanea, Lepocinclis, Leptosira, Lobococcus, Lobocystis, Lobomonas, Luticola, Lynbya, Malleochloris, Mallomonas, Mantoniella, Marssoniella, Martyana, Mastigocoleus, Gastogloia, Melosira, Merismopedia, Mesostigma, Mesotaenium, Micractinium, Micrasterias, Microchaete, Microcoleus, Microcystis, Microglana, Micromonas, Microspora, Microthamnion, Mischococcus, Monocrysis, Monodus, Monomastix, Monoraphidium, Monostroma, Mougeotia, Mougeotiopsis, Myochloris, Myromecia, Myxocarcina, Naegeliella, Nannochloris, Nautococcus, Navicula, Neglectella, Neidium, Nephroclamys, Nephrocystium, Nephrodiella, Nephroselmis, Netrium, Nitella, Nitellopsis, Nitzschia, Nodularia, Nostoc, Ochromonas, Oedogonium, Oligochaetophora, Onychonema, Oocadrium, Oocystis, Opephora, Ophiocystium, Orthoseira, Oscillartoria, Oxyneis, Pachycladella, Palmella, Palmodictyon, Pnadorina, Pannus, Paralia, Pascherina, Paulshulzia, Pediastrum, Pedinella, Pedinomonas, Pedinopera, Pelagodictyon, Penium, Peranema, Peridiniopsis, Peridinium, Peronia, Petroneis, Phacotus, Phacus, Phaeaster, Phaeodermatium,

Phaeophyta, Phaeoshaera, Phaeothamnion, Phormidium, Phycopeltis, Phyllariochloris, Phyllocadium, Phyllomitas, Pinnilaria, Pitophora, Placoneis, Planctonema, Planktophaeria, Planothidium, Plectonema, Pleodorina, Pleurastrum, Pleurocapsa, Pleurocladia, Pleurodiscus, Pleurosigma, Pleurosira, Pleurotaenium, Pocillomanas, Podohedea, Polyblepharides, Polychaetophora, Polyedriella, Polyedriopsis, Polygoniochloris, Polyepidomanas, Polytaenia, Polytoma, Polytomella, Porphyridium, Posteriochromonas, Prasiniochloris, Prasinocladus, Prasinophyta, Praisola, Prochlorophyta, Psammodictyon, Psammothidium, Pseudanabaena, Pseudenoclonium, Psuedocarteria, Pseudochate, Pseudocharacium, Pseudococcomyxa, Pseudodictyosphaerium, Pseudokephyrion, Pseudoncobrysa, Pseudoquadrigula, Pseudophaerocystis, Pseudostaurastrum, Pseudostrausira, Pyrrophyta, Quadrichloris, Quadricoccus, Quadrigula, Radiococcus, Radiobetalum, Raphidiopsis, Raphidocelis, Raphidonema, Raphidophyta, Peimeria, Rhadorderma, Rhabomonas, Rhizoclonium, Rhodomonas, Rhodiphyta, Rhoicosenia, Rhopalodia, Rivularia, Rosenvingiella, Rossithidium, Roya, Scenedesmus, Scherffelia, Schizochlamydeella, Schizochlamys, Schizomeris, Schizothrix, Schroederia, Scolioneis, Scotiella, Scotiellopsis, Scourfieldia, Scytonema, Slenastrum, Selenochloris, Sellaphora, Semiorbis, Siderocelis, Diderocystopsis, Dimonsenia, Siphononema, Sirocladium, Sirogonium, Skeletonema, Sorastrum, Spermatozopsis, Sphaerellocystis, Sphaerellopsis, Sphaerodinium, Sphaeroplea, Sphaerosoma, Spiniferomonas, Spirogyra, Spirotaenia, Spirulina, Spondylomorum, Spondylosium, Sporotetras, Spumella, Staurastrum, Stauerodesmus, Stauroneis, Staurosira, Staurrosiella, Stenopterobia, Stephanocostis, Stephanodiscus, Stephanoporus, Stephanoshaera, Stichoccus, Stichogloea, Sigeoclonium, Stigonema, Stipitococcus, Stokesiella, Stombomonas, Stylochrysalis, Stylodinium, Styloxyis, Stylosphaeridium, Surirella, Sykidion, Symploca, Synechococcus, Synechocystis, Synedra, Synochromonas, Synura, Tabellaria, Tabularia, Teilingia, Temnogametum, Tetmemorus, Tetrachlorella, Tetracyclus, Tetrademus, Tetraedriella, tetraedron, Tetraselmis, Tetraspora, Tetrastrum, Thalassiosira, Thanmiochaete, Thoakochloris, Thorea, Tolypella, Tolypothrix, Trachelomonas, Trachydiscus, Trebouxia, Trentepohlia, Treubaria, Tribonema, Trichodesmium, Tricodiscus, Trochiscia, Tryblionella, Ulothrix, Uroglena, Uronema, Urosolenia, Urospora, Uva, Vacuolaria, Vaucheria, Volvox, Volvulina, Westella, Woloszynskia, Xanthidium, Xanthophyta, Exencoccus, Zygenema, Zygnemopsis, and Zygonium.

[0070] Specific examples of bacteria cells include *Escherichia coli*, *Oligotropha carboxidovorans*, *Pseudomonas sp.* *Alcaligenes eutrophus* (*Cupriavidus necator*), *Bacillus*

licheniformis, *Paenibacillus macerans*, *Rhodococcus erythropolis*, *Pseudomonas putida*, *Lactobacillus plantarum*, *Enterococcus faecium*, *Enterococcus gallinarum*, *Enterococcus faecalis*, *Bacillus subtilis*, *Cupriavidus basilensis*, *Cupriavidus campinensis*, *Cupriavidus gilardi*, *Cupriavidus laharsis*, *Cupriavidus metallidurans*, *Cupriavidus oxalaticus*, *Cupriavidus pauculus*, *Cupriavidus pinatubonensis*, *Cupriavidus respiraculi* and *Cupriavidus taiwanensis*. In some aspects, the bacterium is *Nocardia sp. NRRL 5646*, *Nocardia farcinica*, *Streptomyces griseus*, *Salinispora arenicola*, or *Clavibacter michiganensis*.

[0071] The recombinant cell may be a synthetic cell or a cell produced by a synthetic genome, as described in U.S. Patent Publication 2007/0264688, or 2007/0269862. The cell may be a CHO cell, a COS cell, a VERO cell, a BHK cell, a HeLa cell, a Cv1 cell, an MDCK cell, a 293 cell, a 3T3 cell, or a PC12 cell.

[0072] In some aspects, this application discloses a recombinant cell genetically modified to express one or more heterologous enzymes having 3-ketoacyl-CoA synthase activity. The heterologous enzymes having 3-ketoacyl-CoA synthase activity can comprise enzymes naturally found in *Nocardia brasiliensis* ATCC 700358 (SEQ ID NO: 1) (Designation: Nbra), *Streptomyces sp. KO-3988* (SEQ ID NO: 2) (Designation: SKO3), *Streptomyces cinnamonensis* (SEQ ID NO: 3) (Designation: Scin), *Streptomyces tendae* (SEQ ID NO: 4) (Designation: Sten), *Streptomyces sp. KO-3988* (SEQ ID NO: 5) (Designation: SKO3-2), *Austwickia chelonae* NBRC 105200 (SEQ ID NO: 6) (Designation: Ache), *Streptomyces sp. Mg1* (SEQ ID NO: 7) (Designation: Smg1), *Spirochaeta africana* DSM 8902 (SEQ ID NO: 8) (Designation: Safr), *Streptomyces sp. HKI0576* (SEQ ID NO: 9) (Designation: SHK1), *Streptomyces violaceusniger* Tu 4113 (SEQ ID NO: 10) (Designation: Svio), *Amycolatopsis sp. ATCC 39116* (SEQ ID NO: 11) (Designation: AATC), uncultured bacterium (SEQ ID NO: 12) (Designated Ubac), *Geobacter sulfurreducens* PCA (SEQ ID NO: 13) (Designated Gsul), *Acinetobacter baylyi* DSM 14961 = CIP 107474 (SEQ ID NO: 14) (Designated Abay-2), and/or *Clostridium tetani* E88 (SEQ ID NO: 15) Designation: Ctet).

[0073] In some aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises the amino acid sequence of SEQ ID NO: 1 and corresponds to the polypeptide designated as Nbra. In other aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises a polypeptide having more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than

95%, more than 96%, more than 97%, more than 98%, or more than 99% sequence identity to the amino acid sequence of SEQ ID NO: 1.

[0074] In some aspects, a recombinant cell genetically modified to express the polypeptide designated as Nbra produces fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as Nbra further comprises an engineered biosynthetic pathway to produce fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as Nbra further comprises an engineered biosynthetic pathway to produce FAME.

[0075] In other cases, the recombinant cell expressing heterologous Nbra and comprising an engineered biosynthetic pathway can also produce a mixture of FAMES with different carbon chain lengths. For example, the recombinant cell expressing heterologous Nbra can produce a FAME mixture comprising C6 FAME, C8 FAME, C10 FAME, and/or C12 FAME.

[0076] In some cases, the recombinant cell expressing heterologous Nbra and comprising an engineered biosynthetic pathway can catalyze the addition of malonyl-CoA to a C2-CoA substrate to lead to a C4 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C4-CoA substrate to lead to a C6 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C6-CoA substrate to lead to a C8 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C8-CoA substrate to lead to a C10 fatty acid and/or fatty acid chain product, and/or the addition of malonyl-CoA to a C10-CoA substrate to lead to a C12 fatty acid and/or fatty acid chain product. In other cases, the recombinant cell expressing heterologous Nbra can also comprise one or more additional heterologous polypeptides with 3-ketoacyl-CoA synthase activity (e.g., a NphT7 polypeptide and/or a NphT7 polypeptide mutant as described herein) to increase production of fatty acids and/or fatty acid chain products.

[0077] In some aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises the amino acid sequence of SEQ ID NO: 2 and corresponds to the polypeptide designated as SKO3. In other aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises a polypeptide having more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than

95%, more than 96%, more than 97%, more than 98%, or more than 99% sequence identity to the amino acid sequence of SEQ ID NO: 2.

[0078] In some aspects, a recombinant cell genetically modified to express the polypeptide designated as SKO3 produces fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as SKO3 further comprises an engineered biosynthetic pathway to produce fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as SKO3 further comprises an engineered biosynthetic pathway to produce FAME.

[0079] In other cases, the recombinant cell expressing heterologous SKO3 and comprising an engineered biosynthetic pathway can also produce a mixture of FAMES with different carbon chain lengths. For example, the recombinant cell expressing heterologous SKO3 can produce a FAME mixture comprising C6 FAME, C8 FAME, C10 FAME, and/or C12 FAME.

[0080] In some cases, the recombinant cell expressing heterologous SKO3 and comprising an engineered biosynthetic pathway can catalyze the addition of malonyl-CoA to a C2-CoA substrate to lead to a C4 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C4-CoA substrate to lead to a C6 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C6-CoA substrate to lead to a C8 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C8-CoA substrate to lead to a C10 fatty acid and/or fatty acid chain product, and/or the addition of malonyl-CoA to a C10-CoA substrate to lead to a C12 fatty acid and/or fatty acid chain product. In other cases, the recombinant cell expressing heterologous SKO3 can also comprise one or more additional heterologous polypeptides with 3-ketoacyl-CoA synthase activity (e.g., a NphT7 polypeptide and/or a NphT7 polypeptide mutant as described herein) to increase production of fatty acids and/or fatty acid chain products.

[0081] In some aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises the amino acid sequence of SEQ ID NO: 3 and corresponds to the polypeptide designated as Scin. In other aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises a polypeptide having more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than

95%, more than 96%, more than 97%, more than 98%, or more than 99% sequence identity to the amino acid sequence of SEQ ID NO: 3.

[0082] In some aspects, a recombinant cell genetically modified to express the polypeptide designated as Scin produces fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as Scin further comprises an engineered biosynthetic pathway to produce fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as Scin further comprises an engineered biosynthetic pathway to produce FAME.

[0083] In other cases, the recombinant cell expressing heterologous Scin and comprising an engineered biosynthetic pathway can also produce a mixture of FAMES with different carbon chain lengths. For example, the recombinant cell expressing heterologous Scin can produce a FAME mixture comprising C6 FAME, C8 FAME, C10 FAME, and C12 FAME.

[0084] In some cases, the recombinant cell expressing heterologous Scin and comprising an engineered biosynthetic pathway can catalyze the addition of malonyl-CoA to a C2-CoA substrate to lead to a C4 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C4-CoA substrate to lead to a C6 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C6-CoA substrate to lead to a C8 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C8-CoA substrate to lead to a C10 fatty acid and/or fatty acid chain product, and/or the addition of malonyl-CoA to a C10-CoA substrate to lead to a C12 fatty acid and/or fatty acid chain product. In other cases, the recombinant cell expressing heterologous Scin can also comprise one or more additional heterologous polypeptides with 3-ketoacyl-CoA synthase activity (e.g., a NphT7 polypeptide and/or a NphT7 polypeptide mutant as described herein) to increase production of fatty acids and/or fatty acid chain products.

[0085] In some aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises the amino acid sequence of SEQ ID NO: 4 and corresponds to the polypeptide designated as Sten. In other aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises a polypeptide having more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98%, or more than 99% sequence identity to the amino acid sequence of SEQ ID NO: 4.

[0086] In some aspects, a recombinant cell genetically modified to express the polypeptide designated as Sten produces fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as Sten further comprises an engineered biosynthetic pathway to produce fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as Sten further comprises an engineered biosynthetic pathway to produce FAME.

[0087] In other cases, the recombinant cell expressing heterologous Sten and comprising an engineered biosynthetic pathway can also produce a mixture of FAMES with different carbon chain lengths. For example, the recombinant cell expressing heterologous Sten can produce a FAME mixture comprising C6 FAME, C8 FAME, C10 FAME, and C12 FAME.

[0088] In some cases, the recombinant cell expressing heterologous Sten and comprising an engineered biosynthetic pathway can catalyze the addition of malonyl-CoA to a C2-CoA substrate to lead to a C4 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C4-CoA substrate to lead to a C6 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C6-CoA substrate to lead to a C8 fatty acid and/or fatty acid chain product, the addition of malonyl-CoA to a C8-CoA substrate to lead to a C10 fatty acid and/or fatty acid chain product, and/or the addition of malonyl-CoA to a C10-CoA substrate to lead to a C12 fatty acid and/or fatty acid chain product. In other cases, the recombinant cell expressing heterologous Sten can also comprise one or more additional heterologous polypeptides with 3-ketoacyl-CoA synthase activity (e.g., a NphT7 polypeptide and/or a NphT7 polypeptide mutant as described herein) to increase production of fatty acids and/or fatty acid chain products.

[0089] In some aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises the amino acid sequence of any one of SEQ ID NOs: 5-15 and corresponds to the polypeptides designated as SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 and Ctet, respectively. In other aspects, the heterologous enzyme having 3-ketoacyl-CoA synthase activity comprises a polypeptide having more than 50%, more than 55%, more than 60%, more than 65%, more than 70%, more than 75%, more than 80%, more than 85%, more than 90%, more than 91%, more than 92%, more than 93%, more than 94%, more than 95%, more than 96%, more than 97%, more than 98%, or more than 99% sequence identity to the amino acid sequence of any one of SEQ ID NOs: 5-15.

[0090] In some aspects, a recombinant cell genetically modified to express any one of the polypeptides designated as SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 or Ctet produces fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 or Ctet further comprises an engineered biosynthetic pathway to produce fatty acids and/or fatty acid chain products. In some aspects, the recombinant cell genetically modified to express the polypeptide designated as SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 or Ctet further comprises an engineered biosynthetic pathway to produce FAME.

[0091] In some aspects, each of the heterologous polypeptides with 3-ketoacyl-CoA synthase activity identified above can comprise one or more identified amino acid substitutions within its amino acid sequence to alter a specific activity against a specific CoA substrate when compared to the respective unsubstituted heterologous polypeptide. These heterologous polypeptides with 3-ketoacyl-CoA synthase activity with identified amino acid substitutions can be considered variants or mutants. For instance, one or more identified amino acid substitutions in the variant can alter the specific activity of the respective heterologous polypeptide with 3-ketoacyl-CoA synthase against a C4-CoA substrate. In other words, the specific activity of the respective variant against a C4-CoA substrate can be increased or decreased when compared to the respective polypeptide with unaltered sequence. Therefore, a variant can comprise increased specific activity for certain CoA substrate and can concomitantly produce a certain 3-keto-CoA intermediate. For example, a variant with increased specific activity for a C6-CoA substrate will produce a 3-keto-C8-CoA product leading to C8 fatty acids and/or C8 fatty acid chain products. Likewise, a variant with decreased specific activity against certain CoA may also be desirable to reduce production of certain unwanted fatty acids and/or fatty acid chain products. For example, a variant with decreased specific activity for a C8-CoA substrate will produce less 3-keto-C10-CoA product leading to reduced amounts of C10 fatty acids and/or C10 fatty acid chain products.

[0092] In some aspects, a recombinant cell with a variant with one or more identified amino acid substitutions can comprise increased total FAME production; in other cases, the variant results in decreased total FAME production compared to the wild-type enzyme. A recombinant cell with a variant with one or more identified amino acid substitutions can also comprise altered ratios of FAME production by carbon chain length when compared to the

respective polypeptide with unaltered sequence. For example, a recombinant cell with a variant with one or more identified amino acid substitutions can comprise altered ratios of FAME production by carbon chain length of one or more of C6-FAME (methyl hexanoate), C8-FAME (methyl octanoate), C10-FAME (methyl decanoate), and C12-FAME (methyl dodecanoate).

[0093] In some aspects, the identified amino acid substitutions can also comprise one or more amino acid substitutions at amino acid positions of Nbra, SKO3, Scin, Sten, SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 and Ctet that align to amino acid positions 147 and/or 217 of Npht7 (see, for example, the sequence alignments in Figs. 1 and 2).

[0094] In some aspects, a Nbra (SV) variant with more than one identified amino acid substitutions can comprise an altered specific activity for C2-CoA, C4-CoA, C6-CoA, and C8-CoA substrates when compared to the respective polypeptide with unaltered sequence. In some aspects, a recombinant cell with a Nbra (SV) variant and an engineered biosynthetic pathway can comprise an increased total FAME production of a mixture of C6-FAME (methyl hexanoate), C8-FAME (methyl octanoate), C10-FAME (methyl decanoate), and C12-FAME (methyl dodecanoate) when compared to the respective polypeptide with unaltered sequence. In some cases, the recombinant cell with a Nbra (SV) variant and an engineered biosynthetic pathway can comprise an increased total FAME production when compared to the respective polypeptide with unaltered sequence. It is noted that the wild-type Nbra with an engineered biosynthetic pathway showed little to no ability to produce FAME.

[0095] In some aspects, a Sten (SV) variant with more than one identified amino acid substitution can comprise an altered specific activity for C2-CoA, C4-CoA, C6-CoA, and C8-CoA substrates when compared to the respective polypeptide with unaltered sequence. In some aspects, a recombinant cell with a Sten (SV) variant can comprise a decrease in total FAME production of a mixture of C6-FAME (methyl hexanoate), C8-FAME (methyl octanoate), C10-FAME (methyl decanoate), and C12-FAME (methyl dodecanoate) when compared to the respective polypeptide with unaltered sequence. In some cases, the recombinant cell with a Sten (SV) variant can comprise a decrease in total FAME production when compared to the respective polypeptide with unaltered sequence. It is noted that a recombinant cell with a Sten (SV) variant produces little to no FAME.

[0096] In some aspects, a SKO3 (SV) variant with more than one identified amino acid substitution can comprise an altered specific activity for C2-CoA, C4-CoA, C6-CoA, and C8-CoA substrates when compared to the respective polypeptide with unaltered sequence. In some

aspects, a recombinant cell with a SKO3 (SV) variant and an engineered biosynthetic pathway can comprise an increased total FAME production of a mixture of C6-FAME (methyl hexanoate), C8-FAME (methyl octanoate), C10-FAME (methyl decanoate), and C12-FAME (methyl dodecanoate) when compared to the respective polypeptide with unaltered sequence. In some cases, the recombinant cell with a SKO3 (SV) variant can comprise an increased total FAME production when compared to the respective polypeptide with unaltered sequence.

[0097] In some aspects, a Scin (SV) variant with more than one identified amino acid substitution can comprise an altered specific activity for C2-CoA, C4-CoA, C6-CoA, and C8-CoA substrates when compared to the respective polypeptide with unaltered sequence. In some aspects, a recombinant cell with a Scin (SV) variant and an engineered biosynthetic pathway can comprise a decreased total FAME production of a mixture of C6-FAME (methyl hexanoate), C8-FAME (methyl octanoate), C10-FAME (methyl decanoate), and C12-FAME (methyl dodecanoate) when compared to the respective polypeptide with unaltered sequence. In some cases, the recombinant cell with a Scin (SV) variant can comprise a decreased total FAME production when compared to the respective polypeptide with unaltered sequence.

