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(54) **PRODUCTION OF COMMERCIAL BIODIESEL FROM GENETICALLY MODIFIED MICROORGANISMS**

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C10L 1/19 (2006.01)

(52) **U.S. Cl.** **44/388**

(57) **ABSTRACT**

The invention provides a fermentation and recovery process for the production of biodiesel of commercial grade quality according to commercial and environmental standards (e.g., ASTM ANP, or EPA trace elements and emissions standards), by fermentation of carbohydrates using a genetically modified microorganism. The process provides a direct route for the production of fatty esters, without the need for producing oils which are later chemically transesterified with the concomitant production of large quantities of glycerin and other undesirable side-products.

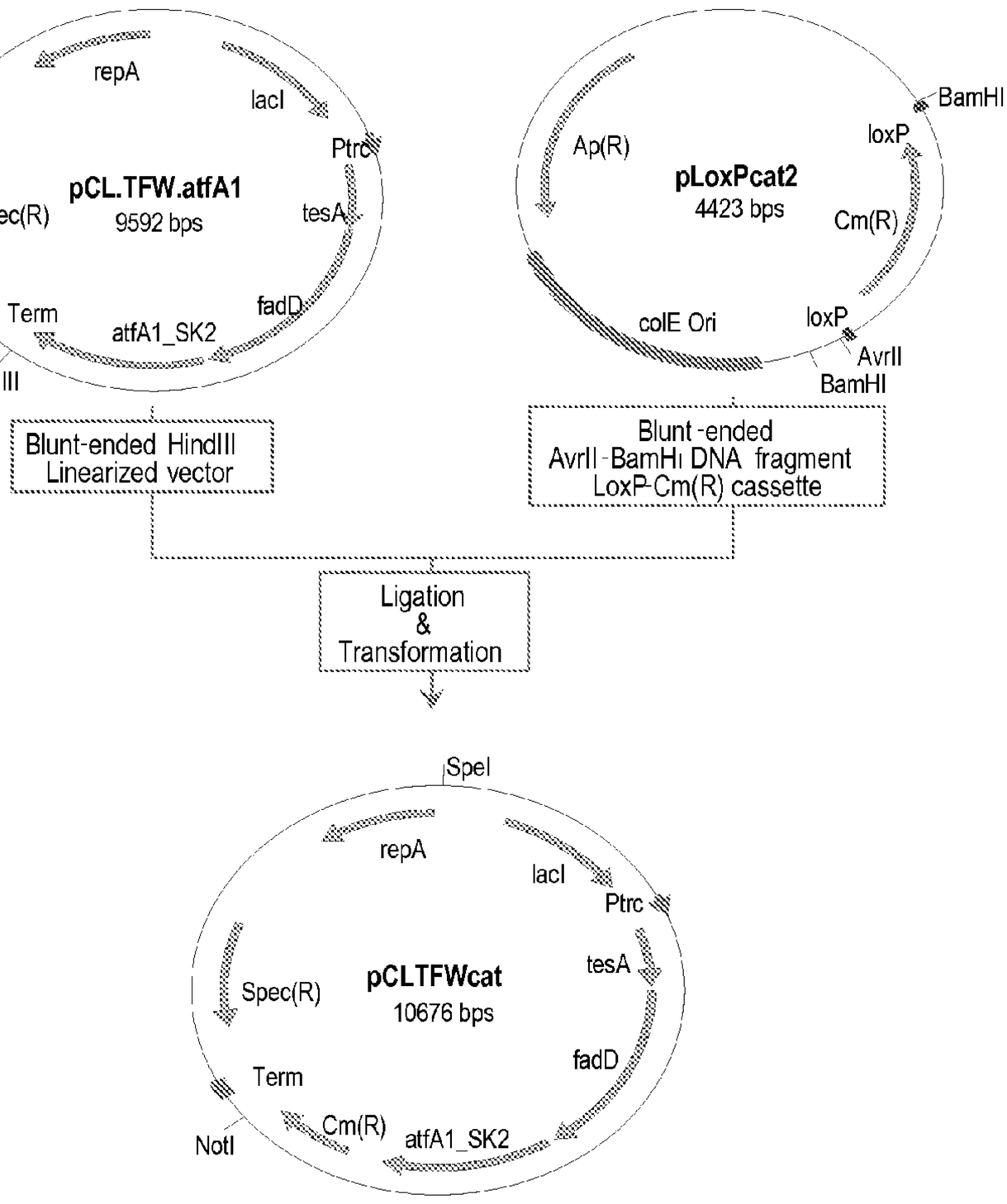
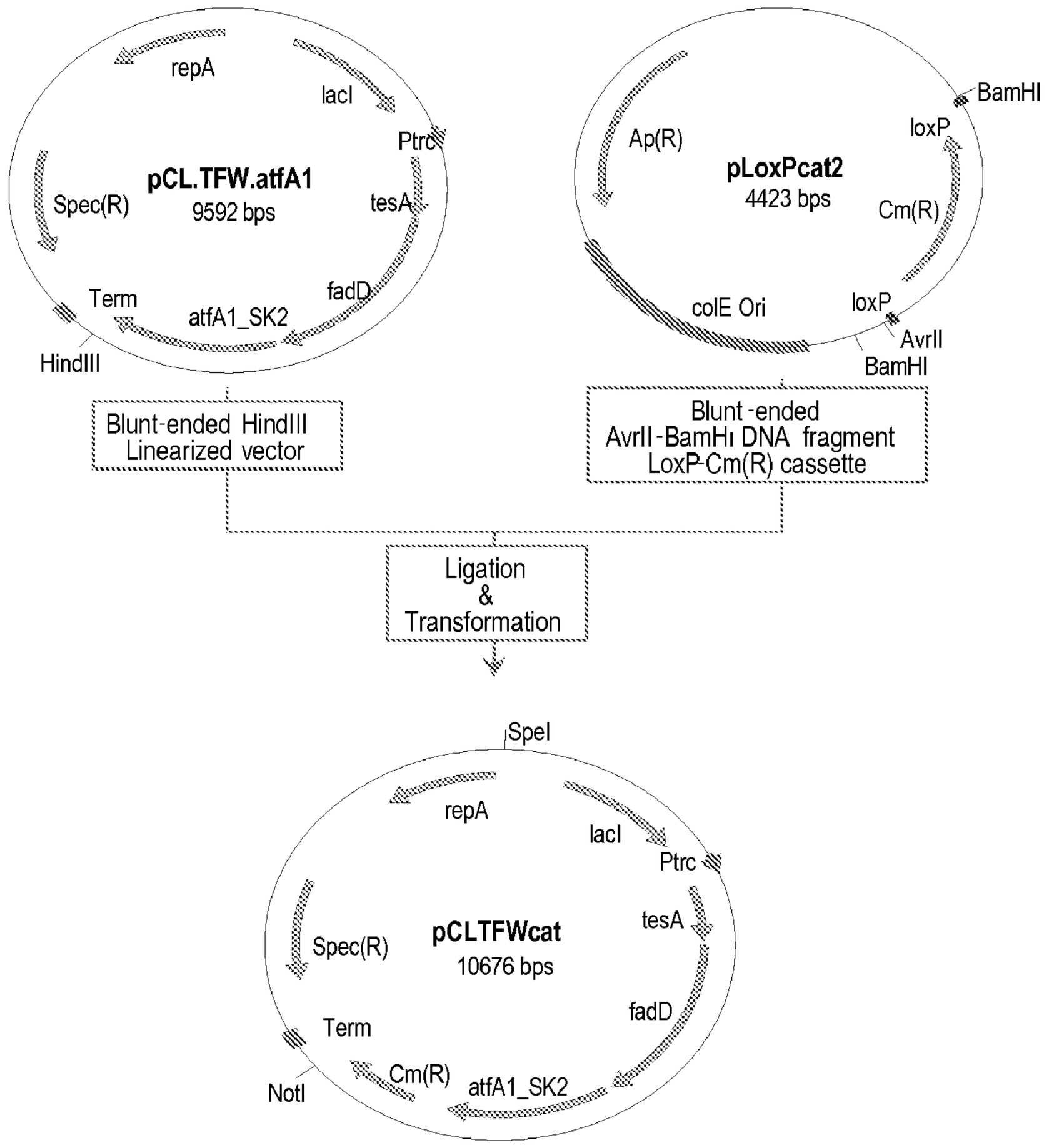


FIGURE 1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colE Ori
loxP
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BamHI
Blunt-ended HindIII Linearized vector
Blunt-ended AvrII-BamHı DNA fragment LoxP-Cm(R) cassette
Ligation & Transformation
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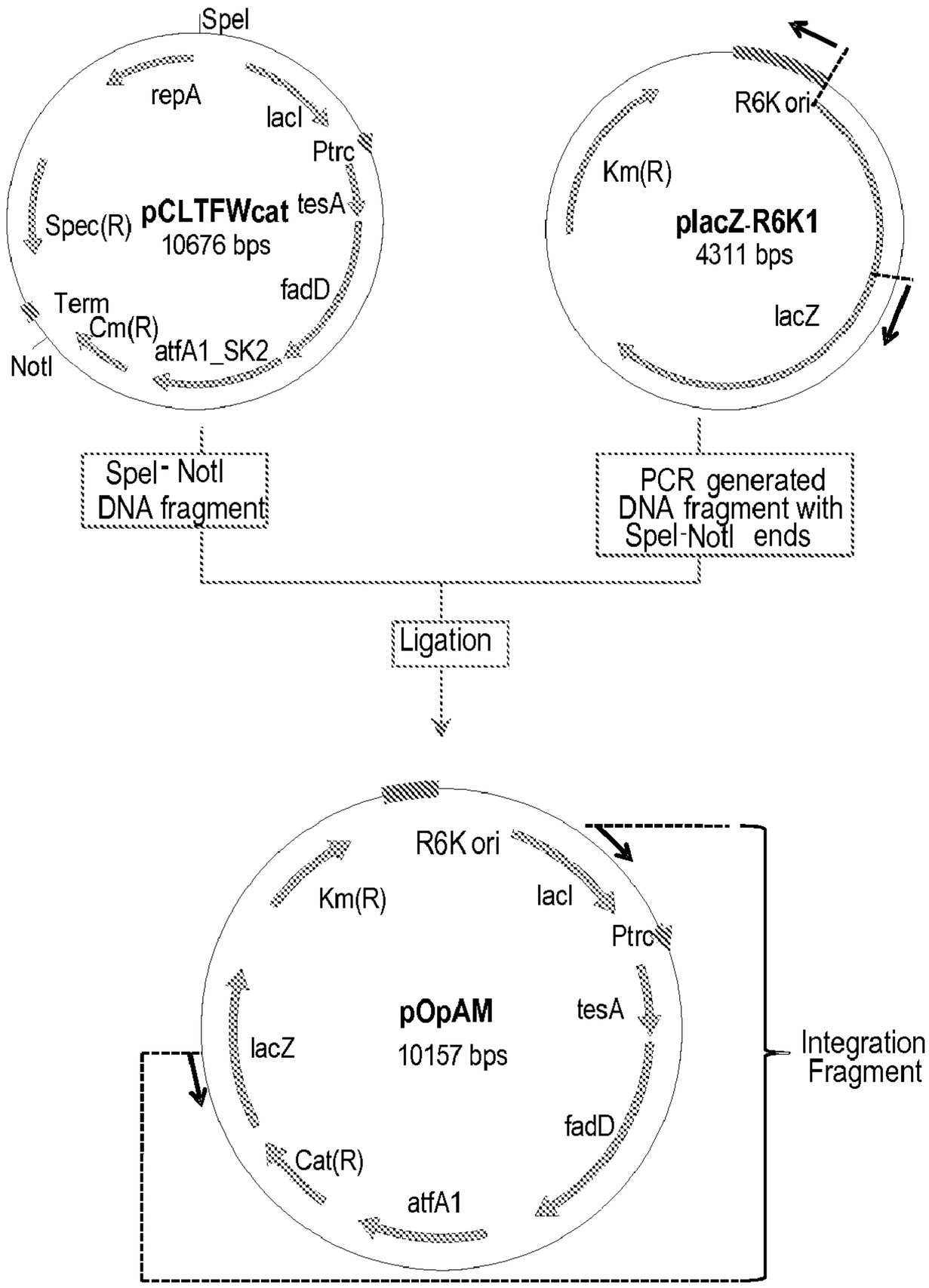
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atfA1_SK2
NotI
R6K ori
Km(R)
placZ-R6K1
4311 bps
lacZ
SpeI - NotI
DNA fragment
PCR generated
DNA fragment with
SpeI-NotI ends
Ligation
R6K ori
Km(R)
lacI
Ptrc
pOpAM
10157 bps
tesA
lacZ
fadD
Cat(R)
atfA1
Integration
Fragment

Figure 2

Figure 3

Final sequence of the entire integration fragment: 6267 bp

GGCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAAC
AAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATT
ACCGAGTCCGGGCTGCGCGTTGGTGCGGATATCTCGGTAGTGGGATACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGTCAAC
CACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATC
AGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTA
ATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCGCGAATTGATCTGGTTTG
ACAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTGGCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTAAATC
ACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGA
AATGAGCTGTTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCGCCGCTGA
GAAAAAGCGAAGCGGCACTGCTCTTTAACAATTTATCAGACAATCTGTGTGGGCACTCGACCGGAATTATCGATTAACTTTATTATTAA
AAATTAAAGAGGTATATATTAATGTATCGATTAAATAAGGAGGAATAAACCATGGCGGACACGTTATTGATTCTGGGTGATAGCCTGAG
CGCCGGGTATCGAATGTCTGCCAGCGCGGCCTGGCCTGCCTTGTTGAATGATAAGTGGCAGAGTAAAACGTCGGTAGTTAATGCCAGCA
TCAGCGGCGACACCTCGCAACAAGGACTGGCGCGCCTTCCGGCTCTGCTGAAACAGCATCAGCCGCGTTGGGTGCTGGTTGAACTGGGC
GGCAATGACGGTTTGCGTGGTTTTCAGCCACAGCAAACCGAGCAAACGCTGCGCCAGATTTTGCAGGATGTCAAAGCCGCCAACGCTGA
ACCATTGTTAATGCAAATACGTCTGCCTGCAAACTATGGTCGCCGTTATAATGAAGCCTTTAGCGCCATTTACCCCAAACTCGCCAAAG
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GTTATCAATCTCTGGTAGATATGTTTGAGCAGTCGGTCGCGCGCTACGCCGATCAACCTGCGTTTGTGAATATGGGGGAGGTAATGACC
TTCCGCAAGCTGGAAGAACGCAGTCGCGCGTTTGCCGCTTATTTGCAACAAGGGTTGGGGCTGAAGAAAGGCGATCGCGTTGCGTTGAT
GATGCCTAATTTATTGCAATATCCGGTGGCGCTGTTTGGCATTTTGCGTGCCGGGATGATCGTCGTAAACGTTAACCCGTTGTATACCC
CGCGTGAGCTTGAGCATCAGCTTAACGATAGCGGCGCATCGGCGATTGTTATCGTGTCTAACTTTGCTCACACACTGGAAAAAGTGGTT
GATAAAACCGCCGTTCAGCACGTAATTCTGACCCGTATGGGCGATCAGCTATCTACGGCAAAAGGCACGGTAGTCAATTTCGTTGTTAA
ATACATCAAGCGTTTGGTGCCGAAATACCATCTGCCAGATGCCATTTCATTTCGTAGCGCACTGCATAACGGCTACCGGATGCAGTACG
TCAAACCCGAACTGGTGCCGGAAGATTTAGCTTTTCTGCAATACACCGGCGGCACCACTGGTGTGGCGAAAGGCGCGATGCTGACTCAC
CGCAATATGCTGGCGAACCTGGAACAGGTTAACGCGACCTATGGTCCGCTGTTGCATCCGGGCAAAGAGCTGGTGGTGACGGCGCTGCC
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FIGURE 3 Continued

GAGTTCCAGCAGCTGGATTTCTCCAGTCTGCATCTTTCCGCAGGCGGAGGGATGCCAGTGCAGCAAGTGGTGGCAGAGCGTTGGGTGAA
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GTGGTAGCATCGGTTTGCCGGTGCCGTCGACGGAAGCCAAACTGGTGGATGATGATGATAATGAAGTACCACCGGGTCAACCGGGTGAG
CTTTGTGTCAAAGGACCGCAGGTGATGCTGGGTTACTGGCAGCGTCCGGATGCTACAGATGAGATCATCAAAAATGGCTGGTTACACAC
CGGCGACATCGCGGTGATGGATGAAGAAGGGTTCCTGCGCATTGTCGATCGTAAAAAAGACATGATTCTGGTTTCCGGTTTTAACGTCT
ATCCCAACGAGATTGAAGATGTCGTCATGCAGCATCCTGGCGTACAGGAAGTCGCGGCTGTTGGCGTACCTTCCGGCTCCAGTGGTGAA
GCGGTGAAAATCTTCGTAGTGAAAAAAGATCCATCGCTTACCGAAGAGTCACTGGTGACCTTTTGCCGCCGTCAGCTCACGGGCTACAA
AGTACCGAAGCTGGTGGAGTTTCGTGATGAGTTACCGAAATCTAACGTCGGAAAAATTTTGCGACGAGAATTACGTGACGAAGCGCGCG
GCAAAGTGGACAATAAAGCCTGATAACTCTAGAAATAATTTAGTTAAGTATAAGAAGGAGATATACATATGAAAGCGCTTAGCCCAGTG
GATCAACTGTTCCTGTGGCTGGAAAAACGACAGCAACCCATGCACGTAGGCGGTTTGCAGCTGTTTTCCTTCCCGGAAGGTGCCGGCCC
CAAGTATGTGAGTGAGCTGGCCCAGCAAATGCGGGATTACTGCCACCCAGTGGCGCCATTCAACCAGCGCCTGACCCGTCGACTCGGCC
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CCAGTGAACGTGAAATGGCTCCGGCTTGGGCGTTCAACACCAAAAAACGCTCCCGCTCACTGCCCAGCAACCCGGTTGACATGGCCTCC
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CAAAAAAGATGAAAACTATGTGTCTATTTTTCAGGCTCCCGACACGATTCTGAATAATACCATCACCGGTTCACGCCGCTTTGCCGCCC
AGAGCTTTCCATTACCGCGCCTGAAAGTTATCGCCAAGGCCTATAACTGCACCATTAACACCGTGGTGCTCTCCATGTGTGGCCACGCT
CTGCGCGAATACTTGATTAGCCAACACGCGCTGCCCGATGAGCCACTGATTGCAATGGTGCCCATGAGCCTGCGGCAGGACGACAGCAC
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TCGAGGAAGCCAAATCCCGCTTCTCGCAGATGAGCCCGGAAGAAATTCTCAATTTCACCGCCCTCACTATGGCTCCCACCGGCTTGAAC
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TGCACAGCTGCAAGGAGTGTATCCAGTATCCATTGCCTTGGATCGCATCGCCCTAAATATCACCCTCACCAGTTATGTAGACCAGATGG
AATTTGGGCTTATCGCCTGCCGCCGTACTCTGCCTTCCATGCAGCGACTACTGGATTACCTGGAACAGTCCATCCGCGAATTGGAAATC
GGTGCAGGAATTAAATAGTAACTCTAGAAATAATTTAAATGGAATTCGAAGCTGATCCATAACTTCGTATAATGTATGCTATACGAAGT
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CATCGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAA

FIGURE 3, continued

GACCGTAAAGAAAAATAAGCACAAGTTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGAATTCCGTATGG
CAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACCCTTGTTACACCGTTTTCCATGAGCAAACTGAAACGTTTTCATCGCTC
TGGAGTGAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGCGTGTTACGGTGAAAACCTGGCCTATTTCCC
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Figure 4:

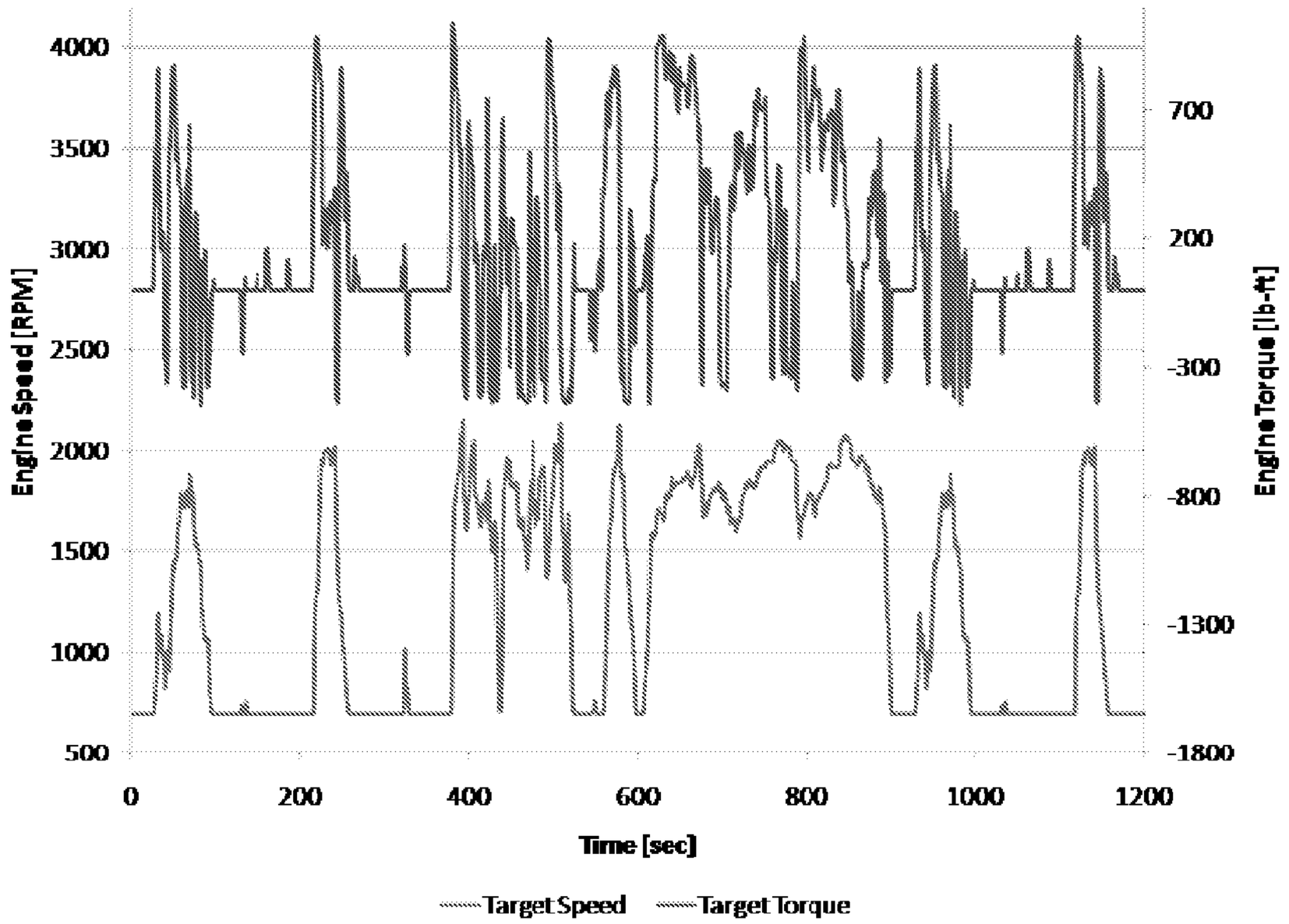
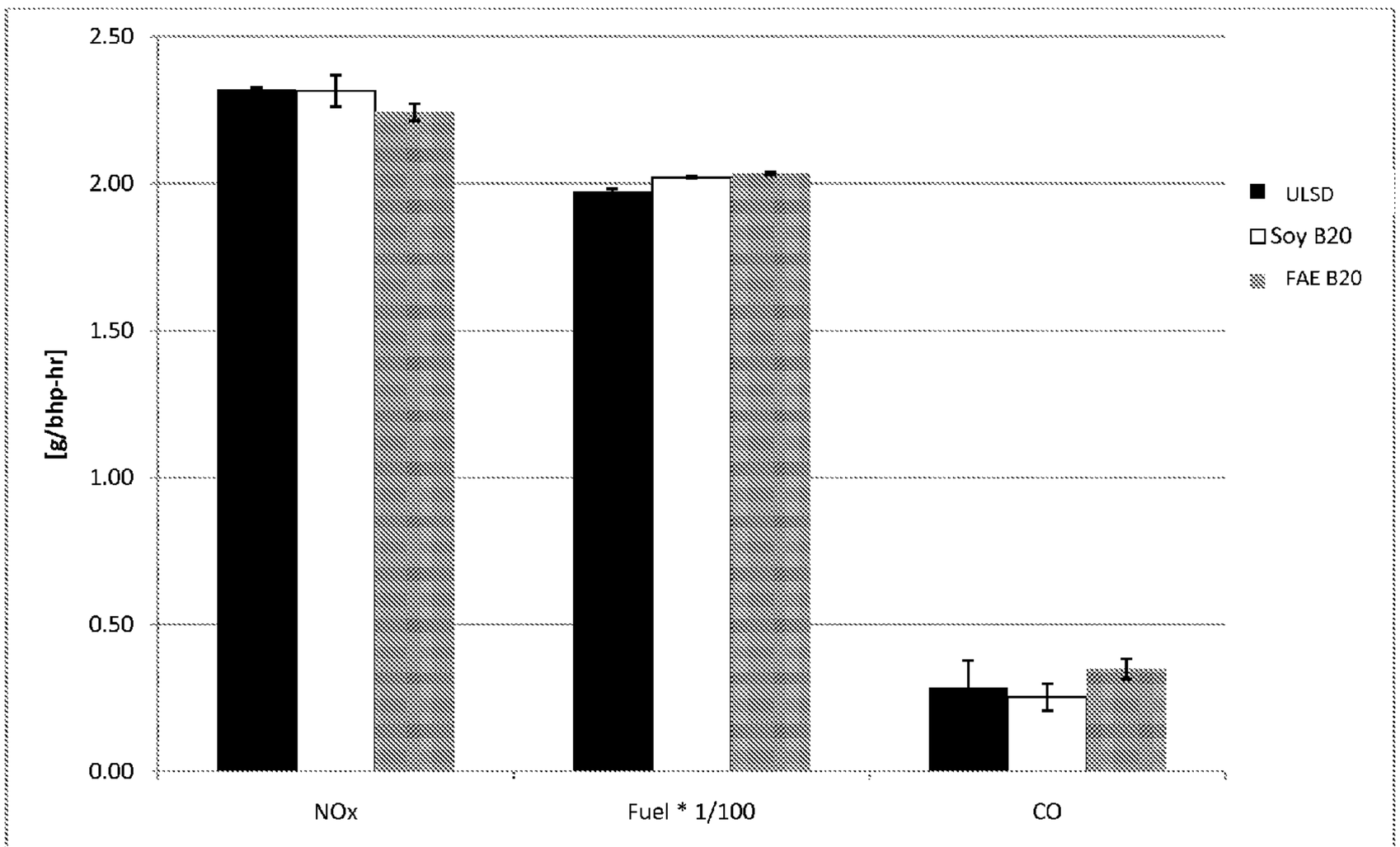

4000
3500
3000
2500
2000
1500
1000
500
Engine Speed [RPM]
700
200
-300
-800
-1300
-1800
Engine Torque [lb-ft]
0
200
400
600
800
1000
1200
Time [sec]
Target Speed
Target Torque

Figure 5: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1.50
1.00
0.50
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[g/bhp-hr]
NOx
Fuel * 1/100
CO
ULSD
Soy B20
FAE B20

Figure 6

Sequence of pLacZ (SEQ ID NO:28)

CTAGTAACGGCCGCCAGTGTGCTGGAATTCAGGCAGTTCAACCTGTTGATAGTACGTACTAAGCTCTCATGTTTCACGTACTAAGCTCT
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FIGURE 6 Continued

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AAACCTCAGTGTGACGCTCCCCGCCGCGTCCCACGCCATCCCGCATCTGACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAATA
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GAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCC

PRODUCTION OF COMMERCIAL BIODIESEL FROM GENETICALLY MODIFIED MICROORGANISMS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Nos. 61/168,293, filed Apr. 10, 2009, 61/266,749, filed Jul. 20, 2009, 61/227,025, filed Jul. 20, 2009, and 61/262,544, filed Nov. 19, 2009, the entire content of each is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] Petroleum is a limited, natural resource found in the Earth in liquid, gaseous, or solid forms. Petroleum is primarily composed of hydrocarbons, which are comprised mainly of carbon and hydrogen. It also contains significant amounts of other elements, such as, nitrogen, oxygen, or sulfur, in different forms.

[0003] Petroleum is a valuable resource, but petroleum products are developed at considerable costs, both financial and environmental. First, sources of petroleum must be discovered. Petroleum exploration is an expensive and risky venture. The cost of exploring deep water wells can exceed $100 million. In addition to the economic cost, petroleum exploration carries a high environmental cost. For example, offshore exploration disturbs the surrounding marine environments.

[0004] After a productive well is discovered, the petroleum must be extracted from the Earth at great expense. Even under the best circumstances, only 50% of the petroleum in a well can be extracted. Petroleum extraction also carries an environmental cost. For example, petroleum extraction can result in large seepages of petroleum rising to the surface. Offshore drilling involves dredging the seabed which disrupts or destroys the surrounding marine environment.

[0005] After extraction, petroleum must be transported over great distances from petroleum producing regions to petroleum consuming regions. In addition to the shipping costs, there is also the environmental risk of devastating oil spills.

[0006] In its natural form, crude petroleum extracted from the Earth has few commercial uses. It is a mixture of hydrocarbons (e.g., paraffins (or alkanes), olefins (or alkenes), alkynes, napthenes (or cylcoalkanes), aliphatic compounds, aromatic compounds, etc.) of varying length and complexity. In addition, crude petroleum contains other organic compounds (e.g., organic compounds containing nitrogen, oxygen, sulfur, etc.) and impurities (e.g., sulfur, salt, acid, metals, etc.).

[0007] Hence, crude petroleum must be refined and purified before it can be used commercially. Due to its high energy density and its easy transportability, most petroleum is refined into fuels, such as transportation fuels (e.g., gasoline, diesel, aviation fuel, etc.), heating oil, liquefied petroleum gas, etc.

[0008] Crude petroleum is also a primary source of raw materials for producing petrochemicals. The two main classes of raw materials derived from petroleum are short chain olefins (e.g., ethylene and propylene) and aromatics (e.g., benzene and xylene isomers). These raw materials are derived from the longer chain hydrocarbons in crude petroleum by cracking the long chain hydrocarbons at considerable expense using a variety of methods, such as catalytic cracking, steam cracking, or catalytic reforming. These raw materials are used to make petrochemicals, which cannot be directly refined from crude petroleum, such as monomers, solvents, detergents, or adhesives.

[0009] One example of a raw material derived from crude petroleum is ethylene. Ethylene is used to produce petrochemicals such as, polyethylene, ethanol, ethylene oxide, ethylene glycol, polyester, glycol ether, ethoxylate, vinyl acetate, 1,2-dichloroethane, trichloroethylene, tetrachloroethylene, vinyl chloride, and polyvinyl chloride. Another example of a raw material derived from crude petroleum is propylene. Propylene is used to produce isopropyl alcohol, acrylonitrile, polypropylene, propylene oxide, propylene glycol, glycol ethers, butylene, isobutylene, 1,3-butadiene, synthetic elastomers, polyolefins, alpha-olefins, fatty alcohols, acrylic acid, acrylic polymers, allyl chloride, epichlorohydrin, and epoxy resins.

[0010] Petrochemicals can be used to make specialty chemicals, such as plastics, resins, fibers, elastomers, pharmaceuticals, lubricants, or gels. Examples of specialty chemicals which can be produced from petrochemical raw materials are: fatty acids, hydrocarbons (e.g., long chain hydrocarbons, branched chain hydrocarbons, saturated hydrocarbons, unsaturated hydrocarbons, etc.), fatty alcohols, esters, fatty aldehydes, ketones, lubricants, etc.

[0011] Specialty chemicals have many commercial uses. Fatty acids are used commercially as surfactants. Surfactants can be found in detergents and soaps. Fatty acids can also be used as additives in fuels, lubricating oils, paints, lacquers, candles, salad oils, shortenings, cosmetics, and emulsifiers. In addition, fatty acids are used as accelerator activators in rubber products. Fatty acids can also be used as a feedstock to produce methyl esters, amides, amines, acid chlorides, anhydrides, ketene dimers, and peroxy acids and esters.

[0012] Hydrocarbons have many commercial uses. For example, shorter chain alkanes are used as fuels. Methane and ethane are the main constituents of natural gas. Longer chain alkanes (e.g., from five to sixteen carbons) are used as transportation fuels (e.g., gasoline, diesel, or aviation fuel). Alkanes having more than sixteen carbon atoms are important components of fuel oils and lubricating oils. Even longer alkanes, which are solid at room temperature, can be used, for example, as a paraffin wax. Alkanes that contain approximately thirty-five carbons are found in bitumen, which is used for road surfacing. In addition, longer chain alkanes can be cracked to produce commercially useful shorter chain hydrocarbons.

[0013] Like short chain alkanes, short chain alkenes are used in transportation fuels. Longer chain alkenes are used in plastics, lubricants, and synthetic lubricants. In addition, alkenes are used as a feedstock to produce alcohols, esters, plasticizers, surfactants, tertiary amines, enhanced oil recovery agents, fatty acids, thiols, alkenylsuccinic anhydrides, epoxides, chlorinated alkanes, chlorinated alkenes, waxes, fuel additives, and drag flow reducers.

[0014] Fatty alcohols have many commercial uses. The shorter chain fatty alcohols are used in the cosmetic and food industries as emulsifiers, emollients, and thickeners. Due to their amphiphilic nature, fatty alcohols behave as nonionic surfactants, which are useful in detergents. In addition, fatty alcohols are used in waxes, gums, resins, pharmaceutical salves and lotions, lubricating oil additives, textile antistatic and finishing agents, plasticizers, cosmetics, industrial solvents, and solvents for fats.

[0015] Esters have many commercial uses. For example, biodiesel, an alternative fuel, is comprised of esters (e.g., fatty acid methyl ester, fatty acid ethyl esters, etc.). Some low molecular weight esters are volatile with a pleasant odor which makes them useful as fragrances or flavoring agents. In addition, esters are used as solvents for lacquers, paints, and varnishes. Furthermore, some naturally occurring substances, such as waxes, fats, and oils are comprised of esters. Esters are also used as softening agents in resins and plastics, plasticizers, flame retardants, and additives in gasoline and oil. In addition, esters can be used in the manufacture of polymers, films, textiles, dyes, and pharmaceuticals.

[0016] Aldehydes are used to produce many specialty chemicals. For example, aldehydes are used to produce polymers, resins, dyes, flavorings, plasticizers, perfumes, pharmaceuticals, and other chemicals. Some are used as solvents, preservatives, or disinfectants. Some natural and synthetic compounds, such as vitamins and hormones, are aldehydes. In addition, many sugars contain aldehyde groups.

[0017] Ketones are used commercially as solvents. For example, acetone is frequently used as a solvent, but it is also a raw material for making polymers. Ketones are also used in lacquers, paints, explosives, perfumes, and textile processing. In addition, ketones are used to produce alcohols, alkenes, alkanes, imines, and enamines.

[0018] In addition, crude petroleum is a source of lubricants. Lubricants derived petroleum are typically composed of olefins, particularly polyolefins and alpha-olefins. Lubricants can either be refined from crude petroleum or manufactured using the raw materials refined from crude petroleum.

[0019] Obtaining these specialty chemicals from crude petroleum requires a significant financial investment as well as a great deal of energy. It is also an inefficient process because frequently the long chain hydrocarbons in crude petroleum are cracked to produce smaller monomers. These monomers are then used as the raw material to manufacture the more complex specialty chemicals.

[0020] In addition to the problems with exploring, extracting, transporting, and refining petroleum, petroleum is a limited and dwindling resource. One estimate of current world petroleum consumption is 30 billion barrels per year. By some estimates, it is predicted that at current production levels, the world's petroleum reserves could be depleted before the year 2050.

[0021] Finally, the burning of petroleum based fuels releases greenhouse gases (e.g., carbon dioxide) and other forms of air pollution (e.g., carbon monoxide, sulfur dioxide, etc.). As the world's demand for fuel increases, the emission of greenhouse gases and other forms of air pollution also increases. The accumulation of greenhouse gases in the atmosphere leads to an increase in global warming Hence, in addition to damaging the environment locally (e.g., oil spills, dredging of marine environments, etc.), burning petroleum also damages the environment globally.