[0098] In some aspects, the recombinant cell can comprise more than one heterologous enzyme having 3-ketoacyl-CoA synthase activity. In other aspects, the recombinant cell can comprise one or more of Nbra, SKO3, Scin, Sten, SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 and Ctet, variant(s) corresponding to I147S and/or F217V (NphT7 numbering) of Nbra, SKO3, Scin, Sten, SKO3-2, Ache, Smg1, Safr, SHK1, Svio, ATTC, Ubac, Gsul, Abay-2 and Ctet, and additional enzymes having 3-ketoacyl-CoA synthase activity (e.g., NphT7 (SEQ ID NO: 16), NphT7(SV) variant (SEQ ID NO: 17), and NphT7(LSVA) variant (SEQ ID NO: 33)). In some cases, the recombinant cell can comprise heterologous 3-ketoacyl-CoA synthase enzymes having complementary specific activities. For example, a heterologous NphT7 comprising high specific activity for C2-CoA substrates can be combined with another heterologous 3-ketoacyl-CoA synthase enzyme. Likewise, a heterologous NphT7(LSVA) variant comprising high specific activity for C4-CoA substrates can be combined with another heterologous 3-ketoacyl-CoA synthase enzyme. In other words, heterologous enzymes with certain high specific activities (e.g., for C2-CoA substrates and/or C4-CoA substrates) can be paired with heterologous enzymes having other high specific activities (e.g., for C6-CoA substrates and/or C8-CoA substrates).

[0099] In some aspects, a recombinant cell comprising heterologous Nbra (SV) variant, with and without heterologous NphT7 comprises an increase in total FAME production compared to a recombinant cell comprising heterologous wild-type Nbra, with or without heterologous NphT7.

[0100] In some aspects, a recombinant cell comprising heterologous Sten and heterologous NphT7 comprises increased total FAME production compared to a recombinant cell comprising heterologous Sten and no heterologous NphT7.

[0101] In some aspects, a recombinant cell comprising heterologous Sten (SV) variant and heterologous NphT7 comprises a decrease in total FAME production compared to a recombinant cell comprising heterologous wild-type Sten and heterologous NphT7. In the absence of NphT7, no FAME was produced from a recombinant cell comprising the heterologous Sten (SV) mutant.

[0102] In some aspects, a recombinant cell comprising heterologous SKO3, with and without NphT7, comprises FAME production. In some aspects, a recombinant cell comprising heterologous SKO3 (SV) variant, with and without heterologous NphT7, comprises FAME production.

[0103] In some aspects, a recombinant cell comprising heterologous Scin, with and without NphT7, comprises FAME production. In some aspects, a recombinant cell comprising heterologous Scin (SV) variant, with and without heterologous NphT7, comprises FAME production.

Engineered Biosynthetic Pathway Reaction Steps

[0104] In some aspects, this application discloses a recombinant cell with an engineered biosynthetic pathway comprising one or more heterologous enzymes that convert chemical precursors and/or substrates into desired chemical products. In some cases, the engineered biosynthetic pathway comprises a pathway of heterologous enzymes to synthesize the desired chemical product. In some instances, individual heterologous enzymes work in a stepwise fashion to convert a precursor into the desired chemical product. The engineered biosynthetic pathway can comprise one or more identified heterologous enzymes having 3-ketoacyl-CoA synthase activity to produce fatty acids and fatty acid chain products of C4-C10 chain length. In other words, the engineered biosynthetic pathway can comprise one or more of Nbra, SKO3, Scin, Sten, SKO3-2, Ache, Smg1, Safr, SHK1, Svio AATC, Ubac, Gsul, Abay-2, Ctet, and/or

their respective variants to produce fatty acids and fatty acid chain products of C4-C10 chain length. The engineered biosynthetic pathway can also comprise additional heterologous enzyme(s) that work in combination with one or more of Nbra, SKO3, Scin, Sten, SKO3-2, Ache, Smg1, Safr, SHK1, Svio AATC, Ubac, Gsul, Abay-2, Ctet, and/or their respective variants to produce fatty acids and fatty acid chain products of C4-C10 chain length.

[0105] In some aspects, the recombinant cell comprises one or more of Nbra, SKO3, Scin, Sten, SKO3-2, Ache, Smg1, Safr, SHK1, Svio AATC, Ubac, Gsul, Abay-2, Ctet, and/or their respective variants to catalyze the reaction of acyl-CoA with malonyl-CoA to produce intermediates to produce fatty acids and fatty acid chain products of C4-C10 chain length. However, the reaction of acyl-CoA with malonyl-CoA produces a 3-ketoacyl-CoA compound that must be reduced to the corresponding acyl compound before it can condense with another molecule of malonyl-CoA to extend the chain. The reduction takes place in three steps, the first being the reduction of the 3-ketoacyl group to the corresponding 3-hydroxyacyl group. The second reaction is a dehydration to the corresponding trans-2-enoylacyl compound, which is reduced in a third step to the corresponding acyl-CoA. The first reaction step is enzymatically catalyzed by a keto-CoA reductase (KCR) enzyme (EC 1.1.1.35). The second step is enzymatically catalyzed by a 3-hydroxy-acyl-CoA dehydratase (3HDh) enzyme (EC 4.2.1.17). Some bifunctional enzymes catalyze both of the first and second step reactions (EC 1.1.1.35 and EC 4.2.1.55). The third reaction step is enzymatically catalyzed by an enoyl-CoA reductase (ECR) enzyme (EC 1.1.1.32).

[0106] Accordingly, the engineered biosynthetic pathway can further comprises at least one of (1) a heterologous KCR gene that encodes for a KCR enzyme; (2) a heterologous 3HDh gene that encodes for a 3HDh enzyme; (3) a heterologous gene that encodes for a bifunctional enzyme that catalyzes both of the first and second reaction steps (EC 1.1.1.35 and 4.1.2.55) and (4) a heterologous ECR gene that encodes for an ECR enzyme. In some aspects, the recombinant cell contains at least (1), (2) and (4) or at least (3) and (4). In some aspects, the recombinant cell further comprises (5) at least one heterologous 3-ketoacyl-CoA synthase gene, different from the 3-ketoacyl-CoA synthases described above, which encodes for a 3-ketoacyl-CoA synthase. In each case, the gene can be under the control of a promoter and/or terminator sequences active in the recombinant cell.

[0107] The KCR enzyme may be, for example, one encoded by a *P. aeruginosa fadB* gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99%

or 100% identical to SEQ ID NO: 36, one encoded by a *P. aeruginosa fadG* gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 102, one encoded by a *C. beijerinckii hbd* gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 101, and others as described in WO 2015/010103.

[0108] The 3HDh enzyme may be, for example, one encoded by a *C. acetobutylicum crt* (short-chain-enoyl-CoA hydratase) gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 99, one encoded by a *P. putida ech* (enoyl-CoA hydratase/aldolase) gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 100, and others as described in WO 2015/010103.

[0109] Suitable bifunctional enzymes that catalyze both the first and second reaction steps include, for example, one encoded by an *E. coli fadB* gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 98; one encoded by an *R. novogicus ech2* gene, and others as described in WO 2015/010103.

[0110] Suitable ECR enzymes include, for example, one encoded by a *T. denticola ter* gene and/or having an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 84.

[0111] The recombinant cell can further include at least one heterologous 3-ketobutyryl-CoA synthase gene, different from the modified 3-ketoacyl-CoA synthases described above, which encodes for a 3-ketobutyryl-CoA synthase. The heterologous 3-ketobutyryl-CoA synthase gene may encode for a 3-ketobutyryl-CoA synthase enzyme that is at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to any of those identified as SEQ ID NOs: 1-120 of WO 2015/10103.

[0112] In some aspects, the heterologous 3-ketobutyryl-CoA synthase gene is a *Streptomyces* Sp CL190 gene and/or a gene that encodes for an NphT7 enzyme that is at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 16.

[0113] In some aspects, the recombinant cell includes at least one gene that encodes for a modified NphT7 enzyme as described in WO 2015/10103. The modified NphT7 enzyme comprises an amino acid sequence having at least 70% but less than 100% sequence identity to SEQ ID NO: 16. The modified NphT7 enzyme may have, for example, one or more amino acid substitutions selected from the group consisting of H100L, I147T, F217V, Y144L, V157F,

G309S, G288S, a PDRP to HFLQ substitution for amino acid residues 86-89, I147F, I147M, I147Q, I147S, I147C, I147E, I147N, I147W, I147D, I147R, I147P, I147L, V196G, I147G, I147H, I147K, I147V, I147A, I147Y, F217G, F217A, F217L, F217I, F217M, F217T, F217P, F217S, F217E, F217L, F217V, F217W, S323A and S323V, and any combination of any two or more thereof.

[0114] In some aspects, the modified NphT7 enzyme comprises at least one amino acid substitution selected from the group consisting of I147V, I147S, I147T, and at least one additional amino acid substitution selected from H100L, F217V, S323A and S323V. In some aspects, the modified NphT7 enzyme corresponds to SEQ ID NO: 33. In some aspects, the modified NphT7 enzyme comprises an I147V, I147S or I147T amino acid substitution and an S323A amino acid substitution (corresponding to SEQ ID NO: 33 in which amino acid 100 is H, amino acid 147 is V, S or T, amino acid 217 is F and amino acid 323 is A). In some aspects, the modified NphT7 enzyme comprises an H100L substitution, an I147V, I147S or I147T amino acid substitution, an F217V substitution and an S323A amino acid substitution (corresponding to SEQ ID NO: 33 in which amino acid residue 100 is L, amino acid residue 147 is V, S or T, amino acid residue 217 is V and amino acid residue 323 is A).

[0115] In some aspects, the recombinant cell includes both of (1) a *Streptomyces* Sp CL190 *nphT7* gene and/or a gene that encodes for an NphT7 enzyme that is at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 16 and (2) a modified NphT7 enzyme having one or more amino acid substitutions selected from the group consisting of H100L, I147T, F217V, Y144L, V157F, G309S, G288S, a PDRP to HFLQ substitution for amino acid residues 86-89, I147F, I147M, I147Q, I147S, I147C, I147E, I147N, I147W, I147D, I147R, I147P, I147L, V196G, I147G, I147H, I147K, I147V, I147A, I147Y, F217G, F217A, F217L, F217I, F217M, F217T, F217P, F217S, F217E, F217L, F217V, F217W, S323A and S323V, and any combination of any two or more thereof. In other aspects, the recombinant cell includes a gene that encodes for an enzyme having SEQ ID NO: 16 and another gene that encodes for an enzyme having SEQ ID NO: 33. In some aspects, the recombinant cell includes a gene that encodes for an enzyme having SEQ ID NO: 16 and another gene that encodes for an enzyme having SEQ ID NO: 33 in which amino acid residue 100 is H or L, amino acid residue 147 is S, T or V, amino acid residue 271 is F or V and amino acid residue 323 is A.

[0116] The recombinant cell can further include at least one heterologous 3-ketoacyl-CoA synthase gene, different from the 3-ketoacyl-CoA synthases described above, which

encodes for a 3-ketoacyl-CoA synthase. The heterologous 3-ketoacyl-CoA synthase gene may encode for a 3-ketoacyl-CoA synthase enzyme that is at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to any of those identified as SEQ ID NO: 1-81, 86-93, 97, 105-116, and 119-171 in PCT/US18/16394.

[0117] In some aspects, the 3-ketoacyl-CoA synthase gene is an *Acinetobacter schindleri* CIP 107287 gene (Designation: Asch) and/or a gene that encodes for a 3-ketoacyl-CoA synthase enzyme that is at least 80%, at least 90%, at least 95%, at least 99% or 100% identical to SEQ ID NO: 18-20, 22 and/or 26.

[0118] In some aspects, the recombinant cell includes at least one gene that encodes for a modified Asch enzyme as described in PCT/US18/16394. The modified Asch enzyme comprises an amino acid sequence having at least 70% but less than 100% sequence identity to SEQ ID NO: 26. The modified Asch enzyme may have, for example, one or more amino acid substitutions selected from the group consisting of T184I, F236L, V268A, V296A, V317A, and S328G, and any combination of any two or more thereof. The modified Asch enzyme may have altered specific activities relative to an unmodified Asch enzyme. For example, a modified Asch enzyme may have amino acid substitutions comprising T184I, F236L, V268A, V296A, V317A, and S328G (SEQ ID NO: 19) and may result in C8 FAME as the most abundant product. A modified Asch enzyme may have amino acid substitutions comprising T184I, V296A, and V268A (SEQ ID NO: 22) and may result in a mixture of C8 FAME and C10 FAME as the most abundant products. A modified Asch enzyme may have amino acid substitutions comprising V296A (SEQ ID NO: 18) and may result in C10 FAME as the most abundant product.

[0119] The recombinant cell can produce one or more enzymes that terminate the acyl elongation cycle and produce a product having the desired chain length. Such a termination enzyme may or may not be heterologous. The selection of termination enzyme may depend on whether the desired product is a fatty acid or a derivative thereof such as a fatty alcohol, a fatty aldehyde, a fatty alkene, a fatty amide, a fatty ester or a fatty alkane.

[0120] The recombinant cell in some aspects includes a heterologous thioesterase gene that encodes for a thioesterase such as an acyl-CoA esterase, in which case the product will be a fatty acid. Suitable thioesterases include those described in Table 11 of WO 2015/101013.

[0121] In some aspects the recombinant cell includes a gene that encodes for an ester synthase, in which case the product typically is a fatty acid ester. Suitable ester synthases have amino acid sequences at least 80%, at least 90%, at least 95%, at least 99% or at least 100%

identical to any of the *Marinobacter aquacolei* Maq1 enzyme (SEQ ID NO: 289 of WO 2015/10103), the *Psychrobacter cryohaloentis* Pcry1 enzyme (SEQ ID NO: 290 of WO 2015/10103), the *Rhodococcus jostii* Rjos1 enzyme (SEQ ID NO: 291 of WO 2015/10103), the *Alcanivorax borkumensis* strain SK2 Abork1 enzyme (SEQ ID NO: 292 of WO 2015/10103) and the *Hahella chejuensis* hche gene (SEQ ID NO: 37). The ester synthase may have an amino acid sequence at least 80%, at least 90%, at least 95%, at least 99% or at least 100% identical to the *Hahella chejuensis* Hche ester synthase (SEQ ID NO: 37).

[0122] The recombinant cell may also include one or more genes that encode for one or more of a fatty acyl-CoA reductase (alcohol or aldehyde forming), a fatty aldehyde reductase, an acyl-ACP reductase, an acyl-CoA:ACP acyltransferase, an acyl-CoA hydrolase, a carboxylic acid reductase, an aldehyde dehydrogenase and/or an acyl-ACP reductase.

[0123] The recombinant cell also may include (A) one or more genes that encode for a carboxyl transferase subunit α enzyme, (EC 6.3.1.2) such as an *E. coli* AccA enzyme or an enzyme that is at least 80%, at least 90%, at least 95% or at least 99% identical thereto; (B) one or more genes that encode for a biotin carboxyl carrier protein, (EC 6.4.1.2) such as an *E. coli* AccB enzyme (SEQ ID NO: 29) or an enzyme that is at least 80%, at least 90%, at least 95% or at least 99% identical thereto; (C) one or more genes that encode for a biotin carboxylase subunit enzyme, (EC 6.3.4.14) such as an *E. coli* AccC enzyme (SEQ ID NO: 30) or an enzyme that is at least 80%, at least 90%, at least 95% or at least 99% identical thereto; (D) a carboxyl transferase subunit β (EC 6.4.1.2), such as an *E. coli* AccD enzyme or an enzyme that is at least 80%, at least 90%, at least 95% or at least 99% identical thereto, (E) a fused *E. coli* AccD subunit and AccA subunit enzyme (SEQ ID NO: 28); or a combination of any two or more thereof. In some aspects, all of (A) – (E) are present.

[0124] In some aspects, the recombinant cell described herein further comprises one or more additional genetic modifications to reduce or eliminate the expression of certain endogenous enzymes in the recombinant cell. Reducing or eliminating the expression of these certain endogenous enzymes in the recombinant cell can increase the production of desired products, such as fatty acids and/or fatty acid chain products. These reduced or eliminated endogenous enzymes include one or more of the following enzymes:

Methylglyoxal synthase (EC 4.2.3.3), for example that encoded by the *E. coli* mgsA gene.

Lactate dehydrogenase (EC 1.1.1.27), for example that encoded by the *E. coli* ldhA gene.

Phosphotransacetylase (EC 2.3.1.8), for example that encoded by the *E. coli pta* gene.

Acetate kinase (EC 2.7.2.1), for example that encoded by *E. coli ackA* gene.

Acyl-CoA synthase (EC 6.2.1.3), for example that encoded by the *E. coli fadD* gene.

Pyruvate formate lyase (EC 2.3.1.54), for example that encoded by the *E. coli pflB* gene.

Pyruvate oxidase (EC 1.2.2.2), for example that encoded by the *E. coli poxB* gene.

Fused acetaldehyde-CoA dehydrogenase (EC 1.2.1.10), for example that encoded by the *E. coli adhE* gene.

Trigger factor (EC 5.2.1.8), for example that encoded by the *E. coli tig* gene.

Restriction endonuclease (EC 3.1.21.3), for example that encoded by the *E. coli hsdR514* gene.

The *atoDAEB* operon.

Acyl-CoA thioesterase (EC 3.1.2.-), for example that encoded by the *E. coli tesB* or *yciA* gene.

Acyl-coenzyme A dehydrogenase (EC 1.3.8.7), for example that encoded by the *E. coli fadE* gene.

3-ketoacyl-CoA thiolase (EC 2.3.1.16), for example that encoded by the *E. coli fadA* gene.

L-ribulokinase (EC 2.7.1.16), for example that encoded by the *E. coli araB* gene.

L-ribulose-5-phosphate-4-epimerase (EC 5.1.3.4), for example that encoded by the *E. coli araD* gene.

Beta-D-galactosidase (EC 3.2.1.23), for example that encoded by the *E. coli lacZ* gene.

Lambda phage lysogen.

Rhamnulose-1-phosphate aldolase (EC 4.1.2.19), for example that encoded by the *E. coli rhaD* gene.

Rhamnulokinase (EC 2.7.1.5), for example that encoded by the *E. coli rhaB* gene.

F mating factor.

Truncated RNase PH (EC 2.7.7.56) *Rph-1* gene.

[0125] Other genetic modifications may be present in the recombinant cell, including any of those described in WO 2015/10103.

[0126] Any heterologous gene may be operatively linked to a promoter and/or terminator sequence that is functional in the recombinant strain. The promoter may be an inducible promoter that functions only under certain conditions. For example, a low phosphate inducible

promoter such as the promoter of the wild-type *E. coli phoE* gene (*PphoE*) is a useful promoter for the 3-ketoacyl synthase gene. Such a promoter is active in a low phosphate environment. Accordingly, a recombinant cell in which the 3-ketoacyl synthase gene is under the control of an *E. coli phoE* promoter or another low phosphate inducible promoter may be cultivated in a fermentation medium containing no more than 25 mM phosphate, especially no more than 20 mM, no more than 2 mM, no more than 1mM, no more than 0.5 mM, or no more than 0.25 mM phosphate. In some aspects, the promoter that is a low phosphate inducible promoter is the promoter for the *pstS* gene (*PpstS*). This promoter may be constructed to include a binding site for Integration Host Factor and is thus designated *PpstSIH* (Lyzen et al., Plasmid 60:125 (2008)).

[0127] Any heterologous gene may be integrated into the genome of the recombinant strain and/or present in one or more plasmids. If integrated into the genome, the heterologous gene may be inserted at a targeted or random location. Transformation methods such as electroporation and chemical methods (including calcium chloride and/or lithium acetate methods) known in the art are suitable. Examples of suitable transformation methods are described, for example, in *Molecular Cloning: A Laboratory Manual*, 4th Ed. Spring Harbor Press 2012. In general, no special transformation methods are necessary to produce the recombinant cells.

[0128] Deletions and/or disruptions of native genes can be performed by transformation methods, by mutagenesis and/or by forced evolution methods. In mutagenesis methods, cells are exposed to ultraviolet radiation or a mutagenic substance, under conditions sufficient to achieve a high kill rate (60-99.9%, preferably 90-99.9%) of the cells. Surviving cells are then plated and selected or screened for cells having the deleted or disrupted metabolic activity. Disruption or deletion of the desired native gene(s) can be confirmed through PCR or Southern analysis methods.

[0129] The recombinant cells described herein are used to produce compounds having a straight-chain alkyl group. The recombinant cells are grown under conditions such that they produce such compounds, and the compounds may be recovered.

[0130] When the recombinant cell is a plant cell, the plant can be grown and the compound having the straight-chain alkyl group can be recovered from the plant or any portion thereof, such as roots, stems, leaves, flowers, seeds, seed pods and the like, in which the compound accumulates during the growth of the plant.

[0131] Single-cell and other microcells can be used in a culturing process to produce such compounds.

[0132] Culturing is performed generally by forming a culture medium that includes at least one carbon source that is capable of being metabolized by the recombinant cell to produce the product compounds and nutrients as may be required by the specific recombinant cell. The nutrients may include, for example, at least one nitrogen source such as yeast extract, peptone, tryptone, soy flour, corn steep liquor, or casein, at least one phosphorus source, one or more vitamins such as biotin, vitamin B12 and derivatives of vitamin B12, thiamin, pantothenate, one or more trace metals and the like. The fermentation medium may also contain additional materials such as anti-foam agents, biocides, buffers and the like.

[0133] In some cases, such as the production of fatty acid esters, the culture medium may also include a reagent that reacts with the straight-chain compound to produce the desired product. In the specific case of fatty acid esters, for example the culture medium preferably contains an alkanol such as methanol, ethanol or a C3-C8 alkanol. The alkanol reacts to produce the corresponding ester. A native or heterologous ester synthase, or other appropriate enzyme, may be expressed by the recombinant cell to catalyze such a reaction.

[0134] Generally, the culture medium is inoculated with the recombinant cell, and the inoculum is cultured in the medium so that the cell density increases to a cell density suitable for production. The culture medium is then maintained at conditions sufficient for the recombinant cells to produce the desired product.

[0135] Suitable culture conditions will of course depend on the requirements of the particular recombinant strain. The temperature of the culture medium may be, for example from 20°C to 70°C, with a temperature of 25 to 40°C being preferred for most recombinant cells.

[0136] The pH of the culture medium may be, for example, from 2.0 to 10.0, from 3.0 to 9.0 or from 6.0 to pH 8.5.

[0137] It is contemplated that the described aspects may be practiced using either batch, fed-batch or continuous processes and that any known mode of bio-production would be suitable.

[0138] The culturing may be performed under aerobic, microaerobic, or anaerobic conditions, as required or can be tolerated by the particular recombinant cell. Generally, no special culturing equipment is needed to perform the fermentation. The equipment may include, for example, a tank suitable for holding the recombinant cell and the culture medium; a line for

discharging contents from the culture tank to an extraction and/or separation vessel; and an extraction and/or separation vessel suitable for removal of the chemical product from cell culture waste.

[0139] The carbon source is one or more carbon-containing compounds that can be metabolized by the recombinant cell as a source of carbon. Examples of suitable carbon sources include sugars such as glucose, sucrose, fructose, lactose, C-5 sugars such as xylose and arabinose, glycerol and polysaccharides such as starch and cellulose. Other suitable carbon sources include fermentable sugars as may be obtained from cellulosic and lignocellulosic biomass through processes of pretreatment and saccharification, as described, for example, in U.S. Patent Publication No. 2007/0031918A1, hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Other suitable carbon sources include high-fructose corn syrup, cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Still other suitable carbon sources include carbon dioxide, carbon monoxide, methanol, methylamine and glucosamine.

[0140] The culturing process may be continued until a titer of the desired product reaches at least 0.01, at least 0.05, at least 0.1, at least 0.25, at least 0.5 or at least 1 g per liter of culture medium (g/L). The fermentation process may be continued until the titer reaches, for example, up to 40, up to 45, up to 50, up to 80, up to 100, or up to 120 g/L. The specific productivity may be, for example, from 0.01 and 0.60 grams of the desired product per gram of cells on a dry weight basis per hour (g chemical product/g DCW-hr). The volumetric productivity achieved may be at least 0.005 g of the desired product per liter per hour (g/L-hr), at least 0.01 g/L-hr, at least 0.1 g/L-hr or at least 0.5 g/L-hr, and may be up to, for example, 10 g/L-hr, up to 5 g/L-hr or up to 1 g/L-hr.

[0141] In some aspects, specific productivity as measured over a 24-hour fermentation (culture) period may be greater than about 0.01, 0.05, 0.10, 0.20, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 or 12.0 grams of chemical product per gram DCW of cells (based on the final DCW at the end of the 24-hour period).

EXAMPLES

[0142] The following examples are provided to illustrate the disclosure, but are not intended to limit the scope thereof. All parts and percentages are by weight unless otherwise indicated.

Overview

[0143] The current FAME pathway takes acetyl-CoA and condenses it with a malonyl-CoA to create acetoacetyl-CoA via the enzyme NphT7. After subsequent keto reduction, dehydration and enoyl reduction, the saturated C4-CoA is then reacted with malonyl-CoA to create a C6-CoA species that also undergoes iterative keto reduction, dehydration and enoyl reduction to make a saturated C6-CoA. The enzyme catalyzing this second reaction is also NphT7, but a variant that has been engineered to accept the saturated C4-CoA by mutagenesis, called NphT7(LSVA). Together these two enzymes can initiate FAME biosynthesis, however only the LSVA variant is needed for FAME production. In this sense, it is necessary for only one version of NphT7 to be present, NphT7(LSVA), since this variant can extend from a C2-CoA to a C6-CoA molecule, whereas the wild-type enzyme can only extend from C2-CoA to C4-CoA molecule. The pathway typically benefits from expression of wild type NphT7, however it is not required. In addition to NphT7 and/or NphT7(LSVA), the current FAME pathway benefits from an additional 3-ketoacyl-CoA synthase, provided by heterologous Asch or one of its variants engineered for increased chain length specificity compared to wildtype Asch.

[0144] Provided herein are enzymes with similar activity to NphT7, but with different substrate specificities.

[0145] Also provided herein are enzymes which can natively catalyze the C2-CoA to C6-CoA reaction without modification (Sten, Sko3, Scin, plus others that show *in vitro* activity on C2-CoA and C4-CoA), which is a function not seen in wild type NphT7.

[0146] A subset of the same mutations introduced into NphT7 that broaden the substrate specificity and extend the catalytic activity from C2-CoA to C6-CoA can also be introduced into the enzymes described herein. These two mutations (called SV) enable some enzymes that otherwise show no ability to produce FAME to now produce FAME in the context of the pathway, and in some cases, behave similarly to NphT7(LSVA), an example of such is provided by Nbra. Thus, the SV mutations are what allow the enzymes to function in the FAME pathway. However, not all of the enzymes behave in this fashion, for example, wild type Sten produces some FAME; however, as a SV mutant it does not.

[0147] Also described herein are enzymes that have the highest activity on C2-CoA, as well as specific enzymes that have the highest activity on C4-CoA.

[0148] Further described are the SV mutations to change the substrate specificity of the enzymes. Although enzymes can have activity on both substrates, C2-CoA and C4-CoA, the mutations can change the relative activity on each substrate and as a consequence influence total FAME production (and the amounts of each individual FAME that is produced).

Example 1.

[0149] The following recombinant *E. coli* strains were used in the following examples as indicated.

[0150] Recombinant Strain 1 is a mutant of the *E. coli* strain designated BW25113, available from the *E. coli* Genetic Strain Center (CGSC#7636; Dept. of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut), having the following additional genetic modifications:

Designation	Description
$\Delta ldhA::frt$	Deletion of native lactate dehydrogenase
$\Delta pflB::frt$	Deletion of native pyruvate formate lyase
$\Delta mgsA::frt$	Deletion of methylglyoxal synthase
$\Delta poxB::frt$	Deletion of pyruvate oxidase
$\Delta pta-ack::frt$	Deletion of phosphotransacetylase and acetate kinase
$\Delta tig::frt$	Deletion of trigger factor protein
$\Delta atoDAEB::frt$	Deletion to disrupt short-chain poly-(R)-3-hydroxybutyrate synthesis
$\Delta fadD::frt$	Deletion of native acyl-CoA synthetase
$\Delta tesB::frt$	Deletion of native thioesterase
$\Delta yciA::frt$	Deletion of native thioesterase
$\Delta adhE$	Deletion of native aldehyde-alcohol dehydrogenase
$P_{pstSIH}-nphT7-ter-TT-loxP$	Insertion of a gene encoding for <i>Streptomyces</i> Sp. CL190 acetoacetyl-CoA synthase (NphT7, SEQ ID NO: 16) and a gene encoding for <i>Treponema denticola</i> enoyl-CoA reductase (ter, SEQ ID NO: 34) under control of <i>E. coli</i> pstSIH promoter and an <i>E. coli</i> terminator at locus of native <i>adhE</i> gene

[0151] Recombinant Strain 2 is a mutant of the BW25113 *E. coli* strain with the following genetic modifications:

Designation	Description
$\Delta ldhA::frt$	Deletion of native lactate dehydrogenase
$\Delta pflB::frt$	Deletion of native pyruvate formate lyase
$\Delta mgsA::frt$	Deletion of methylglyoxal synthase
$\Delta poxB::frt$	Deletion of pyruvate oxidase
$\Delta pta-ack::frt$	Deletion of phosphotransacetylase and acetate kinase
$\Delta tig::frt$	Deletion of trigger factor protein

$\Delta atoDAEB::frt$	Deletion to disrupt short-chain poly-(<i>R</i>)-3-hydroxybutyrate synthesis
$\Delta fadD::frt$	Deletion of native acyl-CoA synthetase
$\Delta tesB::frt$	Deletion of native thioesterase
$\Delta yciA::frt$	Deletion of native thioesterase

[0152] Recombinant Strain 3 is a mutant of the BW25113 *E. coli* strain with the following genetic modifications:

Designation	Description
$\Delta ldhA::frt$	Deletion of native lactate dehydrogenase
$\Delta pfkB::frt$	Deletion of native pyruvate formate lyase
$\Delta mgsA::frt$	Deletion of methylglyoxal synthase
$\Delta poxB::frt$	Deletion of pyruvate oxidase
$\Delta pta-ack::frt$	Deletion of phosphotransacetylase and acetate kinase
$\Delta tig::frt$	Deletion of trigger factor protein
$\Delta atoDAEB::frt$	Deletion to disrupt short-chain poly-(<i>R</i>)-3-hydroxybutyrate synthesis
$\Delta fadD::frt$	Deletion of native acyl-CoA synthetase
$\Delta tesB::frt$	Deletion of native thioesterase
$\Delta yciA::frt$	Deletion of native thioesterase
<i>fabI</i> (ts)	Insertion of modified <i>E. coli fabI</i> gene encoding an enzyme having SEQ ID NO. 35, with the serine at position 241 replaced by phenylalanine (S241F modification), and deletion of the wildtype <i>fabI</i> gene
$\Delta adhE$	Deletion of native aldehyde-alcohol dehydrogenase
$P_{pstSIH-nphT7-ter-TT-loxP}$	Insertion of a gene encoding for <i>Streptomyces</i> Sp. CL190 acetoacetyl-CoA synthase (NphT7, SEQ ID NO: 16) and a gene encoding for <i>Treponema denticola</i> enoyl-CoA reductase (ter, SEQ ID NO: 34) under control of <i>E. coli</i> pstSIH promoter and an <i>E. coli</i> terminator at locus of native <i>adhE</i> gene

[0153] Recombinant Strain 4 is a mutant of the BW25113 *E. coli* strain with the following genetic modifications:

Designation	Description
$\Delta ldhA::frt$	Deletion of native lactate dehydrogenase
$\Delta pfkB::frt$	Deletion of native pyruvate formate lyase
$\Delta mgsA::frt$	Deletion of methylglyoxal synthase
$\Delta poxB::frt$	Deletion of pyruvate oxidase
$\Delta pta-ack::frt$	Deletion of phosphotransacetylase and acetate kinase
$\Delta tig::frt$	Deletion of trigger factor protein
$\Delta atoDAEB::frt$	Deletion to disrupt short-chain poly-(<i>R</i>)-3-hydroxybutyrate synthesis
$\Delta fadD::frt$	Deletion of native acyl-CoA synthetase
$\Delta tesB::frt$	Deletion of native thioesterase
$\Delta yciA::frt$	Deletion of native thioesterase

<i>fabI</i> (ts)	Insertion of modified <i>E. coli fabI</i> gene encoding an enzyme having SEQ ID NO. 35, with the serine at position 241 replaced by phenylalanine (S241F modification), and deletion of the wildtype <i>fabI</i> gene
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[0154] The following recombinant plasmids were used in the following examples as indicated.

[0155] Mutant *E. coli* strains are prepared using standard electroporation methods. In each case, the host strain is transformed with a “Type 1” plasmid and a “Type 2” plasmid as described below.

[0156] Type 1 plasmids are pACYC plasmids containing the p15a origin of replication and a chloramphenicol resistance marker:

[0157] Type 1A: This plasmid includes a gene encoding for a 3-ketoacyl-CoA synthase or a mutated variant. The 3-ketoacyl-CoA synthase genes are as indicated in the specific examples below. This plasmid includes an *E. coli* bifunctional 3-hydroxyacyl-CoA dehydrogenase/dehydratase (*fadB*) gene (SEQ. ID NO. 36) and a *T. denticola* enoyl-CoA (*ter*) gene (SEQ. ID NO. 34) cassette, all under a native *E. coli* pstSIH promoter (SEQ. ID NO. 39) and a native *E. coli* terminator. This plasmid also contains a *Hahella chejuensis* ester synthase gene (SEQ. ID NO. 37) fused to a DNA sequence encoding a protein fragment containing 6 histidine residues and a protease recognition site under an *E. coli* phoE promoter (SEQ. ID NO. 38), and an ACC (acetyl-CoA carboxylase) cassette including fused *E. coli accA* and *accD* genes with a *E. coli tpiA* promoter (SEQ ID NO: 31) and a cassette including the *E. coli accB* and *E. coli accC* genes under an *E. coli rpiA* promoter (SEQ ID NO: 32).

[0158] Type 1B: This plasmid includes a gene encoding for a 3-ketoacyl-CoA synthase or a mutated variant. The 3-ketoacyl-CoA synthase genes are as indicated in the specific examples below. This plasmid also includes an *E. coli* bifunctional 3-hydroxyacyl-CoA dehydrogenase/dehydratase (*fadB*) gene (SEQ ID NO: 36) and a *T. denticola* enoyl-CoA (*ter*) gene (SEQ ID NO: 34) cassette, all under a native *E. coli* pstSIH promoter (SEQ ID NO: 39) and a native *E. coli* terminator.

[0159] Type 2 plasmids are pET Plasmids containing a ColE1 origin of replication and a kanamycin resistance marker:

[0160] Type 2A: This plasmid includes a gene encoding Asch or a variant of Asch with various ratios of C8 and C10 FAME production under an *E. coli* phoE promoter (SEQ ID NO: 38) and an *E. coli* terminator. The Asch genes are as indicated in the specific examples below.

[0161] Type 2B: This plasmid includes a gene encoding Asch or a variant of Asch with various ratios of C8 and C10 FAME production fused to a DNA sequence encoding a protein fragment containing 6 histidine residues and a protease recognition site. The Asch genes are as indicated in the specific examples below. Additionally, this plasmid also contains a *Hahella chejuensis* ester synthase gene (SEQ ID NO: 37) fused to a DNA sequence encoding a protein fragment containing 6 histidine residues and a protease recognition site. Both genes are under an *E. coli* phoE promoter (SEQ ID NO: 38).

[0162] 3-ketoacyl-CoA synthase genes are synthesized based on published polypeptide sequence information for various wild type enzymes, and using codons optimized for expression in *E. coli*. Site-specific mutants of the synthesized 3-ketoacyl-CoA synthase genes are generated by oligonucleotide-directed mutagenesis. The sources of the wild-type genes, the short-hand designations used herein for each of them, and the amino acid sequence of the native enzyme produced by the wild-type genes are as follows:

Source Species/description	GenInfo Identifier (GI)	Designation	Enzyme Encoded (wild type)
<i>Nocardia brasiliensis</i> ATCC 700358/ 3-oxoacyl-ACP synthase	407647804	Nbra	SEQ ID NO: 1
<i>Streptomyces</i> sp. KO-3988/ putative 3-oxoacyl-[acyl-carrier-protein]	57753876	SKO3	SEQ ID NO: 2
<i>Streptomyces cinnamonensis</i> / putative 3-oxoacyl-[acyl-carrier-protein] synthase	312190954	Scin	SEQ ID NO: 3
<i>Streptomyces tendae</i> / putative acetoacetyl-CoA synthase	405779345	Sten	SEQ ID NO: 4
<i>Streptomyces</i> sp. KO-3988/ putative 3-oxoacyl-[acyl-carrier-protein]	86475806	SKO3-2	SEQ ID NO: 5
<i>Austwickia chelonae</i> NBRC 105200/ 3-oxoacyl-[acyl-carrier-protein] synthase III	403737765	Ache	SEQ ID NO: 6
<i>Streptomyces</i> sp. Mg1/ 3-oxoacyl-(acyl-carrier-protein) synthase III	254387377	Smg1	SEQ ID NO: 7
<i>Spirochaeta africana</i> DSM 8902/ 3-oxoacyl-(acyl-carrier-protein) synthase III	383789487	Safr	SEQ ID NO: 8
<i>Streptomyces</i> sp. HKI0576/ 3-oxoacyl-(acyl-carrier-protein)-synthase III	359329645	SHK1	SEQ ID NO: 9
<i>Streptomyces violaceusniger</i> Tu 4113/ 3-oxoacyl-ACP synthase	345011378	Svio	SEQ ID NO: 10

Amycolatopsis sp. ATCC 39116/ 3-oxoacyl-ACP synthase	385679747	AATC	SEQ ID NO: 11
hypothetical protein ACD_77C00103G0010 [uncultured bacterium]	406885125	Ubac	SEQ ID NO: 12
Geobacter sulfurreducens PCA/ 3-oxoacyl-ACP synthase	400756529	Gsul	SEQ ID NO: 13
Acinetobacter baylyi DSM 14961 = CIP 107474/ hypothetical protein F952_01898	480012478	Abay-2	SEQ ID NO: 14
Clostridium tetani E88/ 3-oxoacyl-ACP synthase	28209898	Ctet	SEQ ID NO: 15

*GenInfo Identifier (GI) refers to sequence identification numbers used by the GenBank genetic sequence database at the National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, Maryland, USA.

[0163] Mutations to the amino acid residues encoded by the wild-type genes are designated herein by the shorthand designation for the wild-type enzyme, followed in parentheses by a 3-, 4- or 5 character code consisting of a first letter designating the amino acid residue in the native enzyme, a 1-, 2- or 3-digit number indicating the position of that amino acid residue in the native enzyme, and a final letter designating the amino acid residue in that position in the mutated enzyme. Alternatively, mutations to the amino acid residues encoded by the wild-type genes can also be designated herein by the shorthand designation for the wild-type enzyme, followed in parenthesis by the final letter or letters designating the amino acid residue in that was mutated. The single-letter designations are IUPAC amino acid abbreviations as reported, for example, at *Eur. J. Biochem.* 138:9-37(1984).

[0164] Production of recombinant *E. coli* strains:

[0165] Recombinant *E. coli* strains are prepared using standard electroporation methods. In each case, one of the above described recombinant strains (e.g. Recombinant Strain 1, Recombinant Strain 2, Recombinant Strain 3 or Recombinant Strain 4) is transformed with a "Type 1" plasmid (e.g. Type 1A or Type 1B) and an above-described "Type 2" plasmid (e.g. Type 2A or Type 2B). For each Type 2 plasmid, the 3-ketoacyl-CoA synthase and the promoter for the 3-ketoacyl-CoA synthase gene are as indicated in the specific examples below.

[0166] Small scale fermentation method - shake flask protocol

[0167] A culture of synthetic medium containing salts, glucose, NH₄Cl, and supplemented with vitamins, yeast extract, 35 µg/ml kanamycin and 20 µg/ml chloramphenicol is inoculated with the strain to be tested and grown overnight at 32°C. The OD₆₀₀ of a 1:10 dilution of this culture is determined in order to inoculate a Seed 2 flask to a final OD₆₀₀ of 0.3.

Seed 2 flasks contain 25-30 ml synthetic medium containing salts, glucose, NH₄Cl, and is supplemented with vitamins, yeast extract, 35 µg/ml kanamycin, 20 µg/ml chloramphenicol, and 0-2% methanol. Seed 2 flasks are incubated at 32°C for 6-7 hours.