[0022] Due to the inherent challenges posed by petroleum, there is a need for a renewable petroleum source which does not need to be explored, extracted, transported over long distances, or substantially refined like petroleum. There is also a need for a renewable petroleum source that can be produced economically. In addition, there is a need for a renewable petroleum source that does not create the type of environmental damage produced by the petroleum industry and the burning of petroleum based fuels. For similar reasons, there is also a need for a renewable source of chemicals that are typically derived from petroleum.

SUMMARY OF THE INVENTION

[0023] The invention provides a fermentation and recovery process for the production of biodiesel of commercial grade quality according to commercial standards (e.g., ASTM or ANP) as well as environmental standards (e.g., those promulgated by the United States Environmental Protection Agency (EPA), and similar agencies elsewhere) by fermentation of carbohydrates using a genetically modified microorganism. The process provides a direct route for the production of fatty esters, for example fatty acid esters, and especially fatty acid methyl esters, without the need for producing oils which are later chemically transesterified with the concomitant production of large quantities of glycerol. The biodiesels produced, alone or blended with petroleum diesel according to customary proportions, result in clean emissions profiles and low amounts of impurities and/or undesirable contaminants.

[0024] The invention provides a recombinant cell comprising (a) at least one gene encoding a fatty acid derivative enzyme, which gene is modified such that the gene is overexpressed, and (b) a gene encoding a fatty acid degradation enzyme, which gene is modified such that expression of the gene is attenuated.

[0025] The invention also provides a recombinant cell capable of producing esters, wherein the cell is modified to include at least one exogenous nucleic acid sequence encoding a fatty acid derivative enzyme.

[0026] The invention further provides a recombinant cell comprising (a) an exogenous nucleic acid sequence encoding a thioesterase; (b) an exogenous nucleic acid sequence encoding an acyl-CoA synthase; (c) an exogenous nucleic acid sequence encoding a wax synthase; and (d) a gene encoding a fatty acid degradation enzyme, wherein the gene is modified such that expression of the gene is attenuated.

[0027] The invention additionally provides a composition produced by the recombinant cell as described herein, comprising fatty esters produced from the recombinant cell.

[0028] The invention further provides a fuel composition, including, for example, a biodiesel composition, comprising the fatty esters produced by the recombinant cells in accordance to the description herein. In certain embodiments, the fuel composition also comprises one or more suitable fuel additives.

[0029] The invention also provides a method for producing fatty esters in a recombinant cell comprising (a) obtaining the recombinant cell, (b) culturing the recombinant cell under suitable conditions for expression, and (c) obtaining fatty esters.

[0030] The drawings and examples provided herein are intended solely to illustrate the features of the present invention. They are not intended to be limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

[0031] FIG. **1** is a diagram illustrating the cloning methods used to generate the plasmid pCLTFWcat.

[0032] FIG. **2** is a diagram illustrating the cloning methods used to generate the integration fragment lacZ:: tesA fadD atfA1.

[0033] FIG. **3** is the nucleotide sequence of the integration fragment lacZ:: tesA fadD atfA1.

[0034] FIG. **4** shows the cycle engine speed and torque of the 2008 model year 9.3 L 330 horsepower International MaxxForce 10 engine, which was used in the emissions testing conducted by National Renewable Energy Laboratory of Denver, Colo.

[0035] FIG. **5** indicates and compares the levels of NO_X and CO emissions as well as the levels of fuel consumption by (1) the 2007 Certification Ultra Low Sulfur Diesel (ULSD, Haltermann Product, Channelview, Tex.), which was used as a baseline fuel and a petroleum-based blend stock for the biodiesel blends in the emissions testing; (2) the SOY B20 biodiesel blend; and (3) the FAE B20 biodiesel blend.

[0036] FIG. **6** lists the nucleotide sequence of the plasmid pLacZ (SEQ ID NO:28).

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations and Terms

[0037] The following explanations of terms and methods are provided to facilitate understanding of the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure.

[0038] The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0039] As used herein, the term "alcohol dehydrogenase" (EC 1.1.1.*) is a peptide capable of catalyzing the conversion of a fatty aldehyde to an alcohol (e.g., fatty alcohol). Additionally, one of ordinary skill in the art will appreciate that some alcohol dehydrogenases will catalyze other reactions as well. For example, some alcohol dehydrogenases will accept other substrates in addition to fatty aldehydes. Such non-specific alcohol dehydrogenases are, therefore, also included in this definition.

[0040] As used herein, the term "aldehyde" means a hydrocarbon having the formula RCHO characterized by an unsaturated carbonyl group (C═O). In a preferred embodiment, the aldehyde is any aldehyde made from a fatty acid or fatty acid derivative.

[0041] In one embodiment, the R group is at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbons in length. R can be straight or branched chain. The branched chains may have one or more points of branching. In addition, the branched chains may include cyclic branches. Furthermore, R can be saturated or unsaturated. If unsaturated, the R can have one or more points of unsaturation.

[0042] In one embodiment, the fatty aldehyde is produced biosynthetically.

[0043] Fatty aldehydes have many uses. For example, fatty aldehydes can be used to produce many specialty chemicals. For example, fatty aldehydes are used to produce polymers, resins, dyes, flavorings, plasticizers, perfumes, pharmaceuticals, and other chemicals. Some are used as solvents, preservatives, or disinfectants. Some natural and synthetic compounds, such as vitamins and hormones, are aldehydes.

[0044] As used herein, an "aldehyde biosynthetic gene" or an "aldehyde biosynthetic polynucleotide" is a nucleic acid that encodes an aldehyde biosynthetic polypeptide.

[0045] As used herein, an "aldehyde biosynthetic polypeptide" is a polypeptide that is a part of the biosynthetic pathway of an aldehyde. Such polypeptides can act on a biological substrate to yield an aldehyde. In some instances, the aldehyde biosynthetic polypeptide has reductase activity.

[0046] As used herein, the term "alkane" means a hydrocarbon containing only single carbon-carbon bonds.

[0047] As used herein, an "alkane biosynthetic gene" or an "alkane biosynthetic polynucleotide" is a nucleic acid that encodes an alkane biosynthetic polypeptide.

[0048] As used herein, an "alkane biosynthetic polypeptide" is a polypeptide that is a part of the biosynthetic pathway of an alkane. Such polypeptides can act on a biological substrate to yield an alkane. In some instances, the alkane biosynthetic polypeptide has decarbonylase activity.

[0049] As used herein, an "alkene biosynthetic gene" or an "alkene biosynthetic polynucleotide" is a nucleic acid that encodes an alkene biosynthetic polypeptide.

[0050] As used herein, an "alkene biosynthetic polypeptide" is a polypeptide that is a part of the biosynthetic pathway of an alkene. Such polypeptides can act on a biological substrate to yield an alkene. In some instances, the alkene biosynthetic polypeptide has decarbonylase activity.

[0051] As used herein, the term "attenuate" means to weaken, reduce, or diminish For example, a polypeptide can be attenuated by modifying the polypeptide to reduce its activity (e.g., by modifying a nucleotide sequence that encodes the polypeptide).

[0052] As used herein, the term "base oil" refers to a building block of a lubricant or fuel additive. A base oil is typically used as a solvent for formulating an additive package for. Depending on the grade and/or type of base oil, it may provide a varying degree of performance benefit to an additive package, including, for example, extreme temperature benefits, anti-oxidative benefits, or a suitable pour point. Additive packages are commonly used to improve the service life and performance of finished oil or fuel products.

[0053] As used herein, the term "biocrude" refers to a product derived from biomass, biomass derivatives, or other biological sources that, like petroleum crude, can be converted into other fuels. For example, biocrude can be converted into gasoline, diesel, jet fuel, or heating oil. Moreover, biocrude, like petroleum crude, can be converted into other industrially useful chemicals for use in, for example, pharmaceuticals, cosmetics, consumer goods, industrial processes, and the like.

[0054] Biocrude may include, for example, hydrocarbons, hydrocarbon products, fatty acid esters, and/or aliphatic ketones. In a preferred embodiment, biocrude is comprised of hydrocarbons, for example aliphatic (e.g., alkanes, alkenes, alkynes) or aromatic hydrocarbons.

[0055] As used herein, the term "biodiesel" means a biofuel that can be a substitute of diesel, which is derived from petroleum. Biodiesel can be used in internal combustion diesel engines in either a pure form, which is referred to as "neat" biodiesel, or as a mixture in any concentration with petroleum-based diesel. In one embodiment, biodiesel can include esters or hydrocarbons, such as aldehydes, alkanes, or alkenes.

[0056] As used herein, the term "biofuel" refers to any fuel derived from biomass, biomass derivatives, or other biological sources. Biofuels can be substituted for petroleum based fuels. For example, biofuels are inclusive of transportation fuels (e.g., gasoline, diesel, jet fuel, etc.), heating fuels, and electricity-generating fuels. Biofuels are a renewable energy source.

[0057] As used herein, the term "biomass" refers to a carbon source derived from biological material. Biomass can be converted into a biofuel. One exemplary source of biomass is

plant matter. For example, corn, sugar cane, or switchgrass can be used as biomass. Another non-limiting example of biomass is animal matter, for example cow manure. Biomass also includes waste products from industry, agriculture, forestry, and households. Examples of such waste products that can be used as biomass are fermentation waste, straw, lumber, sewage, garbage, and food leftovers. Biomass also includes sources of carbon, such as carbohydrates (e.g., monosaccharides, disaccharides, or polysaccharides).

[0058] As used herein, the phrase "carbon source" refers to a substrate or compound suitable to be used as a source of carbon for prokaryotic or simple eukaryotic cell growth. Carbon sources can be in various forms, including, but not limited to polymers, carbohydrates, acids, alcohols, aldehydes, ketones, amino acids, peptides, and gases (e.g., CO and CO_2). These include, for example, various monosaccharides, such as glucose, fructose, mannose, and galactose; oligosaccharides, such as fructo-oligosaccharide and galacto-oligosaccharide; polysaccharides such as xylose and arabinose; disaccharides, such as sucrose, maltose, and turanose; cellulosic material, such as methyl cellulose and sodium carboxymethyl cellulose; saturated or unsaturated fatty acid esters, such as succinate, lactate, and acetate; alcohols, such as methanol, ethanol, propanol, or mixtures thereof. The carbon source can also be a product of photosynthesis, including, but not limited to, glucose. A preferred carbon source is biomass. Another preferred carbon source is glucose.

[0059] As used herein, a "cloud point lowering additive" is an additive added to a composition to decrease or lower the cloud point of a solution.

[0060] As used herein, the phrase "cloud point of a fluid" means the temperature at which dissolved solids are no longer completely soluble. Below this temperature, solids begin precipitating as a second phase giving the fluid a cloudy appearance. In the petroleum industry, cloud point refers to the temperature below which a solidified material or other heavy hydrocarbon crystallizes in a crude oil, refined oil, or fuel to form a cloudy appearance. The presence of solidified materials influences the flowing behavior of the fluid, the tendency of the fluid to clog fuel filters, injectors, etc., the accumulation of solidified materials on cold surfaces (e.g., a pipeline or heat exchanger fouling), and the emulsion characteristics of the fluid with water.

[0061] A nucleotide sequence is "complementary" to another nucleotide sequence if each of the bases of the two sequences matches (e.g., is capable of forming Watson Crick base pairs). The term "complementary strand" is used herein interchangeably with the term "complement". The complement of a nucleic acid strand can be the complement of a coding strand or the complement of a non-coding strand.

[0062] The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (e.g., meaning "including, but not limited to,") unless otherwise noted.

[0063] As used herein, the term "conditions sufficient to allow expression" means any conditions that allow a host cell to produce a desired product, such as a polypeptide, aldehyde, or alkane described herein. Suitable conditions include, for example, fermentation conditions. Fermentation conditions can comprise many parameters, such as temperature ranges, levels of aeration, and media composition. Each of these conditions, individually and in combination, allows the host cell to grow. Exemplary culture media include broths or gels. Generally, the medium includes a carbon source, such as glucose, fructose, cellulose, or the like, that can be metabolized by a host cell directly. In addition, enzymes can be used in the medium to facilitate the mobilization (e.g., the depolymerization of starch or cellulose to fermentable sugars) and subsequent metabolism of the carbon source.

[0064] To determine if conditions are sufficient to allow expression, a host cell can be cultured, for example, for about 4, 8, 12, 24, 36, or 48 hours. During and/or after culturing, samples can be obtained and analyzed to determine if the conditions allow expression. For example, the host cells in the sample or the medium in which the host cells were grown can be tested for the presence of a desired product. When testing for the presence of a product, assays, such as, but not limited to, TLC, HPLC, GC/FID, GC/MS, LC/MS, MS, can be used.

[0065] It is understood that the polypeptides described herein may have additional conservative or non-essential amino acid substitutions, which do not have a substantial effect on the polypeptide functions. Whether or not a particular substitution will be tolerated (e.g., will not adversely affect desired biological properties, such as decarboxylase activity) can be determined as described in Bowie et al., *Science (*1990) 247:1306 1310. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, and histidine), acidic side chains (e.g., aspartic acid and glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, and cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, and tryptophan), beta-branched side chains (e.g., threonine, valine, and isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, and histidine).

[0066] As used herein, "conditions that permit product production" refers to any fermentation conditions that allow a production host to produce a desired product, such as acyl-CoA or fatty acid derivatives (e.g., fatty acids, hydrocarbons, fatty alcohols, waxes, or fatty esters). Fermentation conditions usually comprise many parameters. Exemplary conditions include, but are not limited to, temperature ranges, levels of aeration, and media composition. Each of these conditions, individually and/or in combination, allows the production host to grow.

[0067] Exemplary media include broths and/or gels. Generally, a suitable medium includes a carbon source (e.g., glucose, fructose, cellulose, etc.) that can be metabolized by the microorganism directly. In addition, enzymes can be used in the medium to facilitate the mobilization (e.g., the depolymerization of starch or cellulose to fermentable sugars) and subsequent metabolism of the carbon source.

[0068] To determine if the fermentation conditions permit product production, the production host can be cultured for about 4, 8, 12, 24, 36, or 48 hours. During culturing or after culturing, samples can be obtained and analyzed to determine if the fermentation conditions have permitted product production. For example, the production hosts in the sample or the medium in which the production hosts are grown can be tested for the presence of the desired product. Exemplary assays, such as TLC, HPLC, GC/FID, GC/MS, LC/MS, MS, as well as those provided herein, can be used identify and quantify the presence of a product.

[0069] As used herein, "control element" means a transcriptional control element. Control elements include promoters and enhancers. The term "promoter element," "promoter," or "promoter sequence" refers to a DNA sequence that functions as a switch that activates the expression of a gene. If the gene is activated, it is said to be transcribed or participating in transcription. Transcription involves the synthesis of mRNA from the gene. A promoter, therefore, serves as a transcriptional regulatory element and also provides a site for initiation of transcription of the gene into mRNA. Control elements interact specifically with cellular proteins involved in transcription (Maniatis et al., *Science* 236:1237, 1987).

[0070] As used herein, the term "deletion," or "knockout" means modifying or inactivating a polynucleotide sequence that encodes a target protein in order to reduce or eliminate the function of the target protein. A polynucleotide deletion can be performed by methods well known in the art (See, e.g., Datsenko et al., *Proc. Nat. Acad. Sci. USA,* 97:6640-45, 2000 or International Patent Application Nos. PCT/US2007/011923 and PCT/US2008/058788)

[0071] As used herein, the term "demulsifier" refers to a surfactant that breaks an emulsion formed when an oil or a hydrophobic substance (e.g., a fuel) is mixed with water or an aqueous substance. A demulsifier allows the oil and water phases to separate.

[0072] As used herein, the term "a dispersant additive" means a surface active agent added to a suspending medium to promote uniform and maximum separation of extremely fine solid particles, often of colloidal size. A dispersant additive can be used to maintain a suspension of insoluble materials produced from the oxidation and degradation of fuel that occurs when a diesel engine is operated. The dispersant additive can prevent sludge flocculation and precipitation or deposition on metal parts. In a preferred embodiment, ashless dispersant additives are used. An ashless dispersant additive is a dispersant that does not contain metal ions, but typically comprises a material having an oil-soluble polymeric hydrocarbon backbone with functional groups that are capable of associating with the particles to be dispersed. Many ashless dispersant additives are well known in the art. They include, without limitation, carboxylic dispersants, succinimide dispersants, amine dispersants, and Mannich dispersants.

[0073] As used herein, the term "endogenous" means a polynucleotide that is in the cell and was not introduced into the cell using recombinant genetic engineering techniques. For example, a gene that was present in the cell when the cell was originally isolated from nature. A polynucleotide is still considered endogenous if the control sequences, such as a promoter or enhancer sequences which activate transcription or translation, have been altered through recombinant techniques.

[0074] As used herein, the term "ester synthase" means a peptide capable of producing fatty esters. More specifically, an ester synthase is a peptide which converts a thioester to a fatty ester. In a preferred embodiment, the ester synthase converts a thioester (e.g., acyl-CoA) to a fatty ester.

[0075] In an alternate embodiment, an ester synthase uses a thioester and an alcohol as substrates to produce a fatty ester. Ester synthases are capable of using short and long chain thioesters as substrates. In addition, ester synthases are capable of using short and long chain alcohols as substrates.

[0076] Non-limiting examples of ester synthases are wax synthases, wax-ester synthases, acyl CoA:alcohol transacylases, acyltransferases, and fatty acyl-coenzyme A:fatty alcohol acyltransferases. Exemplary ester synthases are classified in enzyme classification number EC 2.3.1.75. A number of these enzymes, as well as other useful enzymes for making the products described herein, have been disclosed in, for example, International Patent Application Nos. PCT/US2007/011923 and PCT/US2008/058788, which are incorporated herein by reference.

[0077] As used herein, the term "exogenous" means a polynucleotide that does not originate from a particular cell as found in nature. For example, "exogenous polynucleotide" could refer to a polynucleotide that was inserted within the genomic polynucleotide sequence of a microorganism or to an extra chromosomal polynucleotide that was introduced into the microorganism. Thus, a non-naturally-occurring polynucleotide is considered to be exogenous to a cell once introduced into the cell. A polynucleotide that is naturally-occurring can also be exogenous to a particular cell. For example, an entire polynucleotide isolated from a first cell can be an exogenous polynucleotide with respect to a second cell if that polynucleotide from the first cell is introduced into the second cell.