[0168] The OD₆₀₀ of a 1:10 dilution of this culture is determined in order to inoculate a production flask to a final OD₆₀₀ of 0.01-0.025. Production flasks contain 25 ml synthetic medium containing salts, glucose, NH₄Cl, and supplemented with vitamins, yeast extract, 35 µg/ml kanamycin, 20 µg/ml chloramphenicol, 1.25-2.5 mM phosphate, 0-2% methanol, 3-6g/l glucose, and 15-40 g/l glycerol. This synthetic medium functions as a limited phosphate medium that promotes the activity of a low phosphate inducible promoter such as the *E. coli* phoE promoter (SEQ. ID NO. 38) or *E. coli* pstSIH promoter (SEQ ID NO: 39). Production flasks are incubated at 32°C. When phosphate is depleted the following additions are made: 2 ml methyl tetradecanoate, 1 ml 12.5% Polysorbate 80 (sold under the trademark name Tween 80), 1-1.25 ml 50% glycerol. Methanol (0-0.5 ml) is added at the beginning of the production assay or right after phosphate depletion. Additional methanol can be added after phosphate depletion to compensate for methanol evaporation. Flasks are incubated at 35°C for a further 24 hours. Samples are taken at 24 hours and extracted with 0.1 % HCl in MTBE (Methyl tert butyl ether). The extracts are analyzed for fatty acid esters by gas chromatography.

[0169] *In vitro* assay for specific activity against different chain length primers

[0170] *In vitro* assays are used to determine a specific activity of a 3-ketoacyl-CoA synthase for different chain length primers. In some aspects, the assay can be used to determine the specific activity of a particular 3-ketoacyl-CoA synthase for a particular chain length primer. For example, the assay can be carried out to test for specific activity for a C6-CoA primer by reacting a C6-CoA primer and malonyl-CoA with a particular 3-ketoacyl-CoA synthase. Magnesium chloride is added to the reaction mixture and complexes between the resulting 3-ketoC8-CoA and magnesium ion are formed. The reaction progress can be monitored by detecting these 3-ketoC8-CoA and magnesium ion complexes at a UV absorbance of 303 nm. These *in vitro* assays can be performed with cell lysates of cells expressing a 3-ketoacyl-CoA synthase and/or an isolated 3-ketoacyl-CoA synthase.

[0171] The presence of 3-ketoC8-CoA products indicates that the assayed 3-ketoacyl-CoA synthase has specific activity for C6-CoA primers and that the assayed 3-ketoacyl-CoA synthase has 3-ketoC8-CoA synthase activity. The specific activity for other chain length primers can be assayed in similar fashion by substituting a different chain length CoA primer.

For example, by substituting a C2-CoA primer, the specific activity for C2-CoA primers can be assayed and the 3-ketoC4-CoA synthase activity can be determined. By substituting a C4-CoA primer, the specific activity for C4-CoA primers can be assayed and the 3-ketoC6-CoA synthase activity can be determined. By substituting a C6-CoA primer, the specific activity for C6-CoA primers can be assayed and the 3-ketoC8-CoA synthase activity can be determined. By substituting a C8-CoA primer, the specific activity for C8-CoA primers can be assayed and the 3-ketoC10-CoA synthase activity can be determined.

[0172] In some aspects, the *in vitro* assay is carried out on isolated 3-ketoacyl-CoA synthase. The 3-ketoacyl-CoA synthase can be isolated by methods known in the art. For example, the 3-ketoacyl-CoA synthase can be overexpressed as a fusion polypeptide with an affinity tag such as a poly-histidine tag and then purified by immobilized metal affinity chromatography. The *in vitro* assay is then carried out on this isolated 3-ketoacyl-CoA synthase by preparing a reaction mixture of CoA primer (0.3 mM), malonyl-CoA (0.3 mM) and 5 mM MgCl₂ in a buffer of 50 mM Tris, pH 8.0. The reaction is initiated by adding the isolated 3-ketoacyl-CoA synthase at various concentrations. As described above, the progress of the reaction can be monitored by detecting 3-ketoCX-CoA and magnesium ion complexes at a UV absorbance of 303 nm, where X represents the chain length of the resulting product. The CoA primer is selected from C2-CoA, C4-CoA, C6-CoA, or C8-CoA. The reactions can be carried out in 96-well microtiter plates.

[0173] The following table shows the *in vitro* specific activity of purified enzymes. Threshold for activity was 0.05 U/mg.

Name Abrev.	C2-CoA Activity (U/mg)	C4-CoA Activity (U/mg)	C6-CoA Activity (U/mg)	C8-CoA Activity (U/mg)
NphT7	5.52	0.00	0.01	0.00
NphT7(LSVA)	5.47	3.03	0.27	0.06
NphT7(SV)	0.86	1.87	0.08	0.07
Nbra	40.16	0.96	0.84	0.63
SKO3	26.51	0.37	0.29	0.18
Scin	23.28	0.53	0.21	0.29
SKO3-2	16.68	0.85	0.46	0.45
Sten	15.39	0.50	0.33	0.22
Ache	4.98	0.39	0.38	0.20
SMgl	4.67	1.36	0.11	0.08
Svio	2.67	0.06	0.03	0.02
AATC	2.47	0.05	0.05	0.04

Safr	1.44	0.22	0.24	0.07
Ubac	1.38	0.08	0.09	0.04
SHK1	0.19	0.65	0.03	0.01
Gsul	0.10	0.15	0.04	0.01
Abay-2	0.09	0.09	0.28	0.64
Ctet	0.05	0.09	0.03	0.01

[0174] These *in vitro* assay results indicate there exists a wide diversity of specific activities and acyl-CoA substrate preferences in these enzymes annotated as 3-ketoacyl-CoA synthases.

Example 2.

[0175] An amino acid sequence alignment of the 3-ketoacyl-CoA synthase enzymes, NphT7 (SEQ ID NO: 16), Sko3 (SEQ ID NO: 2), Scin (SEQ ID NO: 3), Sten (SEQ ID NO: 4), Nbra (SEQ ID NO: 1), FABH_ECOLI (SEQ ID NO: 21), was carried out with the Clone Manager software package (Scientific & Educational Software, Denver, Colorado). The sequence alignment was performed as a global protein sequence alignment with the Scoring Matrix BLOSUM62 and using NphT7 (SEQ ID NO: 16) as the reference sequence (FIG. 1). In the alignment in Figure 1, the SV mutations are marked with the boxes; using the NphT7 numbering, the SV mutations are I147S and F217V.

[0176] An amino acid sequence alignment of the 3-ketoacyl-CoA synthase enzymes, Gsul (SEQ ID NO: 13), SHK1 (SEQ ID NO: 9), Svio (SEQ ID NO: 10), Nbra (SEQ ID NO: 1), Sten (SEQ ID NO: 4), SKO3-2 (SEQ ID NO: 5), Scin (SEQ ID NO: 3), Sko3 (SEQ ID NO: 2), NphT7 (SEQ ID NO: 16), Ache (SEQ ID NO: 6), Smg1 (SEQ ID NO: 7), Safr (SEQ ID NO: 8), FABH_ECOLI (SEQ ID NO: 21), Ctet (SEQ ID NO: 15), Ubac (SEQ ID NO: 12), Abay-2 (SEQ ID NO: 14) was carried out with the Clone Manager software package (Scientific & Educational Software, Denver, Colorado). The sequence alignment was performed as a global protein sequence alignment with the Scoring Matrix BLOSUM62. In the alignment in Figure 2, the SV mutations are marked with the boxes; using the NphT7 numbering, the SV mutations are I147S and F217V.

[0177] FIGS. 1 and 2 show graphical views of the sequence alignment. Each line corresponds to a 3-ketoacyl-CoA synthase enzyme as indicated by the designation. The amino acid position of the first residue of each row is indicated.

Example 3

[0178] The following table and FIG. 3 show the FAME production by NphT7 and its mutants SV and LSVA in a C10 FAME production pathway (the production strain has an Asch gene that produces mainly C10 FAME). This data was generated using recombinant strains 3 and 4 (for + or - chromosomal integration of *nphT7*). These recombinant strains contain plasmid pair type 1B and 2B, where plasmids 1B have *nphT7* variants as indicated in the table and plasmid 2B is the same for all strains (this plasmid 2B contains Asch wild type with a His tag).

Chromosomal <i>nphT7</i>	Plasmid <i>nphT7</i> variant	Total FAME	Methyl Hexanoate (C6) g/L	Methyl Octanoate (C8) g/L	Methyl Decanoate (C10) g/L	Methyl Dodecanoate (C12) g/L
+	<i>nphT7</i> wt	0.13	0.04	0.00	0.03	0.06
+	<i>nphT7</i> (SV)	1.78	0.72	0.07	0.92	0.06
+	<i>nphT7</i> (LSVA)	1.38	0.47	0.05	0.79	0.06
-	<i>nphT7</i> wt	0.08	0.00	0.00	0.01	0.06
-	<i>nphT7</i> (SV)	1.22	0.26	0.04	0.85	0.07
-	<i>nphT7</i> (LSVA)	1.52	0.46	0.04	0.95	0.07

[0179] This data shows that NphT7 wild type in combination with an additional 3-ketoacyl-CoA synthase such as an Asch variant is not sufficient to produce FAME of C6 or longer chain length. However, a mutant of NphT7, such as SV or LSVA, in combination with an Asch variant, produces FAME.

Example 4.

[0180] The table below and FIG. 4 show how the wild type enzymes are different than NphT7. This data shows the wild type homologs in a clean background using recombinant strain 2 which does not have a chromosomal integration of *nphT7*. The recombinant strain contains plasmid pairs 1A and 2A. Plasmids 2A contain the Asch variants that will determine the main FAME species as indicated in the table. For C10 FAME production Asch(V296A) (SEQ ID NO: 20) was used; for C8 FAME production Asch(T184I,F236L,V268A,V296A,V317A,S328G) (SEQ ID NO: 19) was used; for mixed C8/C10 FAME products Asch(T184I) (SEQ ID NO: 20) was used. Plasmids 1A vary based on the NphT7 homologs evaluated, as indicated in the table. As indicated in example 3, similar recombinant strains containing the wild type NphT7 do not produce FAME.

Chromosomal NphT7	Asch target product	WT genes	Total FAME	Methyl Hexanoate (C6) g/L	Methyl Octanoate (C8) g/L	Methyl Decanoate (C10) g/L	Methyl Dodecanoate (C12) g/L
-	C8 FAME	Nbra	0.15	0.03	0.06	0.00	0.06
-	C8 FAME	Sten	0.59	0.00	0.39	0.14	0.06
-	C8 FAME	Sko3	2.47	0.16	2.07	0.18	0.06
-	C8 FAME	Scin	1.89	0.08	1.60	0.15	0.06
-	C10 FAME	Nbra	0.11	0.04	0.00	0.01	0.06
-	C10 FAME	Sten	0.89	0.01	0.00	0.81	0.07
-	C10 FAME	Sko3	2.27	0.37	0.03	1.80	0.06
-	C10 FAME	Scin	2.17	0.28	0.02	1.80	0.06
-	C8/C10 FAME	Nbra	0.23	0.13	0.04	0.00	0.06
-	C8/C10 FAME	Sten	0.95	0.00	0.19	0.69	0.07
-	C8/C10 FAME	Sko3	2.64	0.19	1.53	0.86	0.06
-	C8/C10 FAME	Scin	2.09	0.13	1.09	0.81	0.06

[0181] This data shows that unlike wild type NphT7, three of these wild type enzymes are able to support FAME production (Sten, Sko3, Scin) in combination with an additional 3-ketoacyl-CoA synthase such as an Asch variant. Unexpectedly, wildtype Nbra does not support production of significant amounts of FAME even though the *in vitro* assay results shown in Example 1 would indicate that, unlike wild-type NphT7, wildtype Nbra should be capable of supporting FAME production.

Example 5.

[0182] The following examples show FAME production by strains expressing the wild-type genes or their corresponding SV mutant variants. Variants were evaluated in recombinant strains 1 (with a chromosomal *nphT7* integration) and 2 (no *nphT7* integration in the chromosome). The homologs or the control NphT7(LSVA) were expressed in a plasmid type 1A and were paired with plasmids type 2A containing variant Asch genes that preferentially produce C10 FAME, C8 FAME, or mixed C10 and C8 FAME as indicated in the table, all these without a His tag. For C10 FAME production Asch(V296A) (SEQ ID NO: 18) was used; for C8 FAME production Asch(T184I,F236L,V268A,V296A,V317A,S328G) (SEQ ID NO: 19) was used; for mixed C8/C10 FAME products Asch(T184I) (SEQ ID NO: 20) was used.

a. Nbra/Nbra(SV) (FIG. 5)

Chromosomal NphT7	Asch target product	variant	Total FAME	Methyl Hexanoate (C6) g/L	Methyl Octanoate (C8) g/L	Methyl Decanoate (C10) g/L	Methyl Dodecanoate (C12) g/L
+	C8 FAME	Nbra	0.17	0.05	0.06	0.00	0.06
+	C8 FAME	Nbra(SV)	2.59	0.07	2.04	0.40	0.07
+	C8 FAME	nphT7(LSVA)	3.59	0.36	2.94	0.23	0.06
-	C8 FAME	Nbra	0.15	0.03	0.06	0.00	0.06
-	C8 FAME	Nbra(SV)	0.98	0.00	0.68	0.23	0.07
-	C8 FAME	nphT7(LSVA)	1.82	0.10	1.44	0.21	0.07
+	C10 FAME	Nbra	0.12	0.04	0.00	0.02	0.06
+	C10 FAME	Nbra(SV)	2.92	0.26	0.02	2.58	0.07
+	C10 FAME	nphT7(LSVA)	2.98	0.55	0.03	2.33	0.07
-	C10 FAME	Nbra	0.11	0.04	0.00	0.01	0.06
-	C10 FAME	Nbra(SV)	1.49	0.07	0.00	1.36	0.07
-	C10 FAME	nphT7(LSVA)	1.91	0.13	0.00	1.70	0.07

+	C8/C10 FAME	Nbra	0.18	0.08	0.04	0.00	0.06
+	C8/C10 FAME	Nbra(SV)	3.08	0.11	1.27	1.62	0.07
+	C8/C10 FAME	nphT7(LSVA)	2.80	0.25	1.59	0.88	0.07
-	C8/C10 FAME	Nbra	0.23	0.13	0.04	0.00	0.06
-	C8/C10 FAME	Nbra(SV)	1.66	0.03	0.40	1.16	0.07
-	C8/C10 FAME	nphT7(LSVA)	3.52	0.19	1.65	1.61	0.07

[0183] The wild type Nbra showed no ability to support FAME production in either background with or without NphT7; however the Nbra (SV) showed the ability to produce FAME in both backgrounds. When combined with different Asch variants that preferentially produce C10 FAME, C8 FAME, or mixed C10 and C8 FAME, Nbra(SV) performed similarly to NphT7(LSVA). These results show that the SV mutations in Nbra enable the production of C6-C10 FAME, similar to the NphT7(LSVA).

b. Sten/Sten(SV) (FIG. 6)

Chromo- somal NphT7	Asch target product	variant	Total FAME	Methyl Hexanoate (C6) g/L	Methyl Octanoate (C8) g/L	Methyl Decanoate (C10) g/L	Methyl Dodecanoate (C12) g/L
+	C8 FAME	Sten	1.26	0.00	0.94	0.26	0.06
+	C8 FAME	Sten(SV)	0.78	0.00	0.54	0.18	0.06
+	C8 FAME	nphT7(LSVA)	3.59	0.36	2.94	0.23	0.06
-	C8 FAME	Sten	0.59	0.00	0.39	0.14	0.06
-	C8 FAME	Sten(SV)	0.06	0.00	0.00	0.00	0.06
-	C8 FAME	nphT7(LSVA)	1.82	0.10	1.44	0.21	0.07

+	C10 FAME	Sten	1.52	0.10	0.00	1.36	0.07
+	C10 FAME	Sten(SV)	0.96	0.04	0.00	0.85	0.06
+	C10 FAME	nphT7(LSVA)	2.98	0.55	0.03	2.33	0.07
-	C10 FAME	Sten	0.89	0.01	0.00	0.81	0.07
-	C10 FAME	Sten(SV)	0.12	0.00	0.00	0.06	0.06
-	C10 FAME	nphT7(LSVA)	1.91	0.13	0.00	1.70	0.07
+	C8/C10 FAME	Sten	1.66	0.04	0.61	0.95	0.06
+	C8/C10 FAME	Sten(SV)	0.97	0.00	0.24	0.67	0.07
+	C8/C10 FAME	nphT7(LSVA)	2.80	0.25	1.59	0.88	0.07
-	C8/C10 FAME	Sten	0.95	0.00	0.19	0.69	0.07
-	C8/C10 FAME	Sten(SV)	0.09	0.00	0.00	0.03	0.06
-	C8/C10 FAME	nphT7(LSVA)	3.52	0.19	1.65	1.61	0.07

[0184] Unlike Nbra, the wild type Sten showed the ability to produce FAME in both backgrounds with and without NphT7 when combined with different Asch variants that preferentially produce C10 FAME, C8 FAME, or mixed C10 and C8 FAME. For all Asch contexts the amount of FAME was reduced compared to the NphT7(LSVA), but the result pointed out a clear distinction from NphT7 in that wild type Sten can support FAME production. FAME production by the Sten(SV) variant was lower than by the wild-type Sten in both backgrounds, indicating that the S and/or V mutations are context-specific.

c. Sko3/Sko3(SV) (FIG. 7)

Chromosomal NphT7	Asch target product	variant	Total FAME	Methyl Hexanoate (C6) g/L	Methyl Octanoate (C8) g/L	Methyl Decanoate (C10) g/L	Methyl Dodecanoate (C12) g/L
+	C8 FAME	Sko3	2.41	0.19	2.03	0.13	0.06
+	C8 FAME	Sko3(SV)	2.06	0.08	1.59	0.33	0.06

+	C8 FAME	nphT7(LSV A)	3.85	0.42	3.17	0.20	0.06
-	C8 FAME	Sko3	2.47	0.16	2.07	0.18	0.06
-	C8 FAME	Sko3(SV)	1.26	0.03	0.85	0.31	0.07
-	C8 FAME	nphT7(LSV A)	2.58	0.20	2.08	0.24	0.06
+	C10 FAME	Sko3	1.75	0.32	0.03	1.33	0.06
+	C10 FAME	Sko3(SV)	2.15	0.15	0.00	1.92	0.07
+	C10 FAME	nphT7(LSV A)	3.25	0.53	0.03	2.62	0.07
-	C10 FAME	Sko3	2.27	0.37	0.03	1.80	0.06
-	C10 FAME	Sko3(SV)	1.12	0.05	0.00	1.00	0.07
-	C10 FAME	nphT7(LSV A)	2.03	0.19	0.00	1.77	0.07
+	C8/C1 0 FAME	Sko3	3.07	0.29	1.87	0.85	0.06
+	C8/C1 0 FAME	Sko3(SV)	2.06	0.06	0.75	1.17	0.07
+	C8/C1 0 FAME	nphT7(LSV A)	3.48	0.38	2.02	1.01	0.07
-	C8/C1 0 FAME	Sko3	2.64	0.19	1.53	0.86	0.06
-	C8/C1 0 FAME	Sko3(SV)	1.22	0.00	0.25	0.89	0.07
-	C8/C1 0 FAME	nphT7(LSV A)	3.26	0.16	1.41	1.62	0.07

[0185] Sko3 wild-type demonstrated FAME production in both backgrounds with and without NphT7 when combined with different Asch variants that preferentially produce C10 FAME, C8 FAME, or mixed C10 and C8 FAME. FAME titers were comparable to those found with NphT7(LSVA) in a background without NphT7. Sko3(SV) also showed FAME production in both backgrounds with and without NphT7.

d. Scin/Scin(SV) (FIG. 8)

Chromo-somal NphT7	Asch target product	variant	Total FAME	Methyl Hexanoate (C6) g/L	Methyl Octanoate (C8) g/L	Methyl Decanoate (C10) g/L	Methyl Dodecanoate (C12) g/L
+	C8 FAME	Scin	1.97	0.13	1.67	0.10	0.06
+	C8 FAME	Scin(SV)	1.70	0.03	1.32	0.28	0.07
+	C8 FAME	nphT7(LSVA)	3.85	0.42	3.17	0.20	0.06
-	C8 FAME	Scin	1.89	0.08	1.60	0.15	0.06
-	C8 FAME	Scin(SV)	0.57	0.00	0.35	0.15	0.06
-	C8 FAME	nphT7(LSVA)	2.58	0.20	2.08	0.24	0.06
+	C10 FAME	Scin	2.02	0.36	0.03	1.57	0.06
+	C10 FAME	Scin(SV)	1.83	0.10	0.00	1.66	0.07
+	C10 FAME	nphT7(LSVA)	3.25	0.53	0.03	2.62	0.07
-	C10 FAME	Scin	2.17	0.28	0.02	1.80	0.06
-	C10 FAME	Scin(SV)	0.59	0.00	0.00	0.52	0.07
-	C10 FAME	nphT7(LSVA)	2.03	0.19	0.00	1.77	0.07
+	C8/C10 FAME	Scin	2.15	0.21	1.28	0.59	0.06
+	C8/C10 FAME	Scin(SV)	1.85	0.03	0.50	1.25	0.07
+	C8/C10 FAME	nphT7(LSVA)	3.48	0.38	2.02	1.01	0.07

-	C8/C10 FAME	Scin	2.09	0.13	1.09	0.81	0.06
-	C8/C10 FAME	Scin(SV)	0.74	0.00	0.11	0.57	0.07
-	C8/C10 FAME	nphT7(LSVA)	3.26	0.16	1.41	1.62	0.07

[0186] The wild-type Scin showed ability to support FAME production in both backgrounds with and without NphT7 when combined with different Asch variants that preferentially produce C10 FAME, C8 FAME, or mixed C10 and C8 FAME. Scin (SV) also showed the ability to support FAME production in both backgrounds with and without NphT7. In the background without NphT7, the SV mutation caused a reduction in total FAME produced, similar to Sko3(SV), again indicating that the benefits conferred by one or both of these changes is context-specific.

SEQUENCES

Nbra (SEQ ID NO: 1):

Nocardia brasiliensis ATCC 700358

MNNIAVLGTGSYLPDRIVSNSEVSGADVDSEWIIRKTAIRERRWALPDQATSDLATHAA
SAALDAAGISADEVSAIVVATSTPDHPQPPTAAAFVQHNLGARGASAF'DVNAVCSGFVFAL
SAVEAAIARAGGGYGLVVGADVSRILNPADRRRTVVLF'GDGAGAVVLGPSASGGLRRFGL
HTF'GDLTSLIRVPAGGSRQPYDPAAHELGAQYFTMDGRGVRAF'VNGSLPVLVKQFLHDSG
VAPDDITHLIPHQANGVMLAELAEELGLV'NATMHTTVRYYGNTGAASIPITLDNAARTGG
IRPGD'TVLLVGF'GGGMAVGLTLVEW

Sko3 (SEQ ID NO: 2):

Streptomyces sp. KO-3988

MTDLRILGTGAYVPERIASNDEVAAAAAGVDDAWITAKTGIRERRWAADDQATSDLATAAG
RAALRSAGITAEDLSVIVVATSTPDRPQPPTAAYVQQRLGASDAAAF'DVNAVCSGMVFAL
AAAEGLVTRTGHALVIGADLYSRILNRADRRTVILF'GDGAGAVILGSSVDHGPRI'RH
LHSG'ELSGLIEVPAGGSRMPVDQ'TVLDAGLQYFAMDGRGV'RN'FVCDHLPQLVKGFLHKC
GVVPDDIDHFVPHQANGTMLDSL'FADLELPRATMHRTLTHYANTGAASIPITLDAAARAG
AFNPGDLILMAGFGGMSAGFALVEW

Scin (SEQ ID NO: 3):

Streptomyces cinnamonensis

MTGIAADIAVLGTGAYVPDRIVSNDEVGAPAGVDGEWIVRKTAIRERRWAAPGQATSDLA
VAAGRAALESAGITVQGLSLIVVATSTPDRPQPPTAVYVQQQLGALGAAAF'DVNAVCSGS

VFALSVVEGMLARQGGHALVIGADVSRILNPADRKTVVLF'GDGAGAMVLGPAGGGRAAG
ARVRHVTLLHTV'GELAGLIQVPAGGSRQPADQAVLDAGLQYFAMDGREVRRFVVEQLPQLT
KQFLHEAGVVPDDVAHFVPHQANGVMLD'TVIEDLALS' RATTHLT' LERYGNTGAASIPITL
DAAAREGA'FRPGDLILLAGF'GGGMAAGLALLEW

Sten (SEQ ID NO: 4):

Streptomyces tendae

MSDVRILGTGAYVPDRIVSNDEVGAPAGVDDAWITAKTAIRERRWAATHQATSDLATQAA
RNALDCAGITAAELTVIIVATSTPDRPQPPTAAYVQHNLAATHAAAF'DINAVCSGEIFAL
SAAEGTLARKGGHALIIGADLYSRILNPDRKTTVLF'GDGAGAMILGPATTGPRIRHLLT
HTYGEHSDLIQVPAGGSRPPTTTQTLQQDQHYFTMDGRGVRRFVADHLPRLTKEFLHEAG
TVPDDIRHFIPHQANGVMLDDLFTQLALPHATMHQTLTTYANTGAASIPITLDTAHRNNA
LHPGDLILLAGF'GGGMAAGLALIEW

SK03-2 (SEQ ID NO: 5):

Streptomyces sp. KO-3988

MTEVGILGTGAQVPDRVVSND'EAGAAAGVDDAWVVRKTAIRERRWAAPEQATSDLAAAAG
SAA'RSAGITPDQLSVIVVATSTPDRPQPPTAAYVQQLGAVGAAAF'DVNAVCSGEVFAL
AAAEGILTRRG'HALVIGADVSRILNPADRRTVVLF'GDGAGAMVLGPGRGARVRNLALH
TF'GELAGLIEVPAGGSRPADQAALDAGLQYFTMDGRAVRSFVEDRPLLT'KQFLHDSGV
GPDDIRHFVPHQANGVMLDALFAELALNRAVMHRTLTHYGNTGAASIPITLAEARS'GAF
RPGDLILLAGF'GGGMAVG'FALVEW

Ache (SEQ ID NO: 6):

Austwickia chelonae NBRC 105200

MGVAVWGTGAYVPERIVTND'EVGAPAGVDDAWIRSKTMIRERRWVAENEATSDMATEAGR
RALEAAGITADELSYIVVATSTPDRPQPPTAAYVQHNLAHHAAGF'DMNAVCSGFV'FAGS
TVARMIAATGGYGLVIGADVSRILNPRDRRTVILF'GDGAGAVVLGP'SDGSNHGVLATSL
HTF'GALNDKIMVPAGGSRPPTEDTHYETGLAYFTMEGRAVKEFVLQELPPLVDSFFAETG
ISPQ'EVDHFIPHQANGMMLDELVPTLGLSGARTHRTLEKYGNTGAASVGITLDQAAREGE
LHSGDRIFLAGF'GGGMAAGLALIVW

SMg1 (SEQ ID NO: 7):

Streptomyces sp. Mg1

MSARGIVGTGSYLPEREVS'NKEVAEWCGITAEWIEERTEILTRRYAAPDEATSDLAHAL
TSALRSAGITADRLDYLI'VSTSTGDSPPPTANLVQH'AVGATGAACF'DVNVVCSGFV'FGL
EMAHRLLAARPDGYAAVIGADVSRILDHADHRTTVLLGDGAGAAIVGPVRDGHG'YLGAE
LASRGDAHRLIRVEAGGSRIPTTPQTLADGGHYFRMEGRGV'RD'FVMDHVPPVLDRLLRGA
GLTAADVDFVPHQPNGVLLRGLVDKIGMSHAH'RTLEKYGNVGSASVPVALDDGCRSG
LIRDGDLVVMAGF'GGGMSVGATLMRWQERS

Safr (SEQ ID NO: 8):

Spirochaeta africana DSM 8902

MHSKHTELQEAVITGAGAYVPPRRMSNDELAFGLDTSDEWIFSKTGIHNRHIAAEDQATS
DLGAAAARQALADAGLTPLDIDLILVATSSPDYNGLPSTACVVQDLLGATNAAAMDVAAV
CSGFVYALETARAFARSGSARRILVIGAEIYSRVVDWSDRSTCVLFGDGAGAVVVEESLPE
SAGNTAPRFPRARVLDSVLKSRGSDAQALVRPAGGTRRPVNPNGDNFGADQFLQMDGRKVY
NFAVGAIGEVIQELLTRNGLSLAELDWVIPHQANARILEAAARRLKYPVEQMYSNIAEYA
NTSAASIPIAMNEMAAAGMLKPGQNIITVGFAGGLTYGGNLLRTVV

SHK1 (SEQ ID NO: 9):

Streptomyces sp. HKI0576

MTSAIGILGTGAYLPAREVGNDLALIPDTTAEWILRKTGIRSRRYAAPDEAASDLAAG
AARAALADAGLTADRVDDHIIIVCTSTGDQPLPPTASVVQQLLGAGSAACFDLNAVCAAGFVY
GLEAARGLIAVRPGAHVLLIGADVYSRFLDFTDRRSVLLGDGAGAVVVGAVEDGNGILG
IDLSTRGDAQDLLRIPAGGSRSPASAGTVAEGGHLLRMQGRAVSEFVLENIPPGVDKLLA
GAGVRAEEIDCFVPHQANAVLIRELAARCLADTFLVQVVETYGNTGAASVPIGLDHAVR
EGTVEPGGLVLLSAFGAGMSVGNGLLRWGGGPA

Svio (SEQ ID NO: 10):

Streptomyces violaceusniger Tu 4113

MTDHPVRTPGGRFVGILGTGSYLPAAEAVSNELVAERAGVTAEWIAAKTGIRRRRYAADHE
ATSDLAEEAARAALADAGIRAGQLGWIVVATSTPDHPQPATACLVQHRIGATGAAAFDIN
AVCSGFVFALVTAAGLLAGSGAPAPYALVIGADVYSRIIDRTDRRTAVLFGDGAGAVVLG
PVRHGYGLTGSLTSDGALHELIQVPAGGSRAPASEKTLADGGHFFRMRGRAVGEYVLAE
LPRAIRALLAAHRTDAAGVDHFIPHQANGVLLAKALPDLGLPRARTHLTVAEHGNTSAAS
IPLALDDARRQGVFTDGELLLLAGFGGMSLGAALVRWQDGHGGS

AATC (SEQ ID NO: 11):

Amycolatopsis sp. ATCC 39116

MMKTVVLGTGSYLPPTVLTSTELGDRLTGGQWIVDKTHIRERRVADGSEATSDLATAAA
RRALQSSGTSPDELVLIVATSTPDQPIPATAALVQANISADRAAAFVDVAVCSGFVYAL
VVAHGMLLADDSCRKALVIGADIYSRIIDYDDRTAVLFGDGAGAVVLGKTNADRGHTT
LLGCDGKRADLVQVPAGGSRPASPTLADGLHYFKMIGRPVRELQVMPEVVEQLLKR
AGLTLDDVQHLVPHQANGVMLAELDSALGLAPGVMCRTEVHFHNTGAASVPITLDHAVRG
GQIGEDDRVVLVTFGGGMSWGGALLTWAYTHGEETSHD

Ubac (SEQ ID NO: 12):

hypothetical protein ACD_77C00103G0010 [uncultured bacterium]

MKQLIRNVKIIIGTGSYVPEKIYTNEYLETFTSTSSWIFENIGIKERHIAAPNQATSDLA
TIAGQRAIDDAGIKNEDIDLIIVATATPDRKAPSTAAAFVQHKLNAVNAAAFDMNAVCSGF
LFGMSVASQYIASGVYNNILVIGADTFSRITDWTKRDAVFFGDGAGAVVITSANITEGFL
AYRIYTDQTNEMLGFTIPGGGSEIPLTENNNLDQYFQMNGKSVFASATQALPKAINQVLA
DTGLTIGDIDIMIPHQPSIRILQKTAELIGLPFEKVMTNMDRYANTSGGTIPILLDEVKK
SGKLKRGNIIVLFAAVGSGWTYGASIIKWA

Gsul (SEQ ID NO: 13):

Geobacter sulfurreducens PCA

MMRARIIVGTGSAVPSKVLTNFDLEKMVDTSDEWVTTRTGIEKERRIAVDGEYTSTFATLAA
ERALEMAGVKASDLDLLIVATITPDFPFPATACVVQSNLKATKAAAYDISAACSGFIYAL
AQASNAIRSGSARKALVIGAEVLSRIIDWTDNRNTCLLF'GDGAGAVVLEACDDGHGVLSTH
LHSDGSYWELLYQPGCGNRNPAVQKTLDNRRIYLMQGNVFKLAVRAMEAALDAN
GLTPADISLFIHQANRRIIDAIGKRLGLPGEKVYVNLDRFGNTSAASIPALDEANRSG
RIKPNDVVVFDAFGGGLTWGSALVRW

Abay-2 (SEQ ID NO: 14):

Acinetobacter baylyi DSM 14961 = CIP 107474

MGIRITGTGLYHPAESISNEELVESLNAYVEYYNAENAEDIAAGELPERLGSSAEFIEKA
SGIKSRYVIEKSGVLDPQRLRPRLQERTDDELSIQAEWGVLAQAMENAKVTAEDIDVV
ILACSNMQRPYPYPAVAVEIQSALGIQGYAYDMNVACSAATFGLKQAYDAIQSGARRVLLVN
VEITSGHTDYRSRDCHFIFGDVATASIIIEQTDTKTGFEILNIHLFTQFSNNIRNNFGFLN
RSEDATRDDKQFRQDGRKVFKDVCPLVAKIITAQLEKNQVNPQDIKRFWLHQANLNMNQL
IAKLVLGKEAEAERTPIILDEFANTSSAGVIIALHRTGEEVSEGEFGVICSGAGYSVGS
LLVQKRVA

Ctet (SEQ ID NO: 15):

Clostridium tetani E88

MKNKEVRILSTGKYLPPISISNHDLSKIIDTNDNWIKTRTGIEKRRITKGENTSDLGTKA
ALDALRKGGISPEELDLIIIVATITPDYFTPTSTACIIQRNIKAYNAFAFDISAACSGFTYG
ISIASQFIRNGVAKKVLVIGVETLSKLVDWKDRNTCILF'GDGSGAAILTESNEKGIMNVY
LGSDGRGADLLKCKSSSLTVNSDELKELNSKEEDLENKF IEMDGKEIFKF'AVKVMIKGI
EKVLKDSNLELKDINYIIPHQANLRIIEHVAKKLGIDENKFYININHYGNTSAASIPIAL
AEVDEKGLLKKGDNVILVGF'GAGLTWAASLIKWI

NphT7 (SEQ ID NO: 16):

Streptomyces sp. CL190

MTDVRFRIIGTGAYVPERIVSNDEVGAPAGVDDDWITRKTGIRQRRWAADDQATSDLATA
AGRAALKAAGITPEQLTVIAVATSTPDRPQPPTAAYVQHHLGATGTAAFDVNAVCSGTVF
ALSSVAGTLVYRGGYALVIGADLYSRIILNPADRKTVVLF'GDGAGAMVLGPTSTGTGP'IVR
RVALHTFGGLTDLIRVPAGGSRQPLD'DGLDAGLQYFAMDGREVRRFVTEHLPQLIKGFL
HEAGVDAADISHFVPHQANGVMLDEVF'GELHLP'PRATMHRTVETYGNTGAASIPITMDAAV
RAGSFRPGELVLLAGF'GGGMAASFALIEW

nphT7 (SV) (SEQ ID NO: 17)

Artificial sequence

MTDVRFRIIGTGAYVPERIVSNDEVGAPAGVDDDWITRKTGIRQRRWAADDQATSDLATA
AGRAALKAAGITPEQLTVIAVATSTPDRPQPPTAAYVQHHLGATGTAAFDVNAVCSGTVF
ALSSVAGTLVYRGGYALVIGADLYSRIILNPADRKTVVLF'GDGAGAMVLGPTSTGTGP'IVR
RVALHTFGGLTDLIRVPAGGSRQPLD'DGLDAGLQYVAMDGREVRRFVTEHLPQLIKGFL
HEAGVDAADISHFVPHQANGVMLDEVF'GELHLP'PRATMHRTVETYGNTGAASIPITMDAAV
RAGSFRPGELVLLAGF'GGGMAASFALIEW

Asch (V296A) (SEQ ID NO: 18)

Artificial sequence

MGIRITGTGLFHPTDFITNEELVESLNAYVEQYNLENADKIAAGEIEELRGSSAEFIEKA
SGIKRRYVAEKTGILDPKRLRPLLHERSNDELSIQAEWGVAAAKQAMENAGVTAEDIDVV
ILSCSNIQRAYPALAIEIQ TALGIQGYAYDMNVACSAATFGIKQAADAIKSGARRVLMVN
VEITSGHTDFRSRDCHFIFGDVATASII EETETKTGFEIEDIELFTQF'SNNIRNNFGYLN
LSEVDADIDNNRFRQDGRKVFKEVCPLVAKMITKQLEKNQIEPTNVKRFWLHQANANMNE
LILKLIVGKEHAKSELVPLILDEFANTSSAGV IIALHRTANEVNDGEYGVLC'SFGAGYSV
GSILVKKHVA

Asch (T184I, F236L, V268A, V296A, V317A, S328G) (SEQ ID NO: 19)

Artificial sequence

MGIRITGTGLFHPTDFITNEELVESLNAYVEQYNLENADKIAAGEIEELRGSSAEFIEKA
SGIKRRYVAEKTGILDPKRLRPLLHERSNDELSIQAEWGVAAAKQAMENAGVTAEDIDVV
ILSCSNIQRAYPALAIEIQ TALGIQGYAYDMNVACSAATFGIKQAADAIKSGARRVLMVN
VEIISGHTDFRSRDCHFIFGDVATASII EETETKTGFEIEDIELFTQF'SNNIRNNLGYN
LSEVDADIDNNRFRQDGRKVFKEVCPLAAKMITKQLEKNQIEPTNVKRFWLHQANANMNE
LILKLIVGKEHAKSELAPLILDEFANTGSAGV IIALHRTANEVNDGEYGVLC'SFGAGYSV
GSILVKKHVA

Asch(T184I) (SEQ ID NO: 20)

Artificial sequence

MGIRITGTGLFHPTDFITNEELVESLNAYVEQYNLENADKIAAGEIEELRGSSAEFIEKA
SGIKRRYVAEKTGILDPKRLRPLLHERSNDELSIQAEWGVAAAKQAMENAGVTAEDIDVV
ILSCSNIQRAYPALAIEIQ TALGIQGYAYDMNVACSAATFGIKQAADAIKSGARRVLMVN
VEIISGHTDFRSRDCHFIFGDVATASII EETETKTGFEIEDIELFTQF'SNNIRNNFGYLN
LSEVDADIDNNRFRQDGRKVFKEVCPLVAKMITKQLEKNQIEPTNVKRFWLHQANVNMNE
LILKLIVGKEHAKSELVPLILDEFANTSSAGV IIALHRTANEVNDGEYGVLC'SFGAGYSV
GSILVKKHVA

FabH E. coli (SEQ ID NO: 21)

E. coli

MYTKIIGTGSYLPEQVRTNADLEKMVDTSDEWIVTRTGIRERHIAAPNETVSTMGFEAAT
RAIEMAGIEKDQIGLIVVATTSATHAFPSAACQIQSMLGIKGCPAFDVAAACAGFTYALS
VADQYVKSGAVKYALVVGSDVLARTCDPTDRGTIIIFGDGAGAAVLAASEEPGIISTHLH
ADGSYGELLTLPNADRVNPENSIHLMAGNEVFKVAVTELAHIVDETLAANNLDRSQLDW
LVPHQANLR IISATAKKLGMSMDNVVVTLD RHGNTSAASVPCALDEAVRDGRIKPGQLVL
LEAF'GGGFTWGSALVRF

Asch (T184I, V296A, V268A) (SEQ ID NO: 22)

Artificial sequence

MGIRITGTGLFHPTDFITNEELVESLNAYVEQYNLENADKIAAGEIEELRGSSAEFIEKA

SGIKRRYVAEKTGILDPKRLRPLLHERSNDELSIQAEWGVAAAKQAMENAGVTAEDIDVV
 ILSCSNIQRAYPALAIEIQTALGIQGYAYDMNVACSAATFGIKQAADAIAKSGARRVLMVN
 VEIISGHTDFRSRDCHFIFGDVATASIIETETKTGFEIEDIELFTQFSNNIRNNFGYLN
 LSEVDADIDNNRFRQDGRKVFKEVCPLAAKMITKQLEKNQIEPTNVKRFWLHQANANMNE
 LILKLIVGKEHAKSELVPLILDEFANTSSAGVIALHRTANEVNDGEYGVLCSEFGAGYSV
 GSILVKKHVA

Sko3 (SV) (SEQ ID NO: 23)