[0078] As used herein, the term "fatty acid" means a carboxylic acid having the formula RCOOH. R represents an aliphatic group, preferably an alkyl group. R can comprise between about 4 and about 22 carbon atoms. Fatty acids can be saturated, monounsaturated, or polyunsaturated. In a preferred embodiment, the fatty acid is made from a fatty acid biosynthetic pathway.

[0079] As used herein, the term "fatty acid biosynthetic pathway" means a biosynthetic pathway that produces fatty acids. The fatty acid biosynthetic pathway includes fatty acid enzymes that can be engineered, as described herein, to produce fatty acids, and in some embodiments can be expressed with additional enzymes to produce fatty acids having desired carbon chain characteristics.

[0080] As used herein, the term "fatty acid degradation enzyme" means an enzyme involved in the breakdown or conversion of a fatty acid or fatty acid derivative into another product. A nonlimiting example of a fatty acid degradation enzyme is an acyl-CoA synthase. A number of these enzymes, as well as other useful enzymes for making the products described herein, have been disclosed in, for example, International Patent Application Nos. PCT/US2007/011923 and PCT/US2008/058788, which are incorporated herein by reference. Additional examples of fatty acid degradation enzymes are described herein.

[0081] As used herein, the term "fatty acid derivative" means products made in part from the fatty acid biosynthetic pathway of the production host organism. "Fatty acid derivative" also includes products made in part from acyl-ACP or acyl-ACP derivatives. The fatty acid biosynthetic pathway includes fatty acid synthase enzymes which can be engineered as described herein to produce fatty acid derivatives, and in some examples can be expressed with additional enzymes to produce fatty acid derivatives having desired carbon chain characteristics. Exemplary fatty acid derivatives include for example, fatty acids, acyl-CoAs, fatty aldehydes, short and long chain alcohols, hydrocarbons, fatty alcohols, ketones, and esters (e.g., waxes, fatty acid esters, or fatty esters).

[0082] As used herein, the term "fatty acid derivative enzymes" means all enzymes that may be expressed or overexpressed in the production of fatty acid derivatives. These enzymes are collectively referred to herein as fatty acid

derivative enzymes. These enzymes may be part of the fatty acid biosynthetic pathway. Non-limiting examples of fatty acid derivative enzymes include fatty acid synthases, thioesterases, acyl-CoA synthases, acyl-CoA reductases, alcohol dehydrogenases, alcohol acyltransferases, carboxylic acid reductases, fatty alcohol-forming acyl-CoA reductase, ester synthases, aldehyde biosynthetic polypeptides, and alkane biosynthetic polypeptides. Fatty acid derivative enzymes convert a substrate into a fatty acid derivative. In some examples, the substrate may be a fatty acid derivative which the fatty acid derivative enzyme converts into a different fatty acid derivative. A number of these enzymes, as well as other useful enzymes for making the products described herein, have been disclosed in, for example, International Patent Application Nos. PCT/US2007/011923 and PCT/US2008/058788, which are incorporated herein by reference.

[0083] As used herein, "fatty acid enzyme" means any enzyme involved in fatty acid biosynthesis. Fatty acid enzymes can be expressed or overexpressed in host cells to produce fatty acids. Non-limiting examples of fatty acid enzymes include fatty acid synthases and thioesterases. A number of these enzymes, as well as other useful enzymes for making the products described herein, have been disclosed in, for example, International Patent Application Nos. PCT/US2007/011923 and PCT/US2008/058788, which are incorporated herein by reference.

[0084] As used herein, the term "fatty alcohol" means an alcohol having the formula ROH. In a preferred embodiment, the fatty alcohol is any alcohol made from a fatty acid or fatty acid derivative.

[0085] In one embodiment, the R group is at least about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbons in length. R can be straight or branched chain. The branched chains may have one or more points of branching. In addition, the branched chains may include cyclic branches. Furthermore, R can be saturated or unsaturated. If unsaturated, the R can have one or more points of unsaturation.

[0086] In one embodiment, the fatty alcohol is produced biosynthetically.

[0087] Fatty alcohols have many uses. For example, fatty alcohols can be used to produce many specialty chemicals. For example, fatty alcohols are used as a biofuel; as solvents for fats, waxes, gums, and resins; in pharmaceutical salves, emollients and lotions; as lubricating-oil additives; in detergents and emulsifiers; as textile antistatic and finishing agents; as plasticizers; as nonionic surfactants; and in cosmetics, for examples as thickeners.

[0088] As used herein, the term "fatty ester" means an ester. In a preferred embodiment, a fatty ester is any ester made from a fatty acid to produce, for example, a fatty acid ester. In one embodiment, a fatty ester contains an A side (i.e., the carbon chain attached to the carboxylate oxygen) and a B side (i.e., the carbon chain comprising the parent carboxylate). In a preferred embodiment, when the fatty ester is derived from the fatty acid biosynthetic pathway, the A side is contributed by an alcohol, and the B side is contributed by a fatty acid. Any alcohol can be used to form the A side of the fatty esters. For example, the alcohol can be derived from the fatty acid biosynthetic pathway. Alternatively, the alcohol can be produced through non-fatty acid biosynthetic pathways. Moreover, the alcohol can be provided exogenously. For example, the alcohol can be supplied in the fermentation broth in instances where the fatty ester is produced by an organism that can also produce the fatty acid. Alternatively, a carboxylic acid, such as a fatty acid or acetic acid, can be supplied exogenously in instances where the fatty ester is produced by an organism that can also produce alcohol.

[0089] The carbon chains comprising the A side or B side can be of any length. In one embodiment, the A side of the ester is at least about 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, or 18 carbons in length. The B side of the ester is at least about 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, or 26 carbons in length. The A side and/or the B side can be straight or branched chain. The branched chains may have one or more points of branching. In addition, the branched chains may include cyclic branches. Furthermore, the A side and/or B side can be saturated or unsaturated. If unsaturated, the A side and/or B side can have one or more points of unsaturation.

[0090] In one embodiment, the fatty ester is produced biosynthetically. In this embodiment, first the fatty acid is "activated." Non-limiting examples of "activated" fatty acids are acyl-CoA, acyl-ACP, and acyl phosphate. Acyl-CoA can be a direct product of fatty acid biosynthesis or degradation. In addition, acyl-CoA can be synthesized from a free fatty acid, a CoA, or an adenosine nucleotide triphosphate (ATP). An example of an enzyme which produces acyl-CoA is acyl-CoA synthase

[0091] After the fatty acid is activated, it can be readily transferred to a recipient nucleophile. Exemplary nucleophiles are alcohols, thiols, or phosphates.

[0092] In one embodiment, the fatty ester is a wax. The wax can be derived from a long chain alcohol and a long chain fatty acid. In another embodiment, the fatty ester can be derived from a fatty acyl-thioester and an alcohol. In another embodiment, the fatty ester is a fatty acid thioester, for example fatty acyl Coenzyme A (CoA). In other embodiments, the fatty ester is a fatty acyl panthothenate, an acyl carrier protein (ACP), or a fatty phosphate ester. Fatty esters have many uses. For example, fatty esters can be used as biofuels, surfactants, or formulated into additives that provide lubrication and other benefits to fuels and industrial chemicals.

[0093] As used herein, "fraction of modern carbon" or "f_M" has the same meaning as defined by National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 4990B and 4990C, known as oxalic acids standards HOxI and HOxII, respectively. The fundamental definition relates to 0.95 times the $^{14}C/^{12}C$ isotope ratio HOxI (referenced to AD 1950). This is roughly equivalent to decay-corrected pre-Industrial Revolution wood. For the current living biosphere (e.g., plant material), f_M is approximately 1.1.

[0094] Calculations of "homology" between two sequences can be performed as follows. The sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence that is aligned for comparison purposes is at least about 30%, preferably at least about 40%, more preferably at least about 50%, even more preferably at least about 60%, and even more preferably at least about 70%, at least about 80%, at least about 90%, or about 100% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the

corresponding position in the second sequence, then the molecules are identical at that position (as used herein, amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[0095] The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent homology between two amino acid sequences is determined using the Needleman and Wunsch (1970), *J. Mol. Biol.* 48:444 453, algorithm that has been incorporated into the GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent homology between two nucleotide sequences is determined using the GAP program in the GCG software package, using a NWSgapdna. CMP matrix and a gap weight of about 40, 50, 60, 70, or 80 and a length weight of about 1, 2, 3, 4, 5, or 6. A particularly preferred set of parameters (and the one that should be used if the practitioner is uncertain about which parameters should be applied to determine if a molecule is within a homology limitation of the claims) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0096] Other methods for aligning sequences for comparison are well known in the art. Various programs and alignment algorithms are described in, for example, Smith & Waterman, *Adv. Appl. Math.* 2:482, 1981; Pearson & Lipman, *Proc. Natl. Acad. Sci.* USA 85:2444, 1988; Higgins & Sharp, *Gene* 73:237 244, 1988; Higgins & Sharp, *CABIOS* 5:151-153, 1989; Corpet et al., *Nucleic Acids Research* 16:10881-10890, 1988; Huang et al., *CABIOS* 8:155-165, 1992; and Pearson et al., *Methods in Molecular Biology* 24:307-331, 1994. and Altschul et al., *J. Mol. Biol.* 215:403-410, 1990.

[0097] As used herein, a "host cell" is a cell used to produce a product described herein (e.g., an aldehyde or alkane). A host cell can be modified to express or overexpress selected genes or to have attenuated expression of selected genes. Non-limiting examples of host cells include plant, animal, human, bacteria, cyanobacteria, yeast, or filamentous fungi cells.

[0098] As used herein, the term "hybridizes under low stringency, medium stringency, high stringency, or very high stringency conditions" describes conditions for hybridization and washing. Guidance for performing hybridization reactions can be found, for example, in *Current Protocols in Molecular Biology,* John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Aqueous and nonaqueous methods are described in that reference and either method can be used. Specific hybridization conditions referred to herein are as follows: 1) low stringency hybridization conditions in 6× sodium chloride/sodium citrate (SSC) at about 45° C., followed by two washes in 0.2×SSC, 0.1% SDS at least at 50° C. (the temperature of the washes can be increased to 55° C. for low stringency conditions); 2) medium stringency hybridization conditions in 6×SSC at about 45° C., followed by one or more washes in 0.2×SSC, 0.1% SDS at 60° C.; 3) high stringency hybridization conditions in 6×SSC at about 45° C., followed by one or more washes in 0.2.×SSC, 0.1% SDS at 65° C.; and preferably 4) very high stringency hybridization conditions are 0.5M sodium phosphate, 7% SDS at 65° C., followed by one or more washes at 0.2×SSC, 1% SDS at 65° C. Very high stringency conditions (4) are the preferred conditions unless otherwise specified.

[0099] The term "isolated" as used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs or RNAs, respectively, that are present in the natural source of the nucleic acid. Moreover, an "isolated nucleic acid" includes nucleic acid fragments, such as fragments that are not naturally occurring. The term "isolated" is also used herein to refer to polypeptides, which are isolated from other cellular proteins, and encompasses both purified endogenous polypeptides and recombinant polypeptides. The term "isolated" as used herein also refers to a nucleic acid or polypeptide that is substantially free of cellular material, viral material, or culture medium when produced by recombinant DNA techniques. The term "isolated" as used herein also refers to a nucleic acid or polypeptide that is substantially free of chemical precursors or other chemicals when chemically synthesized.

[0100] As used herein, the "level of expression of a gene in a cell" refers to the level of mRNA, pre-mRNA nascent transcript(s), transcript processing intermediates, mature mRNA (s), and/or degradation products encoded by the gene in the cell.

[0101] As used herein, the term "microorganism" means prokaryotic and eukaryotic microbial species from the domains Archaea, Bacteria and Eucarya, the latter including yeast and filamentous fungi, protozoa, algae, or higher Protista. The term "microbial cell", as used herein, means a cell from a microorganism.

[0102] As used herein, the term "nucleic acid" refers to a polynucleotide, such as deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term also includes analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single (sense or antisense) and double-stranded polynucleotides, ESTs, chromosomes, cDNAs, mRNAs, and rRNAs. The term "nucleic acid" may be used interchangeably with "polynucleotide," "DNA," "nucleic acid molecule," "nucleotide sequence," and/or "gene" unless otherwise indicated herein or otherwise clearly contradicted by context.

[0103] As used herein, the term "operably linked" means that a selected nucleotide sequence (e.g., encoding a polypeptide described herein) is in proximity with a promoter to allow the promoter to regulate expression of the selected nucleotide sequence. In addition, the promoter is located upstream of the selected nucleotide sequence in terms of the direction of transcription and translation. By "operably linked" is meant that a nucleotide sequence and a regulatory sequence(s) are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s).

[0104] The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

[0105] As used herein, "overexpress" means to express or cause to be expressed or produced a nucleic acid, polypeptide, or hydrocarbon in a cell at a greater concentration than is normally expressed in a corresponding wild-type cell. For example, a polypeptide can be "overexpressed" in a recombinant host cell when the polypeptide is present in a greater

concentration in the recombinant host cell compared to its concentration in a non-recombinant host cell of the same species.

[0106] As used herein, "partition coefficient" or "P," is defined as the equilibrium concentration of a compound in an organic phase divided by the concentration at equilibrium in an aqueous phase (e.g., fermentation broth). In one embodiment of a bi-phasic system described herein, the organic phase is formed by the aldehyde or alkane during the production process. However, in some examples, an organic phase can be provided, such as by providing a layer of octane, to facilitate product separation. When describing a two phase system, the partition characteristics of a compound can be described as logP. For example, a compound with a logP of 1 would partition 10:1 to the organic phase. A compound with a logP of −1 would partition 1:10 to the organic phase. By choosing an appropriate fermentation broth and organic phase, an organic fatty acid derivative or product with a high logP value can separate into the organic phase even at very low concentrations in the fermentation vessel.

[0107] As used herein, the term "polypeptide" may be used interchangeably with "protein," "peptide," and/or "enzyme" unless otherwise indicated herein or otherwise clearly contradicted by context.

[0108] As used herein, the term "production host" means a cell used to produce the products disclosed herein. The production host is modified to express, overexpress, attenuate or delete expression of selected polynucleotides. Non-limiting examples of production hosts include plant, algal, animal, human, bacteria, yeast, and filamentous fungi cells.

[0109] As used herein, the term "purify," "purified," or "purification" means the removal or isolation of a molecule from its environment by, for example, isolation or separation. "Substantially purified" molecules are at least about 60% free, preferably at least about 75% free, and more preferably at least about 90% free from other components with which they are associated. As used herein, these terms also refer to the removal of contaminants from a sample. For example, the removal of contaminants can result in an increase in the percentage of a fatty acid derivative or product in a sample. For example, when a fatty acid derivatives or products are produced in a host cell, the fatty acid derivatives or products can be purified by the removal of host cell proteins. After purification, the percentage of fatty acid derivatives or products in the sample is increased.

[0110] The terms "purify," "purified," and "purification" do not require absolute purity. They are relative terms. Thus, for example, when the fatty acid derivatives or products are produced in host cells, a purified fatty acid derivative or product is one that is substantially separated from other cellular components (e.g., nucleic acids, polypeptides, lipids, carbohydrates, or other fatty acid derivatives or products). In another example, a purified fatty acid derivative or purified product preparation is one in which the fatty acid derivative or product is substantially free from contaminants, such as those that might be present following fermentation. In some embodiments, a fatty acid derivative or product is purified when at least about 50% by weight of a sample is composed of the fatty acid derivative or product. In other embodiments, a fatty acid derivative or product is purified when at least about 60%, 70%, 80%, 85%, 90%, 92%, 95%, 98%, or 99% or more by weight of a sample is composed of the fatty acid derivative or product.

[0111] As used herein, the term "recombinant polypeptide" refers to a polypeptide that is produced by recombinant DNA techniques, wherein generally DNA encoding the expressed polypeptide or RNA is inserted into a suitable expression vector and that is in turn used to transform a host cell to produce the polypeptide or RNA.

[0112] As used herein, the term "substantially identical" (or "substantially homologous") is used to refer to a first amino acid or nucleotide sequence that contains a sufficient number of identical or equivalent (e.g., with a similar side chain) amino acid residues (e.g., conserved amino acid substitutions) or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have similar activities.

[0113] As used herein, the term "surfactants" means a substance capable of reducing the surface tension of a liquid in which it is dissolved. A surfactant is typically composed of a water-soluble head and a hydrocarbon chain or tail. The water soluble head is hydrophilic and can be either ionic or non-ionic. The hydrocarbon chain is hydrophobic. Surfactants are used in a variety of products. For example, surfactants are used in the compositions or manufacture of detergents, cleaners, textiles, leather, paper, cosmetics, pharmaceuticals, processed foods, and agricultural products. In addition, surfactants can be used in the extraction and isolation of crude oils.

[0114] There are four major categories of surfactants which are characterized by their uses. Anionic surfactants have detergent-like activity and are generally used for cleaning applications. Cationic surfactants contain long chain hydrocarbons and are often used to treat proteins and synthetic polymers or are components of fabric softeners and hair conditioners. Amphoteric surfactants also contain long chain hydrocarbons, but are typically used in shampoos. Non-ionic surfactants are generally used in cleaning products.

[0115] As used herein, the term "synthase" means an enzyme which catalyzes a synthesis process. As used herein, the term synthase includes synthases, synthetases, and ligases.

[0116] As used herein, the term "transfection" means the introduction of a nucleic acid (e.g., via an expression vector) into a recipient cell by nucleic acid-mediated gene transfer.

[0117] As used herein, the term "transformation" refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous nucleic acid. This may result in the transformed cell expressing a recombinant form of a RNA or polypeptide. In the case of antisense expression from the transferred gene, the expression of a naturally-occurring form of the polypeptide is disrupted.

[0118] As used herein, the term "transport protein" means a polypeptide that facilitates the movement of one or more compounds in and/or out of a cellular organelle and/or a cell. A number of these proteins, as well as other useful proteins for making the products described herein, have been disclosed in, for example, International Patent Application Nos. PCT/US2007/011923 and PCT/US2008/058788, which are incorporated herein by reference.

[0119] As used herein, the term "unrefined, refined and re-refined oils" refers to natural oil, synthetic oil, or a mixture thereof, which may be used as a base oil in blending additive packages. Unrefined oils are those obtained directly from a natural or synthetic source without further purification treatment. Refined oils are similar to the unrefined oils except that they have been further treated in one or more purification steps. These purification steps include, for example, solvent

extraction, secondary distillation, acid or base extraction, filtration, percolation, or other methods well known in the art. Re-refined oils are oils that have been used, but are subsequently treated so that they may be reused. Re-refined oils are also known as reclaimed or reprocessed oils.

[0120] As used herein, a "variant" of polypeptide X refers to a polypeptide having the amino acid sequence of polypeptide X in which one or more amino acid residues is altered. The variant may have conservative changes or nonconservative changes. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without affecting biological activity may be found using computer programs well known in the art, for example, LASERGENE software (DNASTAR).

[0121] The term "variant," when used in the context of a polynucleotide sequence, may encompass a polynucleotide sequence related to that of a gene or the coding sequence thereof. This definition may also include, for example, "allelic," "splice," "species," or "polymorphic" variants. A splice variant may have significant identity to a reference polynucleotide, but will generally have a greater or fewer number of polynucleotides due to alternative splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. The resulting polypeptides generally will have significant amino acid identity relative to each other. A polymorphic variant is a variation in the polynucleotide sequence of a particular gene between individuals of a given species.

[0122] As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of useful vector is an episome (i.e., a nucleic acid capable of extra-chromosomal replication). Useful vectors are those capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids," which refer generally to circular double stranded DNA loops that, in their vector form, are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably, as the plasmid is the most commonly used form of vector. However, also included are such other forms of expression vectors that serve equivalent functions and that become known in the art subsequently hereto.

[0123] As used herein, the term "wax" means a composition comprised of fatty esters. In a preferred embodiment, the fatty ester in the wax is comprised of medium to long carbon chains. In addition to fatty esters, a wax may comprise other components (e.g., hydrocarbons, sterol esters, aliphatic aldehydes, alcohols, ketones, beta-diketones, triacylglycerols, etc.).

[0124] Throughout the specification, a reference may be made using an abbreviated gene name or polypeptide name, but it is understood that such an abbreviated gene or polypeptide name represents the genus of genes or polypeptides. Such gene names include all genes encoding the same polypeptide and homologous polypeptides having the same physiological function. Polypeptide names include all polypeptides that have the same activity (e.g., that catalyze the same fundamental chemical reaction).

[0125] The accession numbers referenced herein are derived from the NCBI database (National Center for Biotechnology Information) maintained by the National Institute of Health, U.S.A. Unless otherwise indicated, the accession numbers are as provided in the database as of April 2009.

[0126] EC numbers are established by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) (available at http://www.chem.qmul.ac.uk/iubmb/enzyme/). 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.

[0127] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0128] Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context.

[0129] Unless otherwise stated, amounts listed in percentage (%) are in weight percent, based on the total weight of the composition.

[0130] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[0131] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

[0132] The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0133] Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Genetically Modified Microorganism

[0134] The invention provides a recombinant cell comprising at least one gene encoding a fatty acid derivative enzyme, which gene is modified such that the gene is overexpressed. In one embodiment, the modified gene encoding a fatty acid derivative enzyme is a gene encoding an acyl-CoA synthase, a thioesterase, an ester synthase, an alcohol acyltransferase, an alcohol dehydrogenase, an acyl-CoA reductase, or a fatty-alcohol forming acyl-CoA reductase. For example, the modified gene encoding a fatty acid derivative enzyme can be a gene encoding an acyl-CoA synthase, a thioesterase, or an ester synthase.

[0135] The acyl-CoA synthase gene can be fadD, fadK, BH3103, yhfL, Pfl-4354, EAV15023, fadD1, fadD2, RPC_4074, fadDD35, fadDD22, faa3p, or a gene encoding ZP_01644857. Preferably, the acyl-CoA synthase gene is fadDD35 from *M. tuberculosis* HR7Rv [NP_217021], yhfL from *B. subtilis* [NP_388908], fadD1 from *P. aeruginosa* PAO1 [NP_251989], a gene encoding ZP_01644857 from *Stenotrophomonas maltophilia* R551-3, or faa3p from *Saccharomyces cerevisiae* [NP_012257].

[0136] The thioesterase gene can be tesA, 'tesA, tesB, fatB, fatB2, fatB3, fatB [M141T], fatA or fatA1.

[0137] The ester synthase gene can be obtained from a variety of organisms including, without limitation, *Acidobacteria, Acidothermus, Acinetobacter, Aeromonas, Alcaligenes, Alcanivorax, Alteromonas, Anaeromyxobacter, Arabidopsis, Bradyrhizobium, Cryptococcus, Erythrobacter, Frankia, Fundibacter, gamma proteobacterium, Hahella, Homo sapiens, Janibacter, Limnobacter, marine gamma proteobacterium, Marinobacter, Methylibium, Microscilla, Moritella, Mus musculus, Mycobacterium, Myxococcus, Natronomonas, Nocardia, Nocardioides, Photobacterium, Plesiocystis, Polaromonas, Psudomonas, Psychrobacter, Reinekea, Rhodococcus, Rhodoferax, Roseiflexus, Saccharomyces, Saccharopolyspora, Salinibacter, Simmodsia, Solibacter, Sphingopyxis, Stigmatella, Streptomyces, Tenacibaculum,* or *Ustilago.* Preferably, the ester synthase gene is wax/dgat. The ester synthase gene also can be obtained from *Mortierella alpina, Cryptococcus curvatus, Alcanivorax jadensis, Acinetobacter* sp. HO1-N or *Rhodococcus opacus.* For example, the ester synthase gene can be a bifunctional ester synthase/acyl-CoA:diacylglycerol acyltransferase from *Simmondsia chinensis, Acinetobacter* sp. strain ADP1, *Alcanivorax borkumensis, Pseudomonas aeruginosa, Fundibacter jadensis, Arabidopsis thaliana,* or *Alkaligenes eutrophus.*

[0138] In another embodiment, the cell comprises a second modified fatty acid derivative enzyme gene, wherein the second gene encodes an acyl-CoA synthase, a thioesterase, or an ester synthase. For example, the cell can comprise a modified gene encoding an acyl-CoA synthase and a modified gene encoding a thioesterase or an ester synthase.

[0139] In another embodiment, the cell comprises a modified gene encoding an acyl-CoA synthase, a modified gene encoding a thioesterase, and a modified gene encoding an ester synthase. The modified gene encoding an ester synthase can be a gene encoding a wax synthase, a wax-ester synthase, an acyl-CoA:alcohol transacylases, an alcohol O-fatty acid acyltransferase, an acyltransferases, or a fatty acyl-coenzyme A:fatty alcohol acyltransferase.

[0140] The invention also provides a recombinant cell capable of producing esters, wherein the cell is modified to comprise at least one exogenous nucleic acid sequence encoding a fatty acid derivative enzyme. In one embodiment, the exogenous nucleic acid sequence encoding a fatty acid derivative encodes an acyl-CoA synthase, a thioesterase, an ester synthase, an alcohol acyltransferase, an alcohol dehydrogenase, an acyl-CoA reductase, or a fatty-alcohol forming acyl-CoA reductase.

[0141] In some embodiments, the cell is modified to comprise at least two, at least three, or at least four exogenous nucleic acid sequences encoding a fatty acid derivative enzyme. In one embodiment, the cell is modified to comprise a first exogenous nucleic acid sequences encoding an acyl-CoA synthase, e.g., fadD, and a second exogenous nucleic acid sequence encoding a thioesterase or an ester synthase.

[0142] In some embodiments, the cell is modified to include at least three exogenous nucleic acid sequences encoding a fatty acid derivative enzyme. For example, the cell can be modified to comprise an acyl-CoA synthase, a thioesterase, and an ester synthase.

[0143] The exogenous nucleic acid sequences can be from *Arthrobacter, Rhodotorula glutinins, Acinetobacter* sp., *Alcanivorax borkumensis, E. coli,* or *Candida lipolytica.* In one embodiment, the exogenous nucleic acid sequence is stably incorporated into the genome of the cell.

[0144] The invention also provides a recombinant cell capable of producing esters. The recombinant cell can comprise an exogenous nucleic acid sequence encoding a thioesterase, an exogenous nucleic acid sequence encoding an acyl-CoA synthase, and an exogenous nucleic acid sequence encoding an ester synthase.

[0145] In some embodiments, the cell optionally comprises a gene encoding a fatty acid degradation enzyme, which gene is modified such that expression of the gene is attenuated. The gene encoding a fatty acid degradation enzyme can be obtained from any organism, for example, from *Saccharomyces cerevisiae, Candida lipolytica, Escherichia coli, Arthrobacter, Rhodotorula glutinins, Acinetobacter, Candida lipolytica, Botryococcus braunii, Vibrio furnissii, Micrococcus leuteus, Stenotrophomonas maltophilia,* or *Bacillus subtilis.* For example, the gene encoding a fatty acid degradation enzyme can be fadD.

[0146] In some embodiments, the cell optionally comprises a gene encoding an outer membrane protein receptor, wherein the gene is modified such that expression of the gene is attenuated. The modified gene encoding an outer membrane protein receptor can be a gene encoding an outer membrane protein receptor for ferrichrome, colicin M, phage T1, phage T5, or phage phi80. The outer membrane protein receptor gene can be obtained from any organism, for example, from *Saccharomyces cerevisiae, Candida lipolytica, Escherichia coli, Arthrobacter, Rhodotorula glutinins, Acinetobacter, Candida lipolytica, Botryococcus braunii, Vibrio furnissii, Vibrio harveyi, Micrococcus leuteus, Stenotrophomonas maltophilia,* or *Bacillus subtilis.* For example, the gene encoding the outer membrane protein receptor is fhuA (or tonA).

[0147] In some embodiments, the cell optionally comprises a gene encoding a DNA-binding transcriptional repressor, wherein the gene is modified such that expression of the gene is attenuated. The DNA-binding transcriptional repressor gene can be obtained from any organism, for example, *Saccharomyces cerevisiae, Candida lipolytica, Escherichia coli, Arthrobacter, Rhodotorula glutinins, Acinetobacter, Candida lipolytica, Botryococcus braunii, Vibrio furnissii, Micrococcus leuteus, Stenotrophomonas maltophilia,* or *Bacillus subtilis.* For example, the modified gene encoding a DNA-binding transcriptional repressor is fabR.

[0148] For example, the cell can comprise a deletion in a gene encoding a fatty acid degradation enzyme, an outer membrane protein, and/or a DNA-binding transcriptional repressor. In one embodiment, the cell comprises an attenuated gene encoding a fatty acid degradation enzyme and an attenuated gene encoding an outer membrane protein receptor.

[0149] The cell can be a *Saccharomyces cerevisiae, Candida lipolytica, Escherichia coli, Arthrobacter, Rhodotorula glutinins, Acinetobacter, Candida lipolytica, Botryococcus braunii, Vibrio furnissii, Micrococcus leuteus, Stenotrophomonas maltophilia* or *Bacillus subtilis* cell. Preferably the cell is an *Arthrobacter* AK 19, *Acinetobacter* sp. strain M-1, *E. coli* B, *E. coli* C, *E. coli* K or *E. coli* W cell. The cell also can be a cyanobacterial cell, such as a *Synechocystis* sp.

PCC6803, a *Synechococcus* sp. PCC7002, or a *Synechococcus elongatus* PCC7942 cell. The cell also can be a plant, animal, or human cell. Preferred cells can be those selected from, for example, *Arabidopsis thaliana, Panicum virgatum, Miscanthus giganteus, Zea mays, Botryococcus braunii, Chlamydomonas reinhardtii, Dunaliela salina, Synchococcus* sp., *Thermosynechococcus elongatus, Chlorobium tepidium, Chloroflexus aurauntieus, Chromatium tepidum, Chromatium vinosum, Rhodospirillum rubrum, Rhodobacter capsulatus,* and *Rhodopseudomonas palusris*. The cell can, in addition, be a cell of a synthetic microorganism such as, for example, synthetic cells produced by synthetic genomes as described in, for example, U.S. Patent Publication Nos.: 2007/0264688, and 2007/0269862. In a further embodiment, the cell can be from those microorganisms that can be engineered to fix carbon dioxide, including, for example, *E. coli, Acetobacter aceti, Bacillus subtilis,* yeast and fungi such as *Clostridium ljungdahlii, Clostridium thermocellum, Penicillium chrysogenum, Pichia pastoris, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pseudomonas fluorescenes,* or *Zymomonas mobilis.*

[0150] In one embodiment, the cell is a microorganism cell from a cyanobacterium, bacterium, yeast, or filamentous fungi. For example, the recombinant cell can be a genetically modified microorganism. In some embodiments, the gene encoding a fatty acid derivative enzyme is codon-optimized, or modified to be optimized for expression in the recombinant cell.

[0151] In a further embodiment, the invention provides a genetically engineered microorganism, which can be cultured under appropriate conditions (e.g., in according to the culture and fermentation conditions described herein) to produce fatty esters. In certain embodiments, the microorganism comprising an exogenous control sequence stably incorporated into the genomic DNA of the microorganism upstream of one or more of at least one of a gene encoding a thioesterase, a gene encoding an acyl-CoA synthase, and a gene encoding an ester synthase, wherein the microorganism produces an increased level of a fatty ester relative to a wild-type microorganism. In certain embodiments, the exogenous control sequence can be a promoter, for example, a developmentally-regulated, organelle-specific, tissue-specific, inducible, constitutive, or cell-specific promoter. In some embodiments, the microorganism can be engineered such that it expresses, relative to a wild type microorganism, a decreased level of at least one of a gene encoding an acyl-CoA dehydrogenase, a gene encoding an outer membrane protein receptor, and a gene encoding a transcriptional regulator of fatty acid biosynthesis. In certain embodiments, the gene encoding an acyl-CoA dehydrogenase is fadE. In certain embodiments, the gene encoding an outer membrane protein receptor is fhuA. In further embodiments, the gene encoding a transcriptional regulator of fatty acid biosynthesis is fabR.

[0152] In some embodiments, the genetically engineered microorganism is selected from a Gram-negative or a Gram-positive bacterium. In alternative embodiments, the genetically engineered microorganism is selected from an *E. coli,* mycrobacterium, *Nocardia* sp., *Nocardia farcinica, Streptomyces griseus, Salinispora arenicola, Clavibacter michiganenesis, Acinetobacter, Alcanivorax, Alcaligenes, Arabidopsis, Fundibacter, Marinobacter, Mus musculus, Pseudomonas, or Simmodsia, Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon,* or *Lipomyces.*

[0153] The invention also provides a method for producing fatty esters in a recombinant cell. The method comprises (a) obtaining a recombinant cell as described herein, (b) culturing the recombinant cell under suitable conditions for expression, and (c) obtaining fatty esters. The production and isolation of fatty esters can be enhanced by employing specific fermentation techniques. For example, a fermentation process was developed to produce a mix of fatty acid methyl esters (FAME) for use as a biodiesel using the recombinant cells described herein.

[0154] In another embodiment, the invention also features a method of producing a fatty ester by culturing the genetically engineered microorganism herein in the presence of a suitable alcohol substrate and isolating the fatty ester.

[0155] A fermentation and recovery process that can be used to produce biodiesel of commercial grade quality is described below. The biodiesel produced by these methods satisfies the ASTM standards and other engine performance standards, and meets the environmental standards set by the EPA and other environmental standard-setting agencies, as well as demonstrates, in a standard diesel engine test, an improved emission profile as compared to a diesel produced using the standard transesterifcation processes.

Fermentation

[0156] The fermentation process can be optimized in lab scale fermentors of 2 to 5 L of volume. The process can then be scaled up in similar ways as those used in any other *E. coli* fermentation, using methods well known to one of ordinary skill in the art.

[0157] For fermentation, *E. coli* cells can be grown in any suitable medium. For example, the medium can comprise 1.5 g/L of KH_2PO_4, 4.54 g/L of K_2HPO_4 trihydrate, 4 g/L of $(NH_4)_2SO_4$, 0.15 g/L of $MgSO_4$ heptahydrate, 20 g/L of glucose, 200 mM of Bis-Tris buffer (pH 7.2), 1.25, and 1.25 mL/L of a vitamin solution. The vitamin solution can comprise, for example, 0.42 g/L of riboflavin, 5.4 g/L of pantothenic acid, 6 g/L of niacin, 1.4 g/L of pyridoxine, 0.06 g/L of biotin, and 0.04 g/L of folic acid.

[0158] An overnight starter culture of any volume (e.g., about 50 mL) can be used to inoculate a larger culture of the same medium, wherein the medium optionally has a reduced glucose concentration (e.g., 5 g/L of glucose) than, for example, the medium described above, in a fermentor with temperature, pH, agitation, aeration and dissolved oxygen controls. The preferred conditions in the fermentor are set at about 32° C., about pH 6.8, and a dissolved oxygen (DO) level of about 30% of saturation. The pH can be maintained by the addition of NH_4OH, which also serves as a nitrogen source for cell growth. When the initial glucose is almost consumed, a feed consisting of, for example, 60% glucose, 3.9 g/L $MgSO_4$ heptahydrate and 10 mL/L of the trace minerals solution is supplied to the fermentor. The trace metals solution can comprise, for example, 27 g/L of $FeCl_3.6H_2O$, 2 g/L of $ZnCl_2.4H_2O$, 2 g/L of $CaCl_2.6H_2O$, 2 g/L of $Na_2MoO_4.2H_2O$, 1.9 g/L of $CuSO_4.5H_2O$, 0.5 g/L of H_3BO_3, and 100 mL/L of concentrated HCl. The feed rate should be set up to match the cells growth rate and avoid accumulation of glucose in the fermentor. By avoiding glucose accumulation, it is possible to reduce or eliminate the formation of by-products such as, for example, acetate, formate and ethanol, which are otherwise typically produced by *E. coli.* In the early phases of the growth, the production of FAME can be induced by the addition of 1 mM IPTG and 20 mL/L of pure methanol. The

fermentation can be continued for a period of about 3 days. Methanol can be added several times during the run to replenish the methanol consumed by the cells for the production of FAME and/or lost by evaporation in the off-gas. The additions should be targeted to maintain the concentration of methanol in the fermentation broth at between about 10 and about 30 mL/L, which serves to insure a good balance between efficient production and avoidance of cell grown inhibition.

[0159] The progression of fermentation can be followed by measuring OD_{600} (optical density at 600 nm), glucose consumption, and/or ester production.

[0160] The fermentation protocol can be scaled up to a larger fermentor (e.g., to a size of about 700 L), which allows the generation of enough biodiesel for quality testing. Analytical methods that can be utilized to continuously monitor the fermentation process, and an exemplary set of suitable methods are described below.