Artificial sequence

MTDLRILGTGAYVPERIASNDEVAAAAGVDDAWITAKTGIRERRWAADDQATSDLATAAG
 RAALRSAGITAEDLSVIVVATSTPDRPQPPTAAYVQRLGASDAAAFDVNAVCSGMVFAL
 AAAEGVLTRTGHALVIGADLYSRSLNRADRRTVILFGDGAGAVILGSSVDHGPRIRHLA
 LHSFGEISGLIEVPAGGSRMPVDQTVLDAGLQYVAMDGRGVRNFVCDHLPQLVKGFLHKC
 GVVPPDDIDHFVPHQANGTMLDSLFADELEPRATMHRTLTHYANTGAASIPITLDAARAG
 AFNPGDLILMAGFGGMSAGFALVEW

Sten(SV) (SEQ ID NO: 24)

Artificial sequence

MSDVRILGTGAYVPDRIVSNDEVGAPAGVDDAWITAKTAIRERRWAATHQATSDLATQAA
 RNALDCAGITAAELTVIIVATSTPDRPQPPTAAYVQHNLAATHAAAFDINAVCSGEIFAL
 SAAEGTLARKGGHALIIGADLYSRSLNPTDRKTTVLFGDGAGAMILGPATTGPRIRHLTL
 HTYGEHSDLIQVPAGGSRPPTTTQTLQQDQHYVTMDGRGVRRFVADHLPRLTKEFLHEAG
 TVPDDIRHFIPHQANGVMLDDLFTQLALPHATMHQTLTTYANTGAASIPITLDTAHRNNA
 LHPGDLILLAGFGGMAAGLALIEW

Nbra(SV) (SEQ ID NO: 25)

Artificial sequence

MNNIAVLGTGSYLPDRIVSNSEVSGADVDSEWIIRKTAIRERRWALPDQATSDLATHAA
 SAALDAAGISADEVSAIVVATSTPDHPQPPTAAYVQHNLGARGASAFDVNAVCSGFVFAL
 SAVEAAIARAGGGYGLVVGADVYSRSLNPADRRTVVLFVDGAGAVVLGPSASGGGLRRFGL
 HTFGDLTSLIRVPAGGSRQPYDPAHELGAQYVTMDGRGVRAFVNGLPVLVKQFLHDSG
 VAPDDITHLIPHQANGVMLAELAEELGLVNATMHTTVRYYGNTGAASIPITLDNAARTGG
 IRPGDTVLLVGFGGMAVGLTLVEW

Asch (SEQ ID NO: 26)

Acinetobacter schindleri CIP 107287

MGIRITGTGLFHPTDFITNEELVESLNAYVEQYNLENADKIAAGEIEELRGSSAEFIEKA
 SGIKRRYVAEKTGILDPKRLRPLLHERSNDELSIQAEWGVAAAKQAMENAGVTAEDIDVV
 ILSCSNIQRAYPALAIEIQTALGIQGYAYDMNVACSAATFGIKQAADAIAKSGARRVLMVN
 VEITSGHTDFRSRDCHFIFGDVATASIIETETKTGFEIEDIELFTQFSNNIRNNFGYLN
 LSEVDADIDNNRFRQDGRKVFKEVCPLVAKMITKQLEKNQIEPTNVKRFWLHQANVMNE
 LILKLIVGKEHAKSELVPLILDEFANTSSAGVIALHRTANEVNDGEYGVLCSEFGAGYSV
 GSILVKKHVA

Scin (SV) (SEQ ID NO: 27)

Artificial sequence

MTGIAADIAVLGTGAYVPDRIVSNDEVGAPAGVDGEWIVRKTAIREERRWAAPGQATSDLA
VAAGRAALESAGITVGQLSLIVVATSTPDRPQPPTAVYVQQQLGALGAAAFDVNAVCSGS
VFALSVVEGMLARQGGHALVIGADVYSRSLNPADRKTIVLFGDGAGAMVLGPAGGGRAAG
ARVRHVTLHTVGEAGLIQVPAGGSRQPADQAVLDAGLQYVAMDGREVRRFVVEQLPQLT
KQFLHEAGVVPDDVAHFVPHQANGVMLDTVIEDLALSRAATHTLTLERYGNTGAASIPITL
DAAAREGAFRPGDLILLAGFGGGMAAGLALLEW

Fused E. coli accD and accA genes:

accDA fusion (SEQ ID NO: 28)

Artificial sequence

MSWIERIKSNITPTRKASIPEGVWTKDCSCGQVLYRAELERNLEVCPKCDHHRMTARNR
LHSLLEDEGSLVELGSELEPKDVLKFRDSKKYKDRLASAQKETGEKDALVVMKGTLYGMPV
VAAAFEFAMGGSMGSSVVGARFVRAVEQALEDNCPLICFSASGGARMQEALMSLMQMAKT
SAALAKMQERGLPYISVLTDPMTGGVSASFAMLGDLNIAEPKALIGFAGPRVIEQTVREK
LPPGFQRFSEFLIEKGAIDMIVRRPEMRLKLASILAKLMNLPAPNPEAPREGVVPPVPDQ
EPEALSGGGGSGGGGSGGGGSGGGGSAASLNFLDFEQPIAELEAKIDSLTAVSRQDEKL
DINIDEEVHRLREKSVELTRKIFADLGAWQIAQLARHPQRPYTLDYVRLAFDEFDELADG
RAYADDKAIVGGIARLDGRFVMIIGHQKGRETKEKIRRNFGMPAPEGYRKALRLMQMAER
FKMPIITFIDTPGAYPGVGAEEERGQSEAIARNLREMSRLGVPVVCVIGEGGSGGALAIG
VGDKVNMLQYSTYSVISPEGCASILWKSADKAPLAAEAMGIIAPRLKELKLIDSIIPEPL
GGAHRNPEAMAAASKAQLLADLADLDVLSTEDLKNRRYQRLMSYGYA

accB protein (SEQ ID NO: 29)

E. coli

MDIRKIKKLIELVEESGISELEISEGEESVRISRAPAASFPVMQQAYAAPMMQQPAQSN
AAAPATVPSMEAPAAAEISGHIVRSPMVGTFYRTPSPDAKAFIEVGQKVNVDGTLTCIVEA
MKMMNQIEADKSGTVKAILVESGQPVFEPLVVIE

accC protein (SEQ ID NO: 30)

E. coli

MLDKIVIANRGEIALRILRACKELGIKTVAVHSSADRDLDKLVLLADETVICIGPAPSVKSY
LNIPAIISAAEITGAVAIHPGYGFLSENANFAEQVERSGFIFIGPKAETIRLMGDKVSAI
AAMKKAGVPCVPGSDGPLGDDMDKNRAIAKRIGYPVIIKASGGGGGRGMRVVRGDAELAQ
SISMTRAEAKAAFSNDMVMEKYLENPRHVEIQVLADGQGNAIYLAERDCSMQRRHQKV
EEAPAPGITPELRRYIGERCAKACVDIGYRGAGTFEFLFENGFEYFIEMNTRIQVEHPVT
EMITGVDLIKEQLRIAAGQPLSIKQEEVHVRGHAVECRINAEDPNTFLPSPGKITRFHAP
GGFGVRWESHYIYAGYTVPPYYDSMIGKLICYGENRDVAIARMKNALQELIIDGIKTNVDL
QIRIMNDENFQHGGTNIHYLEKKLGLQEK

tpiA Promoter (SEQ ID NO: 31)

E. coli

GGTTTGAATAAATGACAAAAAGCAAAGCCTTTGTGCCGATGAATCTCTATACTGTTTCACA

rpiA promoter (SEQ ID NO: 32)

E. coli

GAGTTAACCACGCGGCTTGCCAACGGGGTCTGAATCGCTTTTTTTGTATATAATGCGTGT

nphT7 (LSVA) (SEQ ID NO: 33)

Artificial sequence

MTDVRFRIGTGAYVPERIVSNDEVGAPAGVDDDWITRKTGIRQRRWAADDQATSDLATA
AGRAALKAAGITPEQLTVIAVATSTPDRPQPPTAAYVQHLLGATGTAAFDVNAVCSGTVF
ALSSVAGTLVYRGGYALVIGADLYSRSLNPADRKTVVLF'GDGAGAMVLGPTSTGTGP'IVR
RVALHTFGGLTDLIRVPAGGSRQPLD'TDGLDAGLQYVAMDGREVR'RFVTEHLPQLIKGFL
HEAGVDAADISHFVPHQANGVMLDEVF'GELHLPRATMHRTVETY'GNTGAASIPITMDAAV
RAGSFRPGELVLLAGF'GGGMAAA'FALIEW

Ter (SEQ ID NO: 34)

Treponema denticola

MIVKPMVRNNICLNAHPQGCKKGVEDQIEYTKKRITAEVKAGAKAPKNVLVLGCSNGYGL
ASRITAAGFYGAATIGVSFEKAGSETKYGTPGWYNNLAFDEAAKREGLYSVTIDGDAFSD
EIKAQVIEEAKKKGIKFDLIVYSLASPVRTDPDTGIMHKS'VLKPF'GKTFTGKTVD'PFTGE
LKEISAEPANDEEAAATVKVMGGEDWEVGSNS

fabI S241F (SEQ ID NO: 35)

Artificial sequence

MGFLSGKRILVTGVASKLSIAYGIAQAMHREGAELAF'TYQNDKLKGRVEE'FAAQLGSDIV
LQCDVAEDASIDTMFAELGKVWPKF'DGFVHSIGFAPGDQLDGDYVNAV'TREGFKIAHDIS
SYSF'VAMAKACRSMLNPGSALLT'LSYLGAE'RAIPN'YVMGLAKASLEANVRYMANAMGPE
GVRVNAISAGPIRTLAASGIKDFRKMLAHCEAVTP'IRRTVTI'EDVGNSAAFLCSDLSAGI
FGEVVHVDGGFSIAAMNELELK

fadB (SEQ ID NO: 36)

E. coli

MVYKGD'TLYLDWLEDGIAELVFDAPGSVNKLDTATVASLGEAIGVLEQQSDLKGLLLRSN
KAAFIVGADITEFLSLFLVPEEQLSQWLHFANSVFNRL'EDLPVPTIAAVNGYALGGGCEC
VLATDYRLATPDLRIGLPETKLGIMP'GF'GGSVRMPRMLGADSALEIIAAGKDV'GADQALK
IGLVDGVVKA'EKLV'EGAKAVLRQAINGDLDWKAKRQPKLEPLKLSKIEATMSFTI'AKGMV
AQTAGKHYPAPITAVKTIEAAARFGREEALNLENKSFVPLAHTNEARALVGIFLNDQYVK
GKAKKLT'KDVPKQAAVLGAGIMGGIAYQSAWKGV'PVVMDINDKSLTLGMTEAAKLL
NKQLER'GKIDGLKLAGVISTIHPTLDYAGFDRVDIVVEAVVENPKVKKAVLAETE'QKVRQ
DTVLASNTSTIPISELANALERPEN'FCGMHFFNPVHRMPLVEIIRGEKSSDETI'AKVVAW
ASKMGKTPIVVND'CPGFV'NVRVLF'PYFAGFSQLLRD'GADFRKIDKVM'EKQFGWPMGPAYL

LDVVGIDTAHHAQAVMAAGFPQRMQKDYRDAIDALFDANRF'GQKNGLGF'WRYKEDSKGKP
 KKEEDAAVEDLLAEVSQPKRDFSEEEI IARMMIPMVNEVVRCLEEGI IATPAEADMALVY
 GLGFPPFHGGAFRWLDTLGS AKYLDMAQQYQHLGLPLYEVPEGLRNKARHNEPYPPVEPA
 RPVGDLKTA

Hche WES (SEQ ID NO: 37)

Hahela chejuensis

MTPLSPVDQIFLWLEKRQQPMHVGGGLHIFSFPDDADAKYMTELAQQQLRAYATPQAPFNRR
 LRQRWGRYYWDTDAQFDLEHHFRHEALPKPGRIRELLAHVSAEHSNLMRERPMWECHLI
 EGIRGRRFAVYYKAHHCMLDGVAAMRMCVKSYSFDPTATEMPPIWAISKDVTTPARETQAP
 AAGDLVHSLSQLVEGAGRQLATVPTLIRELGNLLKARDDSDAGLIFRAPPSSILNQRITG
 SRRFAAQSYALERFKAIGKAFQATVNDVVLAVCGSALRNYLLSRQALPDQPLIAMAPMSI
 RQDDSDSGNQIAMILANLGTIADPVRRLTQASARESKERFRQMTPEEAVNYTALTTLA
 PSQLNLLTGLAPKWQAFNVVISNVP'GPNKPLYWNGARLEGMPVSI'PVDYAAALNITLVSY
 RDQLEFGFTACRRTLP'SMQRLLDYIEQ'GIAELEKAAGV

phoE promoter (SEQ ID NO: 38)

E.coli

gatcttgata tcaaacgaac gtttttagcag gactgtcgtc gggtgccaac catctgcgag
 caaagcatgg cgttttgttg cgcgggatca gcaagcctag cggcagttgt ttacgctttt
 attacagatt taataaatta ccacatttta agaattattat taatctgtaa tatactttta
 acaatctcag gttaaaaact ttcctgtttt caacg

pstsIH promoter (SEQ ID NO: 39)

E. coli

aaatcagact gaagacttta tctctctgtc ataaaactgt catattcctt acatataact
 gtcacctggt tgcctatatt tgcttctcgt agccaacaaa caatgcttta tgaatcctcc
 c

[0187] All publications, patents and patent applications, as well accession numbers and the nucleotide and/or protein sequences that can be found therein, are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

WHAT IS CLAIMED IS:

1. An expression system comprising at least one expression cassette comprising a promoter operably linked to a heterologous nucleic acid segment encoding a polypeptide with 3-ketoacyl-CoA synthase activity and having at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-15, wherein the polypeptide catalyzes condensation of acyl-CoA with malonyl-CoA to form 3-ketoacyl-CoA.
2. The expression system of claim 1, wherein the polypeptide with 3-ketoacyl-CoA synthase activity has at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-4.
3. The expression system of claim 1 or 2, wherein the polypeptide catalyzes one or more condensations selected from the group consisting of:
 - a) C2-CoA with malonyl-CoA to form 3-keto-C4-CoA;
 - b) C4-CoA with malonyl-CoA to form 3-keto-C6-CoA;
 - c) C6-CoA with malonyl-CoA to form 3-keto-C8-CoA; and
 - d) C8-CoA with malonyl-CoA to form 3-keto-C10-CoA.
4. The expression system of claim 2, wherein an amino acid residue of the polypeptide with 3-ketoacyl-CoA synthase activity that aligns with amino acid residue 147 of SEQ ID NO: 16 is serine and an amino acid residue that aligns with amino acid residue 217 of SEQ ID NO: 16 is valine.
5. The expression system of any one of claims 1-4, wherein the promoter is an inducible promoter.
6. The expression system of any one of claims 1-5, wherein the promoter is an inducible promoter sensitive to lowering phosphate concentration.
7. The expression system of any one of claims 1-6, wherein the promoter is a PpstSIH promoter or a PphoE promoter.

8. A recombinant cell comprising the expression system of any one of claims 1-7, wherein the recombinant cell produces a fatty acid and/or fatty acid chain product having a chain length of one or more of C4, C6, C8 and/or C10.
9. The recombinant cell of claim 8, wherein the fatty acid chain product comprises one or more of fatty acid ester, fatty alcohol, fatty acid amide and fatty acid amine.
10. The recombinant cell of claim 9, where in the fatty acid ester comprises one or more of fatty acid methyl ester, fatty acid ethyl ester, fatty acid propyl ester, fatty acid butyl ester, fatty acid pentyl ester and fatty acid hexyl ester.
11. The recombinant cell of claim 8, wherein the recombinant cell produces a fatty acid ester of C4, C6, C8, and/or C10 chain length.
12. The recombinant cell of claim 8, wherein the recombinant cell produces a fatty acid ester of C8 and/or C10 chain length.
13. The recombinant cell of any one of claims 11-12, wherein the alkoxy group of the fatty acid ester is derived from a C1, C2, C3, or C4 monoalcohol.
14. The recombinant cell of any one of claims 11-12, wherein the alkoxy group is derived from a C1 or C2 monoalcohol.
15. The recombinant cell of claim 14, wherein the monoalcohol is methanol.
16. The recombinant cell of any one of claims 11-15, further comprising one or more of:
 - a heterologous nucleic acid segment encoding a polypeptide with enoyl-CoA reductase activity;
 - a heterologous nucleic acid segment encoding a polypeptide with bifunctional 3-hydroxyacyl-CoA dehydrogenase/dehydratase activity;

a heterologous nucleic acid segment encoding a polypeptide with ester synthase activity;

a heterologous nucleic acid segment encoding a polypeptide with acetyl-CoA carboxylase activity; and

a heterologous nucleic acid segment encoding a mutant polypeptide with ketoacyl-CoA synthase activity.

17. The recombinant cells of claim 16, wherein the heterologous nucleic acid segment encoding a mutant polypeptide with ketoacyl-CoA synthase activity is an Asch variant selected from the group consisting of Asch (T184I, F236L, V268A, V296A, V317A, S328G); Asch (T184I, V296A, V268A); and Asch (V296A).
18. The recombinant cell of any one of claims 11-17, wherein the recombinant cell is a fungal cell, a bacterial cell, or a plant cell.
19. The recombinant cell of claim 18, wherein the recombinant cell comprises an *Escherichia coli*.
20. The recombinant cell of claim 18, wherein the recombinant cell comprises a yeast cell.
21. A cell culture comprising:
 - a) the recombinant cell of any one of claims 11-20; and
 - b) one or more fatty acids or fatty acid chain products produced by the recombinant cell.
22. A method of producing fatty acids and/or fatty acid chain products comprising growing the recombinant cell of any one of claims 11-20 in a culture medium,
 - wherein the recombinant cell is grown under conditions in which the heterologous nucleic acid segment encoding the polypeptide with 3-ketoacyl-CoA synthase activity is expressed; and
 - the recombinant cell produces a fatty acid and/or a fatty acid chain product.
23. One or more fatty acids or fatty acid chain products of claim 21 for use in products.

24. An isolated 3-ketoacyl-CoA synthase having at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-16 for use in catalyzing the condensation of acyl-CoA with malonyl-CoA to form 3-ketoacyl-CoA.
25. The 3-ketoacyl-CoA synthase of claim 24, wherein said synthase has activity for one or more of C2-CoA, C4-CoA and C6-CoA intermediates.
26. The 3-ketoacyl-CoA synthase of claim 25, wherein said synthase has activity for C2-CoA and C4-CoA intermediates.
27. The 3-ketoacyl-CoA synthase of claim 24, wherein said synthase has activity for C2-CoA, C4-CoA and C6-CoA intermediates.
28. A recombinant cell of claim 8 for use in producing fatty acid and/or fatty acid chain products having a chain length of one or more of C4, C6, C8 and/or C10.
29. An 3-ketoacyl-CoA synthase having at least 80% sequence identity to an amino acid sequence set forth in SEQ ID NO: 1-4, wherein an amino acid residue of the synthase that aligns with amino acid residue 147 of SEQ ID NO: 16 is serine and an amino acid residue that aligns with amino acid residue 217 of SEQ ID NO: 16 is valine.
30. A recombinant cell comprising the 3-ketoacyl-CoA synthase of claim 29, wherein the recombinant cell produces a fatty acid and/or fatty acid chain product having a chain length of one or more of C4, C6, C8 and/or C10.
31. The recombinant cell of claim 30, wherein the recombinant cell produces a fatty acid ester of C4, C6, C8, and/or C10 chain length.
32. The recombinant cell of claim 30, wherein the recombinant cell produces a fatty acid ester of C8 and/or C10 chain length.