Analysis

[0161] Glucose consumption can be analyzed throughout the fermentation process by High Pressure Liquid Chromatography (HPLC). The HPLC analysis can be performed according to methods commonly used in the art for various sugars and organic acids. In an exemplary embodiment, the HPLC conditions can be as follows:

[0162] a. Instrument: Agilent HPLC 1200 Series with Refractive Index detector;

[0163] b. Column: Aminex HPX-87H, 300 mm×7.8 mm;

[0164] c. Column temperature: 350° C.;

[0165] d. Mobile phase: 0.01M H_2SO_4 (aqueous);

[0166] e. Flow rate: 0.6 mL/min;

[0167] f. Injection volume: 20 μL.

[0168] The production of fatty acid methyl and ethyl esters can be monitored and/or analyzed by gas chromatography with a flame ionization detector (GC-FID). The samples from fermentation broth can be extracted with ethyl acetate in a ratio of 1:1 vol/vol. After vigorous vortexing, the samples can be centrifuged and the organic phase can be analyzed by gas chromatography (GC). An exemplary set of analysis conditions are listed below:

[0169] a. Instrument: Trace GC Ultra, Thermo Electron Corporation with Flame ionization detector (FID) detector;

[0170] b. Column: DB-1 (1% diphenyl siloxane; 99% dimethyl siloxane) CO1 UFM 1/0.1/5 01 DET from Thermo Electron Corporation, phase pH 5, FT: 0.4 μm, length 5 m, id: 0.1 mm;

[0171] c. Inlet conditions: 250° C. splitless, 3.8 min 1/25 split method used depending upon sample concentration with split flow of 75 mL/min;

[0172] d. Carrier gas & flow rate: Helium, 3.0 mL/min;

[0173] e. Block temperature: 330° C.;

[0174] f. Oven temperature: 0.5 minute hold at 50° C.; 100° C/minute to 330° C.; 0.5 minute hold at 330° C.;

[0175] g. Detector temperature: 300° C.;

[0176] h. Injection volume: 2 μL; run time/flow rate: 6.3 min/3.0 mL/min (in a splitless method), 3.8 min/1.5 mL/min (in a split 1/25 method), 3.04 min/1.2 mL/min (in a split 1/50 method).

Recovery

[0177] Following fermentation, the broth can be centrifuged to separate the lighter phase containing methyl esters from the heavier phase containing water, salts and the bulk of the microbial biomass. The lighter phase can be centrifuged again to recover the biodiesel. It is also possible to obtain clear biodiesel in a single-step centrifugation and without any pretreatment.

[0178] Centrifugation can be performed using any suitable centrifuge. For example, centrifugation can be performed in disk-stacked continuous centrifuges of pilot scale capacity, (with, for example, a fixed centrifugal force of ~10,000 g), with flows from about 1 to about 5 L per min. Normal adjustments to centrifugation configurations and conditions (including, for example, to gravity ring sizes, back pressure in outlets, flow, etc.) which are well known to one of ordinary skill in the art, can be performed, such that the most favorable separation in terms of recovery efficiency and cleanness of the product is achieved.

[0179] The fermentation broth can be directly centrifuged without any physical or chemical adjustments beforehand. Alternatively, suitable pretreatments can be applied to the light phase to help with the separation during the second centrifugation step. These pretreatments can, in one exemplary embodiment, include the following steps but not necessarily in the listed order:

[0180] a. heating to about 60 to about 80° C.;

[0181] b. adjusting the pH to 2.0 to 2.5 using sulfuric acid; and

[0182] c. addition of suitable demulsifiers (for example, ARB-8285 (Baker Hughes, Houston, Tex.)) to less than 1% of the emulsion/light phase volume.

In a further example, the temperature of step a. can be held for 1 to 2 hrs before the second centrifugation.

[0183] FAME produced from the fermentation broth can be separated by decanting, filtration, or other separation methods known to those of ordinary skill in the art.

Polishing

[0184] The biodiesel obtained from the harvesting step described above has characteristics similar to the commercial standards and environmental benchmarks for biodiesels. The inherent properties of this biodiesel, as well as other purity-related parameters typically would meet the commercial and environmental standards for biodiesel. Those properties include, for example, cetane number, kinematic viscosity, flash point, oxidation stability, copper corrosion, free and total glycerin, methanol, phosphorous, sulfate, K^+ and Na^+ content, trace element content, and emissions profile. Therefore, few if any purification steps for the elimination of other impurities are required. Optional purification steps include, for example, lime washing or acid methylation to eliminate residual free fatty acids, dilute acid washing to remove excess calcium, tangential filtration, washing with water, drying to remove remaining free acids added during methylation or acid washing steps, and using suitable resins to remove of other minor impurities by absorption/adsorption. Not all the optional purification steps are necessary to purify the biodiesel produced every time, and whether one or more of the optional steps are used depends on the characteristics of the product at the end of the fermentation process.

[0185] Small quantities of free fatty acids may be produced during fermentation, and they are separated from the biodiesel along with the esters. The ASTM standards mandate that a biodiesel have a low acid number, as measured by a standard testing method ASTM D 664. Thus, if the free fatty acids level in the FAME after the centrifugation step is, as it typically may be, about 1 to about 2%, one or more of the optional purification steps described above may need to be applied.

Standards are also stringent for the calcium and magnesium contents. Although neither calcium nor magnesium is present in the fermentation product, they can be introduced into the product mixture during the lime wash, making it necessary to perform the dilute acid wash step. In the same manner, excess free acids (e.g., sulfuric, phosphoric, and/or lactic acids) may be introduced to the product mixture during the acid wash or as a catalyst when acid methylation is used as a means to reduce the level of free fatty acids, and they need to be removed from the product mixture by further washing with water. A final treatment with absorbent/adsorbent resins, such as Magnesol™ (The Dallas Group, Whitehouse, N.J.), Amberlist™ BD20 (Dow Chemicals, Philadelphia, Pa.), Biosil™ (Polymer Technology Group, Berkeley, Calif.), or other similar adsorbent/absorption resins, assures elimination of water, methanol, sulfur or other small impurities yet present. Some of the common impurities can also be reduced by modifications made to the fermentation process.

Fatty Esters

[0186] The invention provides a composition produced by a recombinant cell as described herein, wherein the composition comprises fatty esters produced from the recombinant cell.

[0187] As described herein, production hosts can be engineered using known peptides to produce fatty esters from acyl-CoA and alcohols. One of ordinary skill in the art will appreciate that structurally, fatty esters have an A side and a B side (or an A group and a B group, respectively). In some embodiments, the fatty esters comprise, consist essentially of, or consist of the following formula: BCOOA.

[0188] B is an aliphatic group. In some embodiments, B is a carbon chain. In some embodiments, B comprises a carbon chain that is at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 carbons in length. A comprises at least one carbon atom. In some embodiments, A is an aliphatic group. In some embodiments, A is an alkyl group. In some embodiments, the alkyl group comprises, consists essentially of, or consists of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbon atoms. In some embodiments, any of the above B groups can be combined with any of the above A groups. In some embodiments, A comprises, consists essentially of, or consists of a carbon chain having a number of carbons selected from the group consisting of 1, 2, 3, 4, and 5 carbon atoms, while B comprises, consists essentially of, or consists of at least 12, 13, 14, 15, 16, 17, 18, 19, or 20 carbon atoms.

[0189] In some embodiments, the fatty esters comprise a plurality of individual fatty esters. In some embodiments, the methods described herein permit production of a plurality of fatty esters of varied length. In some embodiments, the fatty ester product comprises saturated or unsaturated fatty esters product(s) having a carbon atom content limited to between 5 and 25 carbon atoms. In other words, the invention provides a composition comprising C_5-C_{25} fatty esters (e.g., C_{10}-C_{20} fatty esters, or C_{12}-C_{18} fatty esters).

[0190] In some embodiments, the fatty esters comprise one or more fatty esters having a double bond at one or more points in the carbon chain. Thus, in some embodiments, a 6-, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14-, 15-, 16-, 17-, 18-, 19-, 20-, 21-, 22-, 23-, 24-, 25-, 26-, 27-, 28-, 29-, or 30-carbon chain can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 double bonds, and 1-24 of the aforesaid double bonds can be located following carbon 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, or 29. In some embodiments, a 1-, 2-, 3-, 4-, or 5-carbon chain for A can have 1, 2, 3, or 4 double bonds and 1-4 of the double bonds can be located following carbon 1, 2, 3, or 4. In some embodiments, any of the above A groups can be combined with any of the above B groups.

[0191] In certain preferred embodiments, the B group can have 12, 13, 14, 15, 16, 17, 18 carbon atoms in a chain. In other embodiments, the A group can have one or two carbon atoms.

[0192] In some preferred embodiments, the B group can have one double bond at one or more points in the carbon chain. In more preferred embodiments, the B group can have one double bond at position 7 of the carbon chain, numbering from the reduced end of the carbon chain. One of ordinary skill in the art will recognize that one end of the B group will have a methyl group, and the other end of the B group will have a carboxyl group (C(═O)O—). The end of the B group which is a methyl group is the reduced end of the carbon chain comprising the B group, thus, the double bond is at carbon 7 counting from the methyl group terminus of the B group (e.g., at between carbons 7 and 8 of the B group). The double bond can have any geometry, thus, the double bond in the B group can be cis or trans.

[0193] In some embodiments, the fatty esters comprise straight chain fatty esters. In some embodiments, the fatty esters comprise branched chain fatty esters. In some embodiments, the fatty esters comprise cyclic moieties.

[0194] In certain preferred embodiments, the fatty esters can be selected from the group consisting of methyl dodecanoate, methyl 5-dodecenoate, methyl tetradecanoate, methyl 7-tetradecenoate, methyl hexadecanoate, methyl 9-hexadecenoate, methyl octadecanoate, methyl 11-octadecenoate, and combinations thereof.

[0195] In some embodiments, the fatty ester composition comprises about 5 wt. % or more methyl dedecanoate. In some embodiments, the fatty ester composition comprises about 25% or more methyl dedecanoate. In some embodiments, the fatty ester composition comprises about 5 wt. % to about 25 wt. % methyl dodecanoate.

[0196] In some embodiments, the fatty ester composition comprises about 10 wt. % or less methyl dodec-7-enoate. In some embodiments, the fatty ester composition comprises about 0 wt. % to about 10 wt. % methyl dodec-7-enoate.

[0197] In some embodiments, the fatty ester composition comprises about 30 wt. % or more methyl tetradecanoate. In some embodiments, the fatty ester composition comprises about 50 wt. % or less methyl tetradecanoate. In some embodiments, the fatty ester composition comprises about 30 wt. % to about 50 wt. % methyl tetradecanoate.

[0198] In some embodiments, the fatty ester composition comprises about 10 wt. % or less methyl tetradec-7-enoate. In some embodiments, the fatty ester composition comprises about 0 wt. % to about 10 wt. % methyl tetradec-7-enoate.

[0199] In some embodiments, the fatty ester composition comprises about 15 wt. % or less methyl hexadecanoate. In some embodiments, the fatty ester composition comprises about 0 wt. % to about 15 wt. % methyl hexadecanoate.

[0200] In some embodiments, the fatty ester composition comprises about 10 wt. % or more methyl hexadec-7-enoate. In some embodiments, the fatty ester composition comprises about 40 wt. % or less methyl hexadec-7-enoate. In some embodiments, the fatty ester composition comprises about 10 wt. % to about 40 wt. % methyl hexadec-7-enoate.

[0201] In some embodiments, the fatty ester composition comprises about 15 wt. % or less methyl octadec-7-enoate. In some embodiments, the fatty ester composition comprises about 0 wt. % to about 15 wt. % methyl octadec-7-enoate.

Carbon Chain Characteristics

[0202] In some embodiments, the hydrocarbons, fatty alcohols, fatty esters, and waxes disclosed herein are useful as biofuels and specialty chemicals. The products can be produced such that they contain desired branch points, levels of saturation, and carbon chain lengths. Therefore, these products can be desirable starting materials for use in many applications. One of ordinary skill in the art will appreciate that some of the genes that are used to alter the structure of the fatty acid derivative can also increase the production of fatty acid derivatives.

[0203] Furthermore, biologically produced fatty esters represent a new feedstock for fuels, such as alcohols, diesel and gasoline. Fatty esters have not been produced from renewable sources and, as such, are new compositions of matter. These new fatty esters and fuels can be distinguished from fatty esters and fuels derived from petrochemical carbon on the basis of dual carbon-isotopic fingerprinting. Additionally, the specific source of biosourced carbon (e.g., glucose vs. glycerol) can be determined by dual carbon-isotopic fingerprinting (see, U.S. Pat. No. 7,169,588, which is herein incorporated by reference). The following discussion generally outlines two options for distinguishing chemically-identical materials (that have the same structure, but different isotopes). In some embodiments, this apportions carbon in products by source (and possibly year) of growth of the biospheric (plant) component.

[0204] The isotopes, ^{14}C and ^{13}C, bring complementary information to this examination. The radiocarbon dating isotope (^{14}C), with its nuclear half life of 5730 years, clearly allows one to apportion specimen carbon between fossil ("dead") and biospheric ("alive") feedstocks (see, e.g., Currie, L. A. "Source Apportionment of Atmospheric Particles," Characterization of Environmental Particles, J. Buffle and H. P. van Leeuwen, Eds., 1 of Vol. I of the IUPAC Environmental Analytical Chemistry Series (Lewis Publishers, Inc) (1992) 3, 74). The basic understanding in radiocarbon dating is that the constancy of ^{14}C concentration in the atmosphere leads to the constancy of ^{14}C in living organisms. When dealing with an isolated sample, the age of a sample can be deduced approximately by the relationship $t=(-5730/0.693)\ln(A/A_0)$ (Equation 1) where t=age, 5730 years is the half-life of radiocarbon, and A and A_0 are the specific ^{14}C activity of the sample and of the modern standard, respectively (see, e.g., Hsieh, Y., Soil Sci. Soc. Am J., 56, 460, (1992)). However, because of atmospheric nuclear testing since 1950 and the burning of fossil fuel since 1850, ^{14}C has acquired a second, geochemical time characteristic. Its concentration in atmospheric CO_2, and hence in the living biosphere, approximately doubled at the peak of nuclear testing, in the mid-1960s. It has since been gradually returning to the steady-state cosmogenic (atmospheric) baseline isotope rate ($^{14}C/^{12}C$) of ca. 1.2×10^{12} with an approximate relaxation "half-life" of 7-10 years. The latter half-life cannot be taken literally; rather, one should use the detailed atmospheric nuclear input/decay function to trace the variation of atmospheric and biospheric ^{14}C since the onset of the nuclear age. It is this latter biospheric ^{14}C time characteristic that holds out the promise of annual dating of recent biospheric carbon. ^{14}C can be measured by accelerator mass spectrometry (AMS) with results given in units of "fraction of modem carbon" (f^). f^ is defined by National Institute of Standards and Technology (NIST) Standard Reference Materials (SRMs) 4990B and 4990C, known as oxalic acids standards HOxI and HOxII, respectively. The fundamental definition relates to 0.95 times the $^{14}C/^{12}C$ isotope ratio HOxI (referenced to AD 1950). This is roughly equivalent to decay-corrected pre-Industrial Revolution wood. For the current living biosphere (plant material), IM is approximately 1.1. The stable carbon isotope ratio ($^{13}C/^{12}C$) provides a complementary route to source discrimination and apportionment. The $^{13}C/^{2}C$ ratio in a given biosourced material is a consequence of the $^{13}C/^{12}C$ ratio in atmospheric carbon dioxide at the time the carbon dioxide is fixed and also reflects the precise metabolic pathway. Regional variations also occur. Petroleum, C3 plants (the broadleaf), C4 plants (the grasses), and marine carbonates all show significant differences in $^{13}C/^{12}C$ and their corresponding delta^{13}C values. Furthermore, lipid matter of C3 and C4 plants analyze differently than materials derived from the carbohydrate components of the same plants as a consequence of the metabolic pathway. Within the precision of measurement, ^{13}C shows large variations due to isotopic fractionation effects, the most significant of which for the instant invention is the photosynthetic mechanism. The major cause of differences in the carbon isotope ratio in plants is closely associated with differences in the pathway of photosynthetic carbon metabolism in the plants, particularly the reaction occurring during the primary carboxylation (i.e., the initial fixation of atmospheric CO_2). Two large classes of vegetation are those that incorporate the "C3" (or Calvin-Benson) photosynthetic cycle and those that incorporate the "C4" (or Hatch-Slack) photosynthetic cycle. C3 plants, such as hardwoods and conifers, are dominant in the temperate climate zones. In C3 plants, the primary CO_2 fixation or carboxylation reaction involves the enzyme ribulose-1,5-diphosphate carboxylase and the first stable product is a 3-carbon compound. C4 plants, on the other hand, include such plants as tropical grasses, corn and sugar cane. In C4 plants, an additional carboxylation reaction involving another enzyme, phosphoenol-pyruvate carboxylase, is the primary carboxylation reaction. The first stable carbon compound is a 4-carbon acid which is subsequently decarboxylated. The CO_2 thus released is refixed by the C3 cycle.

[0205] Both C4 and C3 plants exhibit a range of $^{13}C/^{12}C$ isotopic ratios, but typical values are about −10 to −14 per mil (C4) and −21 to -26 per mil (C3) (Weber et al., J. Agric. Food Chem., 45, 2942 (1997)). Coal and petroleum fall generally in this latter range. The ^{13}C measurement scale was originally defined by a zero set by pee dee belemnite (PDB) limestone, where values were given in parts per thousand deviations from this material. The "δ^{13}", values are in parts per thousand (per mil), abbreviated % o, and are calculated as follows:

$$\delta^{13}C=[(^{13}C/^{12}C)\text{sample}-(^{13}C/^{12}C)\text{standard}]/(^{13}C/^{12}C)\text{standard}\times1000$$

[0206] Since the PDB reference material (RM) has been exhausted, a series of alternative RMs have been developed in cooperation with the IAEA, USGS, NIST, and other selected international isotope laboratories. Notations for the per mil deviations from PDB is S^1. Measurements are made on CO_2 by high precision stable ratio mass spectrometry (IRMS) on molecular ions of masses 44, 45, and 46.

[0207] In some embodiments, the inventive fatty esters have a δ^{13} of about −10.9 to about −15.4. In certain other embodiments, the inventive fatty esters have a δ^{13} of −27 to about −24. In yet further embodiments, the inventive fatty esters have a δ^{13} of about −10. In some embodiments, the fatty esters have a δ^{13} of about −28 or greater. (e.g., about −18).

[0208] The fatty esters and the associated biofuels, chemicals, and mixtures can be distinguished from their petrochemical derived counterparts on the basis of ^{14}C (fM) and dual carbon-isotopic fingerprinting, indicating new compositions of matter.

[0209] In some embodiments, the fatty esters described herein have utility in the production of biofuels and chemicals. The new fatty ester based product compositions provided herein additionally can be distinguished on the basis of dual carbon-isotopic fingerprinting from those materials derived solely from petrochemical sources. The ability to distinguish these products is beneficial in tracking these materials in commerce. For example, fuels or chemicals comprising both "new" and "old" carbon isotope profiles can be distinguished from fuels and chemicals made only of "old" materials. Hence, the instant materials can be followed in commerce on the basis of their unique profile.

[0210] In some examples, a biofuel composition is made, which includes a fatty ester having δ^{13} of from about −10.9 to about −15.4, wherein the fatty ester accounts for at least about 85% by volume of biosourced material (derived from a renewable resource such as cellulosic materials and sugars) in the composition. In some embodiments, the fatty ester is additionally characterized as having a δ^{13} of from about −10.9 to about −15.4; and the fatty ester accounts for at least about 85% by volume of biosourced material in the composition. In some embodiments, the fatty ester in the biofuel composition is characterized by having a fraction of modern carbon (fM ^{14}C) of at least about 1, about 1.003, about 1.010, or about 1.5. In some embodiments, the fatty ester in the biofuel composition is characterized by having a fraction of modern carbon (fM ^{14}C) is about 1 to about 1.5 (e.g., about 1.04 to about 1.18, or about 1.111 to about 1.124).

Post Production Processing

[0211] The fatty esters produced during production can be separated from the production media. Any technique known for separating fatty esters from aqueous media can be used. One exemplary separation process provided herein is a two-phase separation process. This process involves processing the genetically engineered production hosts under conditions sufficient to produce a fatty ester (e.g., a fatty ester), allowing the derivative to collect in an organic phase, and separating the organic phase from the aqueous production broth. This method can be practiced in both a batch and continuous production setting.

[0212] The fatty esters produced by the methods described herein will be relatively immiscible in the production broth, as well as in the cytoplasm. Therefore, the fatty esters will collect in an organic phase either intracellularly or extracellularly.

[0213] After completion of the fermentation, the fermentation broth can be centrifuged to separate the lighter phase containing the fatty esters from the heavier phase consisting of water, salts, and the bulk of the microbial biomass. While a single centrifugation step may provide fatty esters suitable for use as a fuel, in some cases a second centrifugation step is carried out to provide a more complete separation of the fatty esters.

[0214] The centrifugation can be carried out using any suitable centrifugation apparatus, many of which are well known in the art. An example of a suitable centrifugation apparatus is a disk-stacked continuous centrifuge having pilot scale capacity, such as the Westfalia™ SA1 (GEA Westfalia Separator, Inc., Northvale, N.J.) or the Alfa-Laval™ LAPX 404 (Alfa Laval AB, Lund, Sweden) centrifuges. Centrifugation can be performed at, for example, centrifugal force of 10,000 g and a flow rate of from about 1 to about 5 L/min

[0215] In some cases, depending on the fermentation characteristics, it may be necessary to provide pretreatments in order to facilitate breaking of an emulsion. An example of a suitable pretreatment includes heating the fermentation broth (e.g., to 60-80° C.), adjusting the pH to 2.0-2.5 with sulfuric acid, and addition of demulsifiers such as phenol-formaldehyde resins, polyamines, polyols, and the like, and then holding the mixture at elevated temperature for 1-2 hrs before centrifugation.

[0216] In some instances, the fatty esters contain as impurities free fatty acids. Removal of free fatty acids from the fatty esters can be accomplished using any suitable method, including lime washing or acid-catalyzed esterification (e.g., methylation). Lime washing can be conducted by heating a mixture comprising fatty esters and free fatty acids and then contacting the mixture with an aqueous slurry of lime (i.e., calcium hydroxide and/or calcium carbonate), followed by centrifugation to separate the purified fatty esters.

[0217] Lime washing can introduce undesirable levels of calcium and/or magnesium ions into the fatty esters. The calcium and/or magnesium ions can be removed from the fatty esters by first washing the fatty esters with dilute acid, such as sulfuric acid, followed by a final water wash. The fatty esters can be separated from the aqueous washes in both steps by centrifugation.

[0218] Acid-catalyzed esterification can be conducted by addition of an alcohol, such as methanol or ethanol, and an acid catalyst, such as sulfuric acid, phosphoric acid, or lactic acid, to the fatty esters, followed by heating of the resulting mixture in order to esterify any free fatty acids present in the fatty esters. Following acid-catalyzed esterification of free fatty acids, the fatty esters can be washed with water as described herein.

[0219] A final treatment of the fatty esters with absorbent/adsorbent resins such as Magnesol™ (The Dallas Group, Inc., Whitehouse, N.J.) or Amberlyst™ BD20 (Dow Chemicals, Philadelphia, Pa.), Biosil™ (Polymer Technology Group, Berkeley, Calif.), and other similar adsorbent/absorption resins can be performed to reduce or eliminate trace amounts of water, methanol, sulfur, or impurities yet present in the fatty esters.

[0220] In some embodiments, the fatty ester composition can be further processed to remove fine solids that might affect fuel injectors or prefilters in engines. In some embodiments, the fatty ester composition can also be processed to remove species that have poor volatility and that could lead to deposit formation in engines. In some embodiments, traces of sulfur compounds that may be present can be removed. Examples of suitable treatments include washing, adsorption, distillation, filtration, centrifugation, settling, and coalescence.

[0221] In other embodiments, the fatty esters can be subjected to lime washing followed by cross flow filtration, also referred to as tangential flow filtration, in place of centrifugation. In these embodiments, a mixture of fatty esters can be treated with an aqueous lime slurry and then pumped through the lumen of a cylindrical ceramic membrane. The fatty esters that permeate the ceramic membrane are collected as the product.

[0222] Accordingly, in certain embodiments, the present invention also pertains to A method of producing the fatty ester compositions of the invention, comprising: (a) culturing the microorganism under conditions sufficient to allow expression; and (b) obtaining the fatty esters. In certain embodiments, the obtaining the fatty esters comprises one or more polishings. In certain embodiments, the one or more polishings comprise one or more of the following steps: (a) a lime wash, (b) an acid methylation, (c) a dilute acid wash, (d) a tangential filtration, (e) a water wash, (f) a final drying, and (g) an adsorption or adsorption with suitable resins. In certain embodiments, the obtaining the fatty esters comprises one or more separations. For example, the one or more separations comprise one or more of the following steps: (a) centrifugations, (b) decantations, (c) distillations, and (d) filtrations. In further embodiments, the obtaining the fatty esters comprise one or more pretreatments. For example, the one or more pretreatments comprise one or more acid pretreatments. In another example, the one or more pretreatments comprise one or more heat pretreatments.

Biodiesel Fuel Performance Standards

[0223] The American Society for Testing and Materials ("ASTM") has published a standard specification for biodiesel (B 100) Grades S15 and S500 for use as a blend component for middle distillate fuels, with the specification designated as D 6751. The amount of biodiesel present in any fuel mix is designated using a "B" factor. For example, 100% biodiesel is labeled B 100. A fuel mixture containing 20% biodiesel is labeled B20. The D 6751 specification provides upper limits or ranges for minor components found in biodiesels such as sulfur, phosphorous, calcium and magnesium, sodium and potassium, and carbon residue, as well as specifications for distillation temperature, cetane number, and viscosity. The ASTM D 6751 biodiesel standard must be met in order for a biodiesel to be suitable for use as an engine fuel in the United States.

[0224] Similar to the United States, other countries and regions of the world, including, for example, the European Union, also publish standard specifications for biodiesel used in their jurisdictions. Specifically, the European Union's biodiesel standards closely track the ASTM D 6751 standards.

[0225] For example, the Brazilian Agencia Nacional do Petroleo, Gas Natural e Biocombustíveis ("ANP") has published ANP 7 which describes the specifications for biodiesel to be used in Brazil. The ANP 7 specification provides upper limits or ranges for minor components found in biodiesels such as micro carbon residue, sulfated ash, glycerin, sodium, potassium, calcium and magnesium, phosphorus, methanol, iodine, and sulfur. In addition, ANP 7 provides specifications for biodiesel characteristics, such as acid number, oxidation stability, ester content, ignition delay, density of liquids at 20° C., viscosity, flash point, corrosion, and cold filter plugging. The ANP biodiesel standard must be met in order for a biodiesel to be suitable for use as an engine fuel in Brazil. The cetane number is one of the most commonly cited indicators of diesel fuel quality. The cetane number measures the readiness of the fuel to autoignite when injected into a diesel engine. Unlike a gasoline engine, a diesel engine operates without the use of spark ignition of the fuel/air mixture. Generally, the cetane number is dependent on the composition of the fuel and can impact engine startability, noise level, and exhaust emissions. A commonly used test procedure for determination of cetane number is designated as ASTM D 613.

[0226] The fatty ester produced as described herein desirably contain low levels of impurities.

[0227] In some embodiments, the fatty ester produced as described herein contain less than or equal to about 10 mg/kg (e.g., less than or equal to about 10 mg/kg, less than or equal to about 9 mg/kg, less than or equal to about 8 mg/kg, less than or equal to about 7 mg/kg, less than or equal to about 6 mg/kg, less than or equal to about 5 mg/kg, less than or equal to about 4 mg/kg, less than or equal to about 3 mg/kg, less than or equal to about 2 mg/kg, or less than or equal to about 1 mg/kg) of total calcium and magnesium combined.

[0228] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 500 ppm (e.g., less than or equal to about 500 ppm, less than or equal to about 400 ppm, or less than or equal to about 300 ppm, less than or equal to about 200 ppm, less than or equal to about 100 ppm, less than or equal to about 50 ppm, less than or equal to about 25 ppm, or less than or equal to about 20 ppm, less than or equal to about 15 ppm, less than or equal to about 10 ppm, less than or equal to about 8 ppm, less than or equal to about 6 ppm, less than or equal to about 5 ppm, less than or equal to about 4 ppm, less than or equal to about 3 ppm, less than or equal to about 2 ppm) of sulfur.

[0229] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 0.02 wt. % (e.g., less than or equal to about 0.02 wt. %, less than or equal to about 0.015 wt. %, less than or equal to about 0.012 wt. %, less than or equal to about 0.01 wt. %, less than or equal to about 0.008 wt. %, less than or equal to about 0.006 wt. %, less than or equal to about 0.004 wt. %, less than or equal to about 0.002 wt. %, less than or equal to about 0.001 wt. %, less than or equal to about 0.0005 wt. %) of sulfated ash.

[0230] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 0.05 vol. % (e.g., less than or equal to about 0.04 vol. %, or less than or equal to about 0.03 vol. %, or less than or equal to about 0.02 vol. %, or less than or equal to about 0.01 vol. %) of water and sediment.

[0231] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 0.02 wt. % (e.g., less than or equal to about 0.02 wt. %, less than or equal to about 0.018 wt. %, less than or equal to about 0.015 wt. %, less than or equal to about 0.012 wt. %, less than or equal to about 0.01 wt. %, less than or equal to about 0.008 wt. %, less than or equal to about 0.006 wt. %, less than or equal to about 0.004 wt. %, less than or equal to about 0.002 wt. %) of free glycerin.

[0232] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 0.38 wt. % (e.g., less than or equal to about 0.38 wt. %, less than or equal to about 0.35 wt. %, less than or equal to about 0.30 wt. %, less than or equal to about 0.25 wt. %, less than or equal to about 0.20 wt. %, less than or equal to about 0.15 wt. %, less than or equal to about 0.10 wt. %, less than or equal to about 0.05 wt. %, less than or equal to about 0.04 wt. %, less than or equal to about 0.03 wt. %, less than or equal to about 0.02 wt. %, less than or equal to about 0.01 wt. %) of total glycerin.

[0233] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 10 mg/kg (e.g., less than or equal to about 10 mg/kg, less than or equal to about 9 mg/kg, less than or equal to about 8 mg/kg, less than or equal to about 7 mg/kg, less than or equal to about 6 mg/kg, less than or equal to about 5 mg/kg, less than or equal to about 4 mg/kg, less than or equal to about 3 mg/kg, less than or equal to about 2 mg/kg, less than or equal to about 1 mg/kg) of phosphorous.

[0234] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 10 mg/kg (e.g., less than or equal to about 10 mg/kg, less than or equal to about 9 mg/kg, less than or equal to about 8 mg/kg, less than or equal to about 7 mg/kg, less than or equal to about 6 mg/kg, less than or equal to about 5 mg/kg, less than or equal to about 4 mg/kg, less than or equal to about 3 mg/kg, less than or equal to about 2 mg/kg, less than or equal to about 1 mg/kg, or less than or equal to about 0.5 mg/kg) of total sodium and potassium combined.

[0235] The fatty esters produced as described herein desirably have a total contamination in middle distillates of about 24 mg/kg or less (e.g., about 22 mg/kg or less, about 20 mg/kg or less, about 18 mg/kg or less, about 16 mg/kg or less, about 14 mg/kg or less, about 12 mg/kg or less, about 10 mg/kg or less).

[0236] The fatty esters produced as described herein desirably have a carbon residue of about 0.1 wt. % or less (e.g., about 0.1 wt. % or less, about 0.08 wt. % or less, about 0.06 wt. % or less, about 0.05 wt. % or less, about 0.04 wt. % or less, about 0.03 wt. % or less, about 0.02 wt. % or less, about 0.01 wt. % or less, about 0.005 wt. % or less, about 0.002 wt. %).

[0237] Suitable test methods for determination of impurities as described herein are set forth in the Table below.

TABLE

Impurity	Test Method(s)
Calcium and Magnesium (combined)	EN 14538, UOP 389
Sulfur	D 5453, D 7039
Sulfated Ash	D 874, EN 3987
Water and Sediment	D 2709, D 1796
Free Glycerin	D 6584
Total Glycerin	D 6584
Phosphorous	D 4951, EN 14107
Sodium and Potassium (combined)	EN 14108, EN 14109, EN 14538, UOP 391
Total Contamination in Middle Distillates	EN 12662
Carbon Residue	D 4530

[0238] The fatty esters produced as described herein desirably have a kinematic viscosity of equal to about 3.5 mm^2/s or higher (e.g., equal to about 3.5 mm^2/s or higher, about 3.2 mm^2/s or higher, about 3.0 mm^2/s or higher, about 2.8 mm^2/s or higher, about 2.5 mm^2/s or higher, about 2.2 mm^2/s or higher, about 2.0 mm^2/s or higher, about 1.9 mm^2/s or higher). In an alternative embodiment, the fatty esters produced as described herein desirably have a kinematic viscosity of less than or equal to about 6.0 mm^2/s (e.g., less than or equal to about 6.0 mm^2/s, less than or equal to about 5.0 mm^2/s, less than or equal to about 4.0 mm^2/s, less than or equal to about 3.5 mm^2/s, less than or equal to about 3.0 mm^2/s, less than or equal to about 2.0 mm^2/s). In a further embodiment, the fatty esters produced as described herein desirably have a kinematic viscosity of between about 3.0 and 6.0 mm^2/s (e.g., between about 3.0 and 5.5 mm^2/s, between about 3.0 and 5.0 mm^2/s, between about 3.0 and 4.5 mm^2/s, between about 3.0 and 4.0 mm^2/s). The kinematic viscosity can be determined by use of test method D 445 or EN 3104.

[0239] The fatty esters produced as described herein desirably have an acid number of less than or equal to about 0.80 mg KOH/g (e.g., less than or equal to about 0.80 mg KOH/g, less than or equal to about 0.70 mg KOH/g, less than or equal to about 0.60 mg KOH/g, less than or equal to about 0.50 mg KOH/g, less than or equal to about 0.40 mg KOH/g, less than or equal to about 0.30 mg KOH/g, less than or equal to about 0.20 mg KOH/g, less than or equal to about 0.10 mg KOH/g, less than or equal to about 0.05 mg KOH/g). The acid number can be determined by use of test methods D 664, D 3242, D 974, EN 14104.

[0240] The fatty esters produced as described herein desirably have a boiling point at 760 mm Hg of about 360° C. or lower (e.g., about 350° C. or lower, about 340° C. or lower, about 330° C. or lower, or about 325° C. or lower).

[0241] The fatty esters produced as described herein desirably have a cetane number of about 40 or higher (e.g., about 41 or higher, about 42 or higher, about 45 or higher, about 47 or higher, about 50 or higher). The cetane number can be determined by use of test methods D 613 or D 6890.

[0242] The fatty esters produced as described herein desirably have an oxidation stability of about 3 hours or longer (e.g., about 3 hours or longer, about 4 hours or longer, about 5 hours or longer, about 6 hours or longer, about 7 hours or longer). The oxidation stability can be determined using any suitable method, for example, by using test method EN 14112.

[0243] The fatty esters produced as described herein desirably have a cloud point of about 10° C. or lower (e.g., about 8° C. or lower, about 5° C. or lower, about 4° C. or lower, about 3° C. or lower, about 2° C. or lower, about 1° C. or lower, about 0° C. or lower, about −1° C. or lower, about −2° C. or lower, about −3° C. or lower about −4° C. or lower about −5° C. or lower). The cloud point is the temperature at which wax crystals begin to form in a petroleum product as it is cooled. The cloud point can be determined using any suitable method, for example, by using test method D 2500 or D 6890.

[0244] The fatty esters produced as described herein desirably have a density of liquid at 15° C. of about 860 kg/m^3 or more (e.g., about 860 kg/m^3 or more, about 865 kg/m^3 or more, about 870 kg/m^3 or more, about 875 kg/m^3 or more, about 880 kg/m^3 or more, about 885 mg/m^3 or more, about 890 kg/m^3 or more, about 895 kg/m^3 or more). In an alternative embodiment, the fatty esters produced as described herein desirably have a density of liquid of about 900 kg/m^3 or less (e.g., about 900 kg/m^3 or less, about 890 kg/m^3 or less, about 880 kg/m^3 or less, about 870 kg/m^3 or less, about 865 kg/m^3 or less). In a further embodiment, the fatty esters produced as described herein desirably have a density of liquid at 20° C. of about 865 kg/m^3 or more (e.g., about 865 kg/m^3 or more, about 870 kg/m^3 or more, about 875 kg/m^3 or more, about 878 kg/m^3 or more). In yet a further embodiment, the fatty esters produced as described herein desirably have a density of liquid at 20° C. of about 880 kg/m^3 or less (e.g., about 880 kg/m^3 or less, about 875 kg/m^3 or less, about 870 kg/m^3 or less, about 868 kg/m^3 or less). The density of liquid at 20° C. can be determined using any suitable method, for example, by using test method D 1298, D 4052, EN 3675, or EN 12185.