33. The recombinant cell of any one of claims 31-32, wherein the alkoxy group is derived from a C1, C2, C3, or C4 monoalcohol.
34. The recombinant cell of any one of claims 31-32, wherein the alkoxy group is derived from a C1 or C2 monoalcohol.
35. The recombinant cell of claim 34, wherein the monoalcohol is methanol.

nphT7 protein	1	mtdvrfrii--gtgayvperivsndevgapagvdddwitrktgirrrwaaddqatsdlataagraalka
Sko3 protein	1	mtd--lril--gtgayvperiasndevaaaagvddawitaktairrrwaaddqatsdlataagraalrs
Scin protein	1	mtgiaadiavltgtgayvdrivsndevgapagvddgawivrtktairrrwaaddqatsdlavaagraales
Sten protein	1	msdv--ril--gtgayvdrivsndevgapagvddawitaktairrrwaathqatsdlataaarnaldc
Nbra protein	1	--mnniavl--gtgsylpdrivsnsevgsgadvsdwilrktairrrwalpdqatsdlathaasaalda
FABH_ECOLI	1	---mytkii--gtgsylpeqvrtnadlekmdvtsdwivtrtgirerhiaapnetvstmgfeaatraiem
nphT7 protein	69	agitpeqltviavatstpdrrppptaayvqhlgatgtaafdnvavcsgtvfalssv--agtlvyrgg--y
Sko3 protein	67	agitaedlsvivvatstpdrrppptaayvqrlgasdaaafdnvavcsgmvfalaaa--egvlttrtg--h
Scin protein	71	agitvgqlslivvatstpdrrppptaayvqqqlgalgaaafdnvavcsghvfalsvv--egmlarqgg--h
Sten protein	67	agitaaeltviiivatstpdrrppptaayvqhlaathaafdnvavcsgeifalsaa--egtarkgg--h
Nbra protein	67	agisadevsaiivvatstpdhqpptaafvqhnlqargasafdnvavcsghvfalsaveaaiaraggg--y
FABH_ECOLI	66	agiekdqiglivvattsatthafpsaacqigsmigikgcpafdvaaacagfyal--sv--adqyvksqavky
nphT7 protein	136	alvigadlysrilnpadkrktvvlfgdgagavmlgptstgt--gpivrrvalhtfggltdlirvpaggsrq
Sko3 protein	134	alvigadlysrilnradrtvvlfgdgagavilgs--svdh--gprirhlahsfegelsglievpaggsrm
Scin protein	138	alvigadvysrilnpadkrktvvlfgdgagavmlpgagggaraagrvrhtlhtvgelagliqvpaggsrq
Sten protein	134	aliiigadlysrilnptdrktvvlfgdgagavmlgp--att--gprirhitlhtygehsdliqvpaggsrl
Nbra protein	135	glvvgadvysrilnpadrtvvlfgdgagavmlgpsasg----glrrfglhtfgdltslirvpaggsrq
FABH_ECOLI	134	alvvgsdvlarbcpdtrgtiilifgdgagaavl---aase--epgiisthlhadgsygelldtlpnaadrn
nphT7 protein	204	pltdgldaglcylfandgrevrfrvtehlpqlikgflheagvdaadishfvphqangvmldevfgelhlp
Sko3 protein	201	pvdqtvldaglcylfandgrgvnrfvcdhlplqlvkglhkcgvpddidhfvphqangtmldslfadlelp
Scin protein	208	padqavldaglcylfandgrevrfrvveqlplqtkflheagvppddvahfvphqangvmldeviedials
Sten protein	200	ptttqtlqqdqhyflmdgrgvrrfvadhlpriitkeflheagtvppddirhfvphqangvmlddlftqlalp
Nbra protein	200	pydpaahelgaqylfmdgrgvrafvngslpvlvkqflhdsgvapddithiiphqangvmlaelaelglv
FABH_ECOLI	199	pensihl-----lmagnevfkvavtelahivdetlaannldrsqldwlvphqanlriisatakklgms
nphT7 protein	274	ratmhrtvetyngtgaasipitmdaavragfsfpgelvlvlagfgggmaasfaliew
Sko3 protein	271	ratmhrtlthyantgaasipitldaaaragafnpgdlilmlagfgggmsagfalvew
Scin protein	278	ratthltleryngtgaasipitldaaaregafpddlillagfgggmaaglaliew
Sten protein	270	hatmhqtlttyantgaasipitldtahrnnaipgdlillagfgggmaaglaliew
Nbra protein	270	natmhrtvryyngtgaasipitldnaartggirpddtvlvlgfgggmavgltlvew
FABH_ECOLI	262	mdnvvvtldrhngntsaaavpcaldeavrdgrikpgqlvlleafgggftwgsalvrf

FIG. 1

```

Gsul      1 -----mmrarivgtgsavpskvltndfde-----kmvdttsd-
SHK1      1 -----mtsa---igilgtgaylparevgndela-----alipdtt-
Svio      1 mtdhvpvrtpggrpvgilgtgsylpaeavsnelva-----eragvta-
Nbra      1 -----mnniavlgtgsylpdrivsnsevg-----sgadvds-
Sten      1 -----msdvrlgtgayvpdrivsndevg-----apagvdd-
SKO3-2    1 -----mt---evgilgtgaqvpdrvsndeag-----aaagvdd-
Scin      1 -----mtgiaadiavlgtgayvpdrivsndevg-----apagvdg-
Sko3      1 -----mt---dlrilgtgayvperiasndeava-----aaagvdd-
NphT7     1 -----mtdvr--friigtgayvperivsndevg-----apagvdd-
Ache      1 -----mgvavwgtgayvperivtndevg-----apagvdd-
SMg1      1 -----msar---givgtgsylperevsnkeva-----ewcgita-
AATC      1 -----mmktvvlgtgsylpvtststlg-----drlgt-g-
Safr      1 ---mhskhtelqeavitgagayvpprrmsndela-----gfldtsd-
FABH_ECOLI 1 -----mytkiigtgsylpeqvrtndle-----kmvdttsd-
Ctet      1 -----mknkevrilstgkylppisisnhdls-----kiidtnd-
Ubac      1 -----mkqlirnvkiigtgsyvpekiytneyle-----tfistts-
Abay-2    1 -----mgiritgtglyhpaesisneelveslnayveyynaenaekiaagelper

Gsul      32 ----e-wvttrtgikerrria-----vdgeyt--stfatlaaeralem
SHK1      33 ----aewilrktgirsrrya-----apdeaa--sdlagaaraalad
Svio      42 ----e-wiaaktgirrerrya-----adheat--sdlaveaaraalad
Nbra      32 ----e-wiirktairerrwa-----lpdqat--sdlathaasaalda
Sten      32 ----a-witaktairerrwa-----athqat--sdlatqaarnaldc
SKO3-2    32 ----a-wvvrktairerrwa-----apeqat--sdlaaaagsaalrs
Scin      36 ----e-wivrktairerrwa-----appgat--sdlavaagraales
Sko3      32 ----a-witaktgirerrwa-----addgat--sdlataagraalrs
NphT7     34 ----d-witrktgirqrrwa-----addgat--sdlataagraalka
Ache      31 ----a-wirsktmirerrwv-----aeneat--sdmateagrralea
SMg1      32 ----e-wieerteiltrrya-----apdeat--sdlaaahaltsalrs
AATC      31 ----gqwivdkthirerrva-----dgseat--sdlataaarralqs
Safr      39 ----ewifsktgihnrhia-----aedgat--sdlgaaaarqalad
FABH_ECOLI 31 ----ewivtrtgirerhia-----apnetv--stmgfeaatraiem
Ctet      34 ----nwiktrtgiekrr-i-----tkgent--sdlgtkaaldalrk
Ubac      36 ----s-wifenigikerhia-----apnqat--sdlatiagqraidd
Abay-2    50 lgssaefiekasgiksryvieksqvgldpqrlrprlqertddelsiqawgvlaakqamen

```

FIG. 2A

Gsul	67	agvkasldllivatitpdf-pfpatacvvqsnlkatkaaaydisaacsgfiyalaaqsn
SHK1	69	agltadrvdhiivctstgd-qplpptasvvqqllgagsaacfdlnavcagfvvgleaarg
Svio	77	agiragqlgwivvatstpd-hpqpataclvqhrigatgaaafdinavcsgfvfalvtaag
Nbra	67	agisadevsaivvatstpd-hpqpptaafvqhnlgargasafdvnavcsgfvfalsavea
Sten	67	agitaaeltviivatstpd-rpqpptaayvqhnlaathaaafdinavcsgeifalsaaeg
SKO3-2	67	agitpdqlsvivvatstpd-rpqpptaayvqqllgavgaaafdvnavcsgevfalaaaeg
Scin	71	agitvgqlslivvatstpd-rpqpptavvqqlgalgaaafdvnavcsgsvfalsvveg
Sko3	67	agitaedlsvivvatstpd-rpqpptaayvqqlrgasdaafdvnavcsgmvfalaaaeg
NphT7	69	agitpeqltviavatstpd-rpqpptaayvqhhlgatgtaafdvnavcsgtvfalssvag
Ache	66	agitadelsyivvatstpd-rpqpptaayvqhnlgahhaagfdmnnavcsgfvfagstvar
SMg1	67	agitadrllylivststgd-spppntanlvqhavgatgaacfdvnavcsgfvfglemahr
AATC	67	sgtspdeldivivatstpd-qpipataalvqanisadraaafdvnavcsgfvyalvvhg
Safr	74	agltpldidlilvatsspdynlplstacvvqdlkgatnaaamdvaavcsgfvyaletara
FABH_ECOLI	66	agiekdqiglivvat-tsathafpsaacqigsmlgikgcpafdvaaacagftyalsvadq
Ctet	68	ggispeeldliivatitpdyf-tpstaciiqrnikaynafafdisaacsgftygisiasq
Ubac	71	agiknedidliivatstpd-rkapstaafvqhklnavnaaafdmnavcsgflfgmsvasq
Abay-2	110	akvtaedidvvilacsnmq-rpyavaveiqsalgiqgy-aydmnvacsatfglkqayd
Gsul	126	airsgsar--k--alvigaevlsrildwtldrntcllfgdgagavvleacddg--hgvlst
SHK1	128	liavr--pgah--vlligadvysrildftdrsavllgdgagavvvgavedng-----
Svio	136	llagsgapapy--alvigadvysrildrtdrtaavlfgdgagavvlgp----vrhg----
Nbra	126	aia--ragggy--glvvgadvysrildnpadrtrtvvlfgdgagavvlgpsasgg-----
Sten	126	tla--rk-ggh--aliigadlysrlidnptdrktvvlfgdgagamilgpa---ttg----
SKO3-2	126	ilt--rr-ggh--alvigadvysrildnpadrtrtvvlfgdgagavvlgp---gr--g----
Scin	130	mia--rq-ggh--alvigadvysrildnpadrktvvlfgdgagavvlgpagggraag----
Sko3	126	vlt--rt-ggh--alvigadlysrlidnradrtrtvvlfgdgagavvlgssvd--hg----
NphT7	128	tlv--yr-ggy--alvigadlysrlidnpadrktvvlfgdgagavvlgptstg--tg----
Ache	125	mia---atggy--glvigadvysrildnprdrtrtvvlfgdgagavvlgps-dgsnhg----
SMg1	126	llaa--rpdgy--aavigadvysrildhadhrtrtvllgdgagaaivgpvrvg--hg----
AATC	126	mlladdsc-rk--alvigadiysrildyddrtrtavvlfgdgagavvlgkt-----na----
Safr	134	farsgsar--r--ilvigaeiysrildvdsdrstcvlfgdgagavvveslpesagntaprf
FABH_ECOLI	125	yvksgevsk--y--alvvgdsrlartcdptdrgtiiifgdgagavvlaasee-----
Ctet	127	firngvak--k--vlvigvetlsklvdwkdrrntcilfgdgsgaailtesnekgi-----
Ubac	130	yi----asgvynnilvigadtfsrltd-wtkrdavffgdgagavvi--tsaniteg----
Abay-2	168	ai-----qsgarrvllvnveitschtdyrsrdchfifgdvatasileqtd-----

FIG. 2B

Gsul	180	----hl----hsdgsywel-lyqpgcggnrpavqktl-----ddrriylmmqgnevfk
SHK1	178	--ilgi-dl-strgdaqdl-iripaggsrspasagtv-----aeggghlrmqgravse
Svio	186	ygltdgs-ll-tdsgalhel-iqvpaggsrapasektl-----adgghffrmrgravge
Nbra	175	--lrrf-gl-htfgdltsl-irvpaggsrqpydpaah-----elgaqyftmdgrgvra
Sten	173	prihl-tl-htygehsdl-iqvpaggsrlptttqtl-----qqdqhyftmdgrgvrr
SKO3-2	172	arvrnl-al-htfgelagl-ievpaggsrrpadqaal-----daglqyftmdgravrs
Scin	181	arvrhv-tl-htvgelagl-iqvpaggsrqpadqavl-----daglqyfamdgreivr
Sko3	174	prihl-al-hsfgeisgl-ievpaggsrmpvdqtv-----daglqyfamdgrgvrr
NphT7	177	pivrrv-al-htfggldtl-irvpaggsrqpldtqtl-----daglqyfamdgreivr
Ache	175	--vlat-sl-htfgalndk-imvpaggsrlptedthy-----etglayftmegravke
SMg1	176	ylgael-a---srgdahrl-irveaggsripttpqtl-----adgghyfrmeigrvrd
AATC	174	drghl-tllgdcgkradl-vqvpaggsrrpasqtl-----adglhyfkmigrpvre
Safr	190	prarvldsvlksrgsdaqa-lvrpaggtrrpvnpgdn-----fgadqlqmdgrkvyn
FABH_ECOLI	172	pgi--isthlhadgsygel-ltlpnadrvnpensi-----hltmagnevfk
Ctet	177	-----mnvylgsdgrgdl-lkcksssltvnsdelkellnskeedlenkfiemdgeifk
Ubac	179	----flayriytdtqnemlgftipgggseipltennd-----qyfqmngksvfa
Abay-2	213	-----tktgfeilnlhftqfsnnir-nnfgflnrse-datrdkqfrqdgkvfk
Gsul	224	lavramedaalealdangltpadislfiphqanrriidaigkrl--glpgekvyvn---l
SHK1	226	fvlenippgvdkllagagvraeeidcfvphqanavlirelaarc--gladtplvqv---v
Svio	236	yvlaelprairallaahrtdaagvdhfvphqangvllakalpdl--glprarthlt---v
Nbra	223	fvngslpvlvkqflhdsgvapddithliphqangvmlaelael--glvnatmhtt---v
Sten	223	fvadhlprltkeflheagtpddirhfiphqangvmlddlftql--alphatmhqt---l
SKO3-2	222	fvedrlplltkqflhdsgvgpddirhfiphqangvmlldalfael--alnrvvmhrt---l
Scin	231	fvveqlpqltkqflheagvvpddvahfvphqangvmldeviedl--alsratthlt---l
Sko3	224	fvcdhlplqlvkqflhkcgvpddidhfiphqangtmldslfadl--elpratmhrt---l
NphT7	227	fvtehlplqlkqflheagvdaadishfvphqangvmldevfgel--hlpratmhrt---v
Ache	223	fvlgelpllvdsffaetgispqevdhfvphqangmldelvpvl--glsgarthrt---l
SMg1	224	fvmdhvpplvdrllrgagltadvdhfvphqangvmlldvldki--gmshahthrt---l
AATC	225	lagqvmpevveqllkragltlddvqhlvphqangvmlaelasal--glapgvmcrt---v
Safr	242	favgaigevigeqlltrnglsaelldwviphqanarileaaaarl--kypveqmysn---i
FABH_ECOLI	215	vavtelahivdetlaannldrsqldwlvphqanlriisatakkl--gmsmdnvvt---l
Ctet	231	favkvmikgiekvkdsnlkldinyiiphqanlriehvakkkl--gidenkfyin---i
Ubac	226	satqalpkainqvladtgtitgdidimiphqpsirilqktaeli--glpfekvmtn---m
Abay-2	262	dvcplvakiitaqleknqvnppdikrfwlhqan-lnmqliaklvlgke-aeaertpiil

FIG. 2C

Gsul	279	drfgntsaasiplaldeanrsgrikpndvvvfdafgggltwgsalvrw-----
SHK1	281	etygntgaasvpigldhavregtvepgglvllsafgagmsvgngllrwgggpa-----
Svio	291	aehgntsaasiplalddarrqgvftdgelllllagfgggmslgaalvrwqdgghgs----
Nbra	278	ryygntgaasipitldnaartggirpgdtvllvgfgggmavgltlvew-----
Sten	278	ttyantgaasipitltdtahrnnalhpgdlilllagfgggmaaglaliw-----
SKO3-2	277	thygntgaasipitlaeaarsgafrpgdlilllagfgggmavgfalvew-----
Scin	286	erygntgaasipitldaaaregafrpgdlilllagfgggmaaglaliew-----
Sko3	279	thyantgaasipitldaaaragafnpgdlilmagfgggmsagfalvew-----
NphT7	282	etygntgaasipitmdaavragfrpgelvlllagfgggmaasfaliew-----
Ache	278	ekygntgaasvgitldqaaregelhsgdriflagfgggmaaglaliw-----
SMg1	279	ekygnvgsasvpvalddgcrsglirdgdlvvmagfgggmsvgatlmrwqers-----
AATC	280	ehfgntgaasvpitldhavrggqigeddrvvlvtfgggmswggalltwaythgeetshd
Safr	297	aeyantsaasipiamnemaagmlkpgqniitvgfgaglttyggnlrtvv-----
FABH_ECOL	270	drhgntsaasvpcaldeavrdgrikpgqlvllleafgggftwgsalvrf-----
Ctet	286	nhygntsaasipialaevdekglkkgdnvilvgfgagltwaaslikwi-----
Ubac	281	dryantsggtipilldevkksgklkrqgnivlfaavgsqwtvgasiikwa-----
Abay-2	320	defantssagviialhrtgee--vsegefgvicsfgagysvgsllvqkrva-----

FIG. 2D

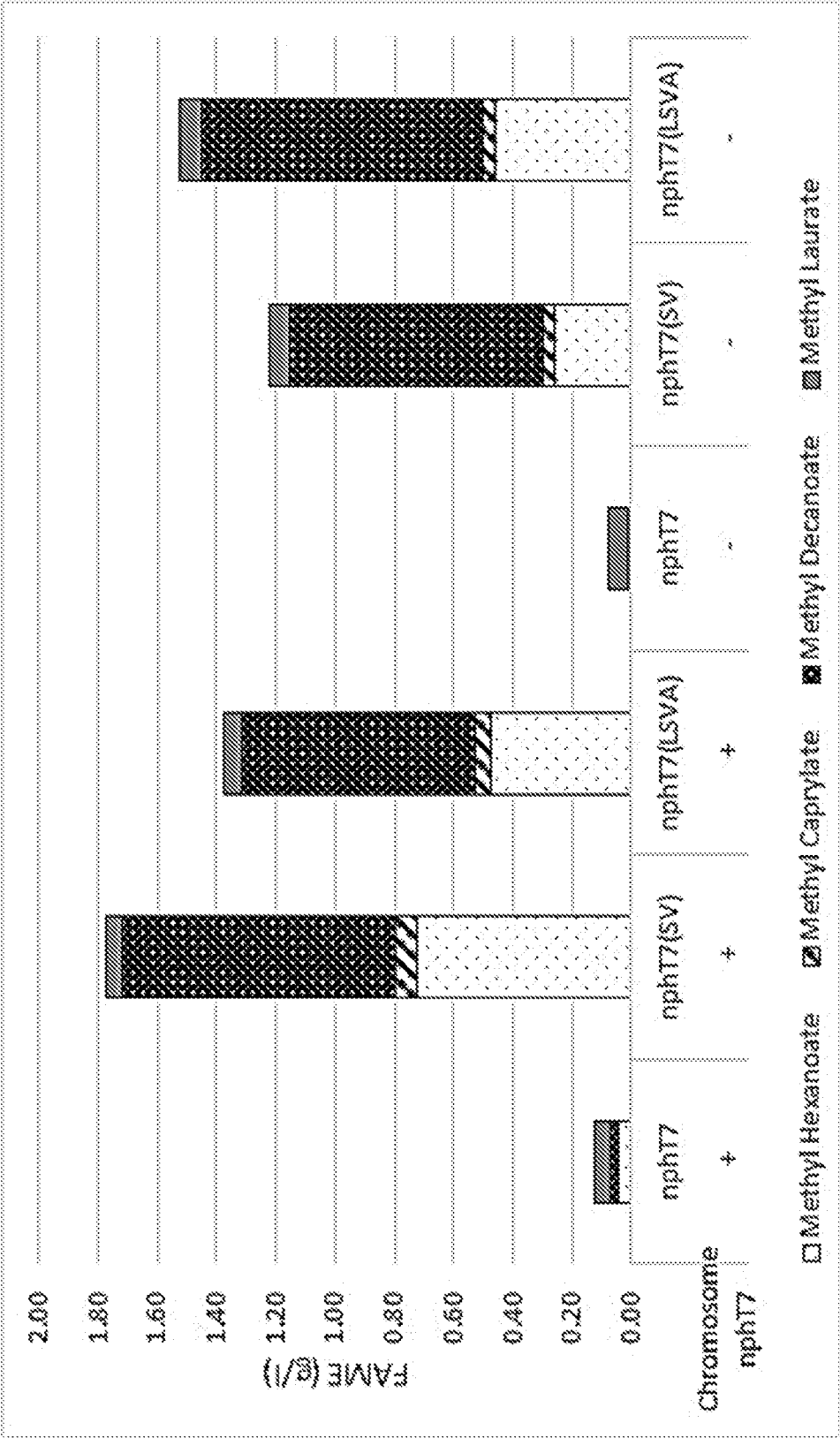


FIG. 3

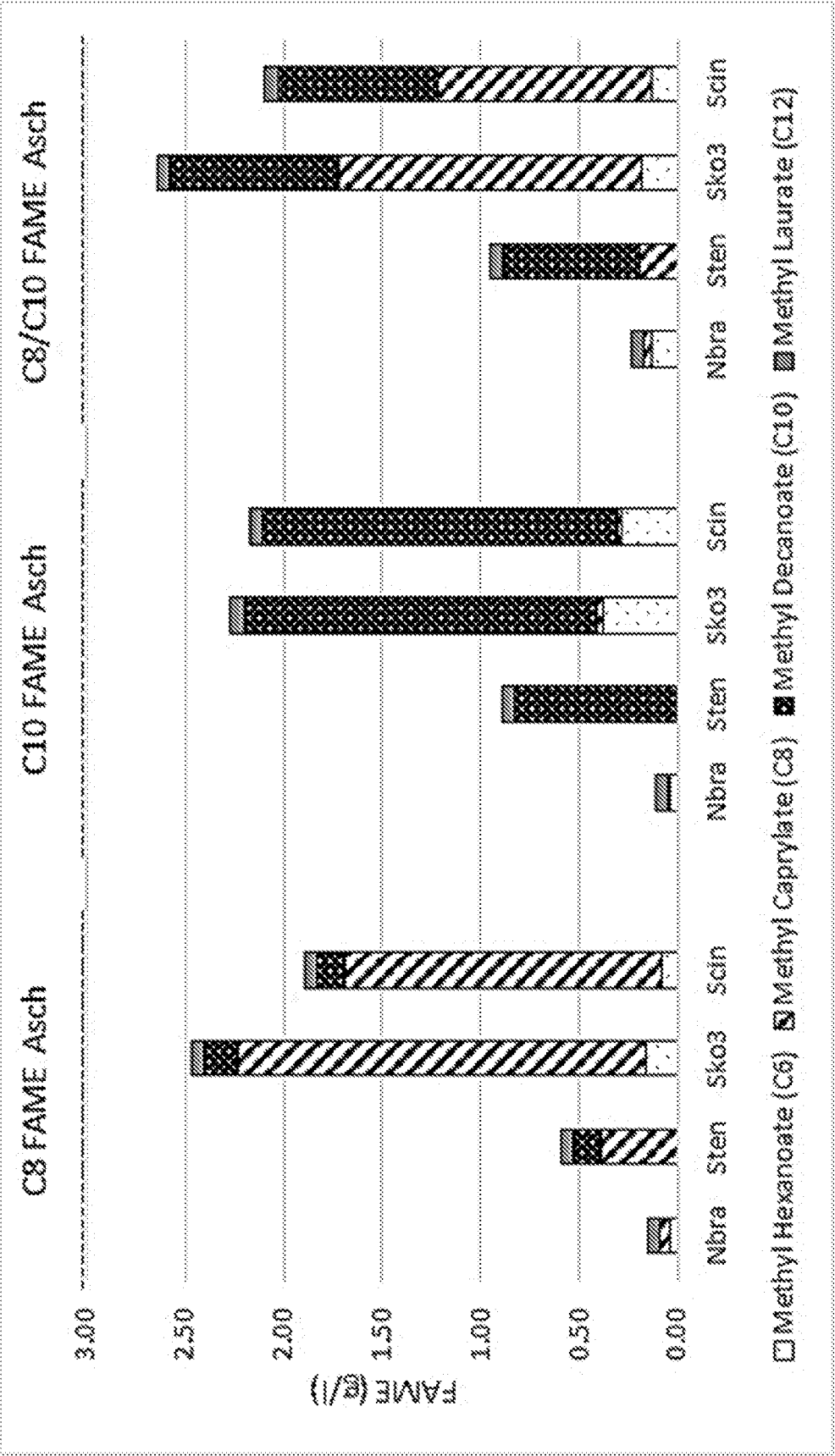
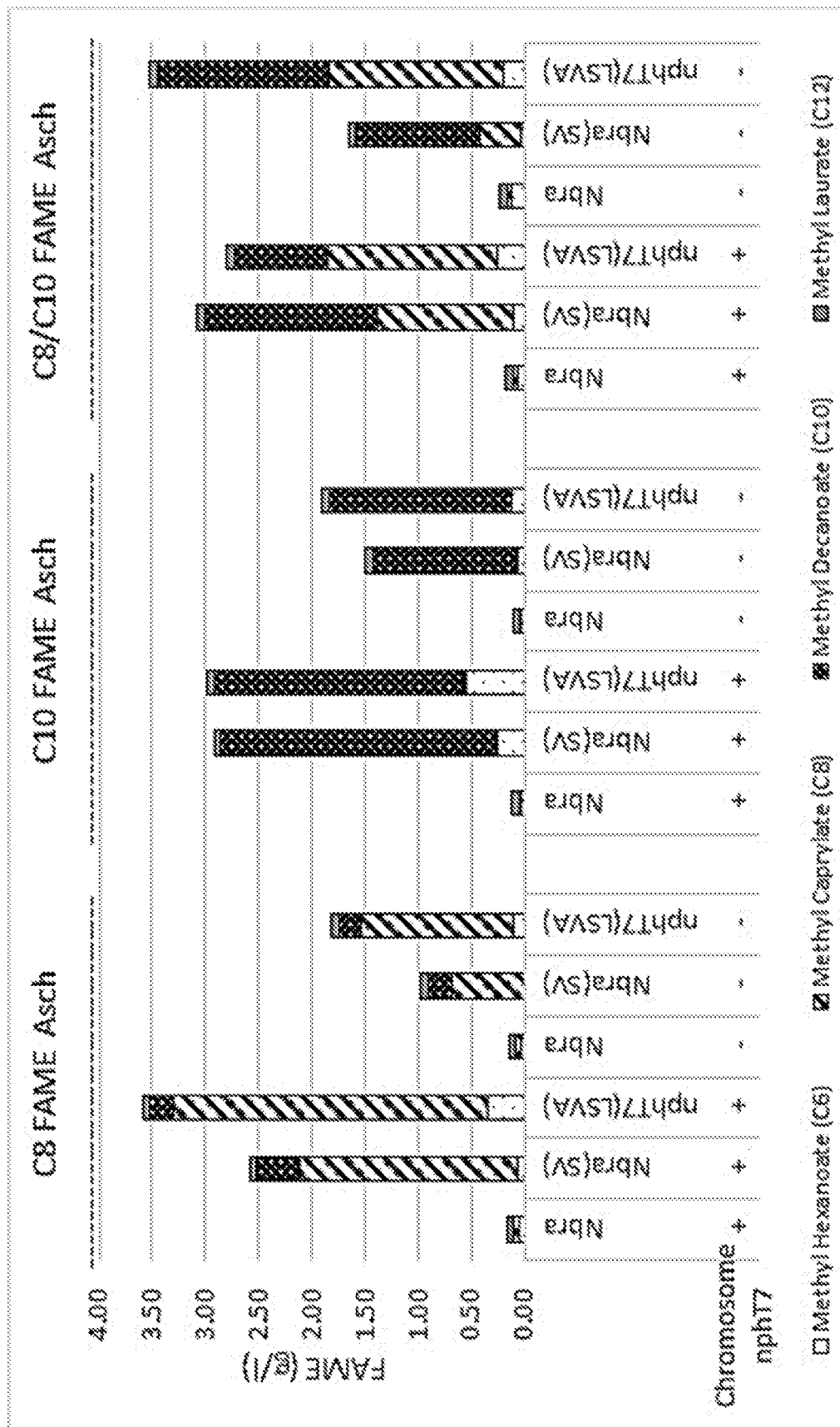


FIG. 4



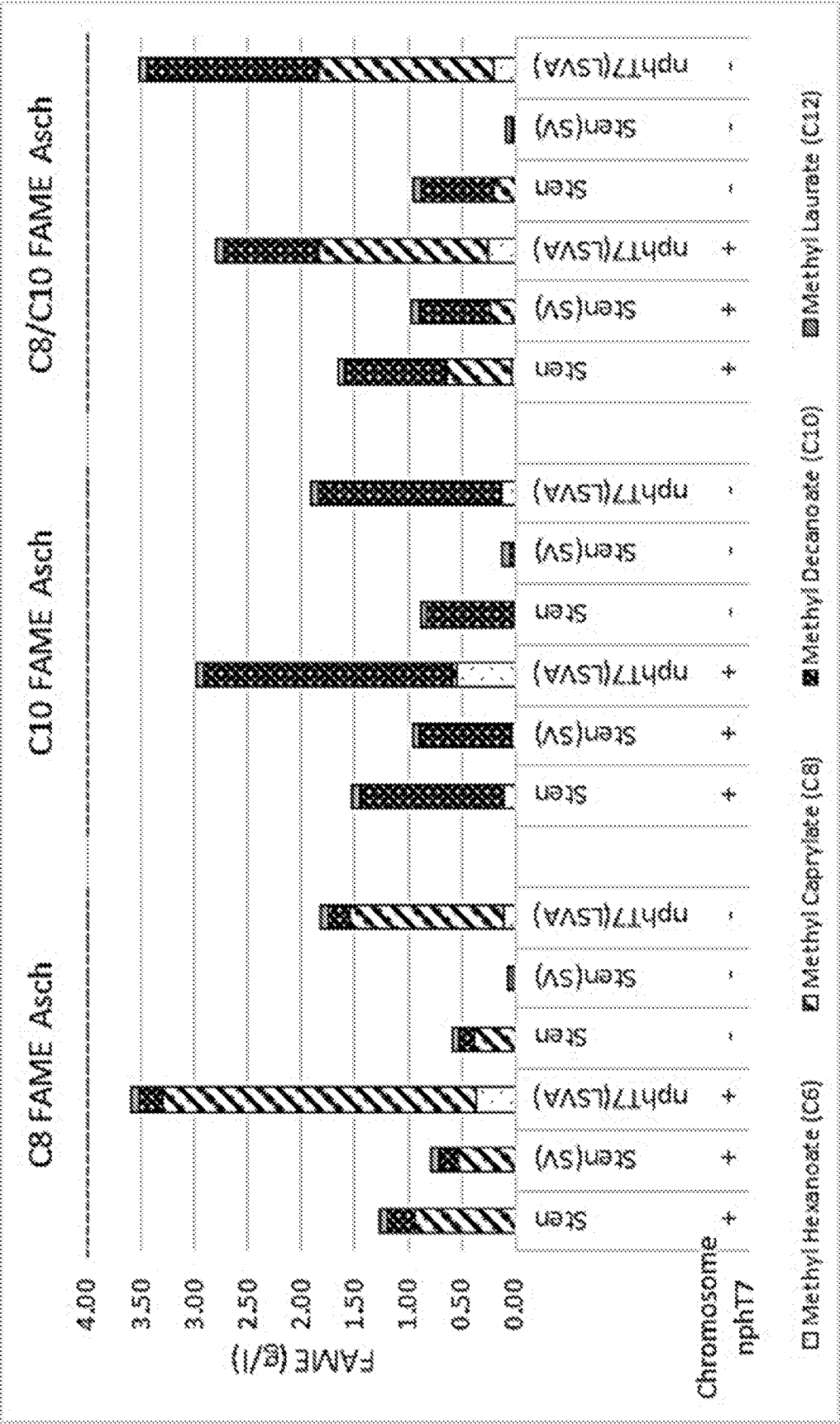


FIG. 6

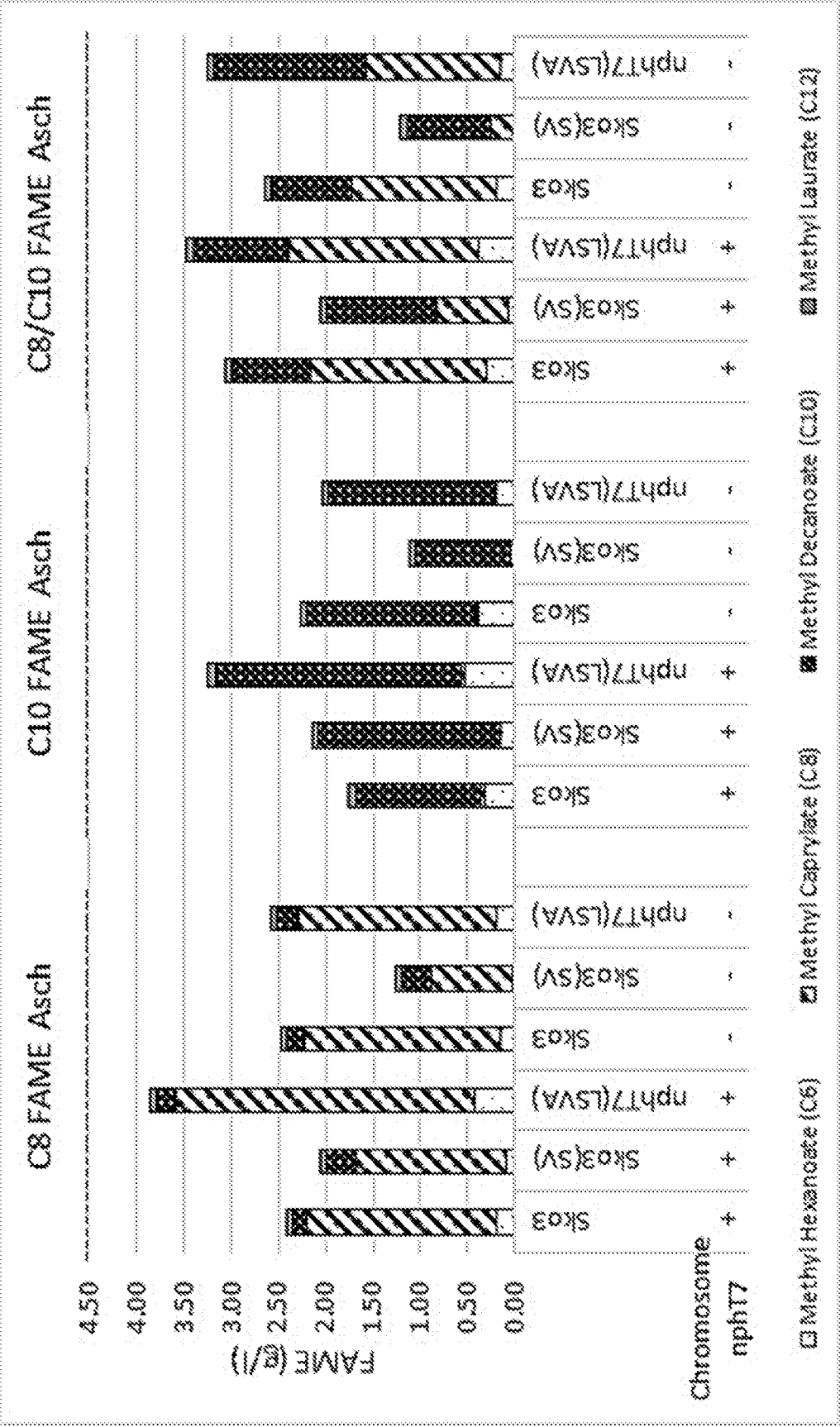


FIG. 7

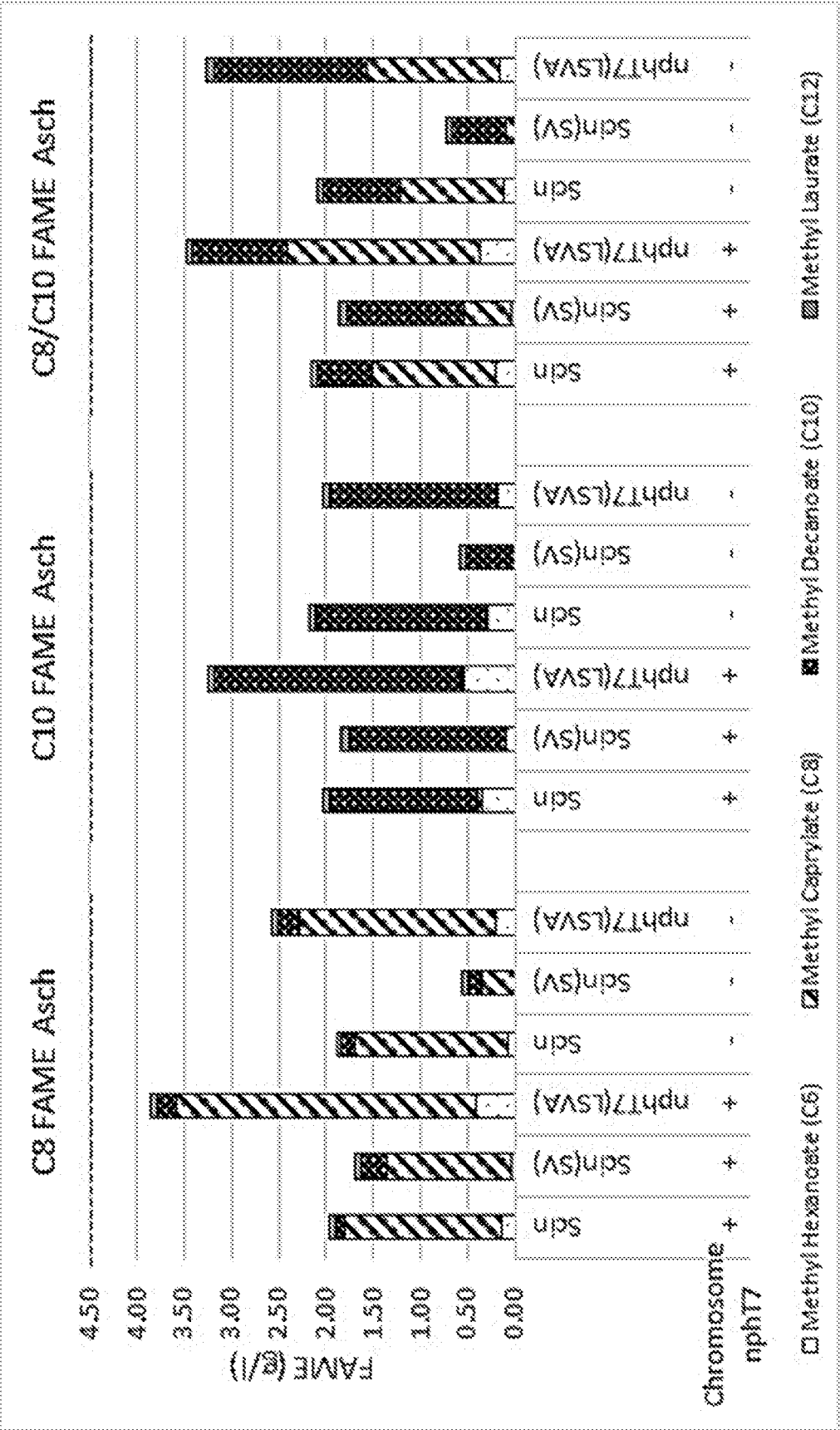


FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/035801

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12N 1/13; C12N 1/15; C12N 1/19; C12N 1/21; C12N 5/10; C12N 9/00; C12N 9/02 (2019.01)

CPC - C12N 9/0006; C12N 9/001; C12N 9/1029; C12N 9/16; C12N 9/88; C12N 15/52; C12P 7/40; C12P 7/52; C12P 7/6409; C12P 7/6436; C12Y 203/0118; C12Y 203/01194 (2019.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 435/134; 435/252.3 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/010103 A2 (OPX BIOTECHNOLOGIES, INC. et al) 22 January 2015 (22.01.2015) entire document	1-4, 24-27, 29-35
A	US 2015/0275242 A1 (OSTERHOUT et al) 01 October 2015 (01.10.2015) entire document	1-4, 24-27, 29-35
A	→ KAWASAKI et al. "Presence of Copalyl Diphosphate Synthase Gene in an Actinomycete Possessing the Mevalonate Pathway," The Journal of Antibiotics, 25 November 2004 (25.11.2004), Vol. 57, No. 11, Pgs. 739-747. entire document	1-4, 24-27, 29-35
A	WO 2017/214133 A2 (PROVIVI, INC.) 14 December 2017 (14.12.2017) entire document	1-4, 24-27, 29-35
A	→ SEEGER et al. "The biosynthetic genes for prenylated phenazines are located at two different chromosomal loci of Streptomyces cinnamomensis DSM 1042," Microbial Biotechnology, 08 December 2010 (08.12.2010), Vol. 4, No. 2, 252-262. entire document	1-4, 24-27, 29-35
A	US 2017/0211102 A1 (CALYSTA, INC.) 27 July 2017 (27.07.2017) entire document	1-4, 24-27, 29-35

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

22 August 2019

Date of mailing of the international search report

16 SEP 2019

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450
Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/035801

Box No. 1 **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☒ forming part of the international application as filed:
 ☒ in the form of an Annex C/ST.25 text file.
 ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs:1-20 were searched.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/035801

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-23, 28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.