[0245] The fatty esters produced as described herein desirably have a flash point of about about 100° C. or higher (e.g., about 110° C. or higher, about 120° C. or higher, about 130° C. or higher, about 140° C. or higher). The flash point can be determined using any suitable method, for example, by using test method D 93 or EN 3679.

[0246] The fatty esters produced as described herein desirably have a total ester content of about 96.5 wt. % or more (e.g., about 96.6 wt. % or more). The total ester content can be determined using any suitable method, for example, by using test method EN 14103. An exemplary B 100 biodiesel of the present invention comprising the fatty esters produced as described herein has a total ester content of about 97.5 wt. % or more.

[0247] The fatty esters produced as described herein desirably have a cold filter plugging point of about 5° C. or lower (e.g., about 4° C. or lower, about 2° C. or lower, about 0° C. or lower, about −2° C. or lower, about −3° C. or lower, about −4° C. or lower, about −5° C. or lower). The cold filter plugging point can be determined using any suitable method, for example, by using test method D 6371 or EN 116.

[0248] The fatty esters produced as described herein desirably have a copper strip corrosion rating of class 3 or lower (e.g., class 3 or lower, class 2 or lower, or class 1) in a standard copper strip test, for example, using test method ASTM D 130.

[0249] The fatty esters produced as described herein desirably have a methanol or ethanol level of about 0.5 wt. % or lower (e.g., about 0.5 wt. % or lower, about 0.4 wt. % or lower, about 0.3 wt. % or lower, about 0.2 wt. % or lower, about 0.1 wt. % or lower, about 0.08 wt. % or lower, about 0.05 wt. % or lower, about 0.04 wt. % or lower, about 0.03 wt. % or lower, about 0.02 wt. % or lower), as measured in a standard method, for example, EN 14110.

[0250] The fatty esters produced as described herein desirably has an iodine value of about 120 g/100 g or less (e.g., about 120 g/100 g or less, about 110 g/100 g or less, about 100 g/100 g or less, about 95 g/100 g or less, about 90 g/100 g or less, about 85 g/100 g or less, about 80 g/100 g or less, about 75 g/100 g or less, about 70 g/100 g or less), as measured in a standard test to determine the level of unsaturation in the fatty ester content of a fuel, such as, for example, EN 14111.

Environmental Standards

[0251] The United States Environmental Protection Agency ("EPA") sets purity and emissions standards for all diesel fuels, including biodiesel fuels, and related products marketed in the United States. By requiring producers and importers of fuels or additives to register their product with the EPA, the agency acts within the authority provided by section 211 of the Clean Air Act, (42 U.S.C. §7401 et seq. (1970)), to regulate fuels and fuel additives, to obtain information about emissions and health effects when appropriate, and to reduce the risk to public health from exposure to their emissions.

Trace Elements

[0252] The fatty esters produced as described herein desirably contain substantially lower levels of trace elements than those in other biofuels derived from triglycerides, such as fuels derived from vegetable oils and fats. Specifically, the crude fatty ester biofuels described herein (prior to mixing with other fuels, such as, for example, petroleum-based fuels) desirably contain less heavy metal elements such as, for example, copper than crude biodiesels derived from other biomass, such as, for example, those derived from soy. The crude fatty ester biofuels described herein also desirably contain less transesterification catalyst than petrochemical diesel or biodiesel. For example, the fatty ester can contain less than about 2%, 1.5%, 1.0%, 0.5%, 0.3%, 0.1%, 0.05%, or 0% of a transesterification catalyst or an impurity resulting from a transesterification catalyst. In certain embodiments, the fatty ester produced according to the disclosures herein contains no impurity resulting from a transesterification catalyst. Non-limiting examples of transesterification catalysts include hydroxide catalysts, such as NaOH, KOH, and LiOH; and acidic catalysts, such as mineral acid catalysts and Lewis acid catalysts, Non-limiting examples of catalysts and impurities resulting from transesterification catalysts include tin, lead, mercury, cadmium, zinc, titanium, zirconium, hafnium, boron, iron, aluminum, phosphorus, arsenic, antimony, bismuth, calcium, magnesium, strontium, uranium, potassium, sodium, lithium, and combinations thereof.

[0253] Furthermore, the crude fatty ester biofuels described herein contains low amounts of other trace elements, including, for example, chromium, molybdenum, nitrogen, and halogen ions, and therefore posing little or no health and environmental threat as biodiesel fuels.

[0254] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 0.02 ppm of copper (e.g., less than or equal to about 2 ppm, less than or equal to about 0.019 ppm, or less than or equal to about 0.0188 ppm, or less than or equal to about 0.0186 ppm of copper).

[0255] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 2 ppm of boron (e.g., less than or equal to about 2 ppm, less than or equal to about 1.9 ppm of boron, less than or equal to about 1.8 ppm of boron, less than o equal to about 1.7 ppm of boron, less than or equal to about 1.6 ppm of boron).

[0256] In some embodiments, the fatty esters produced as described herein contain less than or equal to about 2 ppm of chromium (e.g., less than or equal to about 2 ppm, less than or equal to about 1.9 ppm, less than or equal to about 1.8 ppm, less than or equal to about 1.7 ppm, less than or equal to about 1.6 ppm, less than or equal to about 1.5 ppm of chromium).

[0257] In certain embodiments, the fatty esters produced as described herein contain less than or equal to about 5 ppm of iron (e.g.,less than or equal to about 5 ppm, less than or equal to about 4 ppm, less than or equal to about 3.5 ppm, or less than or equal to about 3.3 ppm of iron).

[0258] In certain embodiments, the fatty esters produced as described herein contain less than or equal to about 2 ppm of molybdenum (e.g., less than or equal to about 2 ppm, less than or equal to about 1.9 ppm, less than or equal to about 1.8 ppm, less than or equal to about 1.7 ppm, less than or equal to about 1.6 ppm, less than or equal to about 1.5 ppm of molybdenum).

[0259] In certain embodiments, the fatty esters produced as described herein contain less than or equal to about 35 ppm of nitrogen (e.g., less than or equal to about 35 ppm, less than or equal to about 34 ppm, less than or equal to about 33 ppm, less than or equal to about 32 ppm, less than or equal to about 31 ppm, less than or equal to about 29 ppm of nitrogen). Alternatively, the fatty esters produced as described herein contain less than or equal to about 1.0% of nitrogen (e.g., less than or equal to about 0.9%, less than or equal to about 0.8%, less than or equal to about 0.7%, less than or equal to about 0.6%, less than or equal to about 0.5% of nitrogen).

[0260] In certain embodiments, the fatty esters produced as described herein contain less than or equal to about 35 ppm of total halogens (e.g., less than or equal to about 35 ppm, less than or equal to about 34 ppm, less than or equal to about 33 ppm, less than or equal to about 32 ppm, or less than or equal to about 31 ppm of total halogens).

[0261] In certain embodiments, the fatty esters produced as described herein contain less than or equal to about 2.5 ppm of zinc (e.g., less than or equal to about 2.5 ppm, less than or equal to about 2.4 ppm, less than or equal to about 2.3 ppm, less than or equal to about 2.2 ppm, or less than or equal to about 2.1 ppm of zinc).

Emissions

[0262] Evaporative emissions from operating diesel engines include hydrocarbon (HC) vapors that escape from a fuel tank or permeate through hoses and connections in diesel engines. Evaporative emissions are regulated by the EPA because they contribute to the formation of ground-level ozone, a key component of smog. Combustion emissions are released through vehicle tailpipes or equipment exhaust systems when fuel is burned in a diesel engine. They include CO, NO_X and particulate matters (PM), which are regulated by the EPA because they impact ground level ozone and human health. In recent years, as these emissions are increasingly recognized as hazardous to the environment and human health, the EPA has imposed aggressively and incrementally stricter standards on diesel fuels manufactured and sold in the United States. For example, in 1984, the NO_X emission upper limit for heavy duty diesel engines was 10.7 gram per brake horsepower hour (g/bhp-hr). But by 1991, that upper limit was reduced to 5 g/bhp-hr; by 2004, to 2 g/bhp-hr. In another example, in 1984, the upper limit for PM for heavy duty diesel engines was 0.6 g/bhp-hr, but by 1991, that upper limit was reduced 0.25 g/bhp-hr; by 1994, to 0.10 g/bph-hr.

[0263] As used herein, the term "emission" or "emit" refers to the total amount of substances discharged from a standard diesel engine run under standard testing conditions. The emission may include the substances discharged from the diesel engine via evaporative emission and combustion emission.

[0264] The fatty esters described herein, after blended/formulated into B20 biodiesels, emit less NO_X and HC than a certified Ultra Low Sulfur Diesel (ULSD, Haltermann Products, Channelview, Tex., 2007 certification), and a B20 biodiesel formulated with 20% of a biodiesel derived from soy. Thus, in some embodiments, the fatty esters produced in accordance with the present disclosures have cleaner or comparable emissions profile of known pollutants as compared to biodiesels derived from other sources.

[0265] NO_X gases are formed when oxygen and nitrogen in the air react with each other during combustion. The most abundant pollutant, nitric oxide (NO) oxidizes in the atmosphere to form nitrogen dioxide (NO_2), which can oxidize to form ozone or particles known as $PM_{2.5}$. The formation of NO_X is most common when there are high temperatures and excess oxygen. Because NO_X is most abundant when combustion temperatures are high and hydrocarbon or total hydrocarbon (THC) and CO are most abundant when temperatures are low, there is a trade-off among these emissions.

[0266] In some embodiments, a fatty ester composition the fatty esters described herein emits NO_X at about 2.3 g/bph-hr or less (e.g., at about 2.3 g/bph-hr or less, at about 2.2 g/bph-hr or less, at about 2.1 g/bph-hr or less). In certain embodiments, the fatty esters described herein emits about 2 to about 2.3 g/bph-hr of NO_X (e.g., about 2.15 to about 2.2 g/bph-hr).

[0267] In certain embodiments, a B20 biodiesel blended with the fatty esters described herein achieves at least about 2.0% reduction of NO_X emission (e.g., at least about 2.5%, at least about 2.8%, at least about 3.0%, at least about 3.2%, or at least about 3.3% reduction in NO_X emission) as compared to the baseline certified petroleum-based diesel fuel ULSD. Alternatively, a B20 biodiesel blended with the fatty esters described herein achieves at least about 2.0% reduction (e.g., at least about 2.5%, at least about 2.8%, at least about 3.0%, or at least about 3.1%) of NO_X emission as compared to a B20 biodiesel blended with soy-derived biodiesel.

[0268] Hydrocarbon pollution results when unburned or partially burned fuel is emitted from the engine as exhaust and when fuel evaporates directly into the atmosphere. Hydrocarbon pollutants also react with NO_X in the presence of sunlight to form ozone. In some embodiments, the fatty esters described herein emits less than or equal to about 2 g/bhp-hr of total hydrocarbon (THC) (e.g., less than or equal to about 2 g/bph-hr, less than or equal to about 1.8 g/bhp-hr, less than or equal to about 1.5 g/bhp-hr, less than 1.0 g/bhp-hr, or less than or equal to about 0.5 g/bhp-hr) of total hydrocarbon (THC).

[0269] In certain embodiments, a B20 biodiesel blended with the fatty esters described herein achieves at least about 90% reduction of THC emission (e.g., at least about 95%, at least about 100%, at least about 105%, at least about 110%, at least about 115%, or at least about 120% reduction in THC emission) as compared to the THC emission of the baseline certified all petroleum-based diesel fuel ULSD. Alternatively, a B20 biodiesel blended with the fatty esters described herein achieves at least about 50% reduction (e.g., at least about 55%, at least about 60%, at least about 62%, or at least about 65% reduction) in THC emission as compared to the THC emission of a B20 biodiesel blended with soy-derived biodiesel.

[0270] Particulate matter (PM) is a common pollutant emitted by diesel-fueled vehicles and industrial equipment. PM is typically made up of small particles that contain a variety of chemical components. Larger particles are visible as smoke or dust, and settle out relatively rapidly. Smaller particles, such as $PM_{2.5}$, can be suspended in the air for long periods of time and inhaled into the lungs by humans and animals. Low levels of PM, however, can be effectively removed using particulate filters or other after-treatment devices suitable for removing diesel soot. In some embodiments, the fatty esters described herein emits equal to or less than about about 0.007 g/bhp-hr of PM (e.g., equal to or less than about 0.007 g/bhp-hr, equal to or less than about 0.006 g/bhp-hr, equal to or less than about 0.005 g/bhp-hr, equal to or less than about 0.004 g/bhp-hr, equal to or less than about 0.003 g/bhp-hr, equal to or less than about 0.002 g/bhp-hr) of PM. In some embodiments, the fatty esters as described herein emits about 0.001 to about 0.007 g/bhp-hr (e.g., about 0.001 to about 0.006, about 0.001 to about 0.005, about 0.001 to about 0.004) or PM.

[0271] In certain embodiments, a B20 biodiesel blended with the fatty esters described herein produced a somewhat increased amount (e.g., about 90% more PM) of particulates as compared to the amount of particulates generated by the baseline certified all petroleum-based diesel fuel ULSD, but a comparable level (e.g., about 10 to 15% more) of PM as compared to the amount of particulates generated by a B20 biodiesel blended with soy-derived biodiesel.

[0272] Carbon monoxide forms when the carbon in the fuel is not burned completely due to a lack of oxygen. The level of CO produced is typically not a concern unless the fuel and the engine using it are operated at high altitudes where less oxygen is present to promote combustion. In some embodiments, the fatty esters described herein emits about 0.4 g/bhp-hr or less (e.g., about 0.3 g/bph-hr or less, about 0.25 g/bph-hr or less) of CO. In some embodiments, the fatty esters described herein emits about 0.25 to about 0.40 g/bhp-hr (e.g., about 0.25 to about 0.35 g/bhp-hr, about 0.25 to about 0.3 g/bhp-hr) of CO.

[0273] In certain embodiments, a B20 biodiesel blended with the fatty esters described herein emits a comparable amount (e.g., about 10% to about 25% increase) of CO produced as compared to the amount of CO generated by the baseline certified fuel ULSD, but a somewhat increased level (e.g., about 30% to 40% increase) of CO as compared to the amount of CO generated by a B20 biodiesel blended with soy-derived biodiesel.

Other Harmful Substances

[0274] The fatty esters produced as described herein desirably contains substantially lower levels of certain toxic chemical substances that are known to be harmful to the human or animal health, such as, for example, are carcinogenic. An exemplary harmful substance that is present at low or negligible level is benzene, which is a known carcinogen and neurotoxin. The fatty esters and compositions described herein contains less than about 15 ppm (e.g., less than about 12 ppm, less than about 10 ppm) of benzene.

Fuel Compositions

[0275] The fatty esters described herein can be used as a fuel. One of ordinary skill in the art will appreciate that, depending upon the intended purpose of the fuel, different fatty esters can be produced and used. For example, for motor fuel intended to use in cold climates, a branched fatty ester can be desirable. Moreover, the fatty ester-based fuels can be combined with other fuels or fuel additives to produce fuels having desired properties.

[0276] The fatty esters described herein can also be blended with other biofuels, which refer to any fuel derived from biomass such as, for example, plant matters, animal matters, waste products from industry, agriculture, forestry, and households, as well as sources of carbon, such as carbohydrates. Corn, sugar cane, and switchgrass are examples of plant matters that can be used as biomass from which the other biofuel may be derived. Cow manure or other animal wastes are examples of animal matters that can serve as biomass. In limited circumstances, certain Fischer-Tropsch fuels can also serve as the other biofuel if they are derived from biomass using the catalyzed gasification process and/or the Fischer-Tropsch process.

[0277] The fatty esters described herein can alternatively or additionally be blended with fuels derived from non-biomass sources, including, for example, fuels derived from coal, natural gas, and fossil. These fuels may include, for example, petroleum-based diesel, and Fischer-Tropsch diesel fuel made from gasification of coal and natural gas.

[0278] In certain embodiments, a biofuel composition of the invention comprises petroleum diesel. In some embodiment, the biofuel composition comprises about 95% or less of petroleum diesel. For example, the biofuel composition comprises about 95% or less, about 90% or less, about 85% or less, about 80% or less, about 75% or less, about 70% or less, about 65% or less, about 60% or less, about 55% or less, about 50% or less, about 45% or less, about 40% or less, about 35% or less, about 30% or less, about 25% or less, or about 20% or less of petroleum diesel.

[0279] The fatty esters described herein can be blended with other fuels in customary proportions. The amount of biodiesel (or fuel derived from biomass) present in any fuel mix is designated using a "B" factor. Accordingly, a fuel that is 100% biodiesel is labeled B 100, whereas a fuel mixture containing equal to or no more than 20% biodiesel is labeled B20. It is within the present invention that a B100 biodiesel comprises about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more of the fatty esters described here, with the remaining part of the B 100 biodiesel being one or more diesels derived from other types biomass or derived from biomass using methods that differ from the ones described herein. Also, a B20 biodiesel may comprise about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20% of the fatty esters described here, with about 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0% of one or more diesels derived from other types of biomass or derived from biomass using methods that differ from the ones described here. In some embodiments, a B20 biodiesel of the present invention is a biodiesel composition comprising equal to about 20% of the fatty esters produced in accordance with the present description. In alternative embodiments, a B20 biodiesel of the present invention comprises up to about 20% but no more than about 20% of the fatty esters produced according to the description herein, for example, comprise about 10-20%, about 12-20%, about 14-20%, about 16-20%, about 18-20%, or about 20% of the fatty esters produced according to the descriptions herein. In a particular embodiment, a B20 biodiesel of the present invention comprises about 20% of the fatty esters produced according to the description herein.

[0280] In certain embodiments, the present invention features a biofuel composition comprising a fatty ester produced in accordance with the description herein. In some embodiments, the biofuel composition of the present invention further comprises suitable fuel additives that not only afford improved performance but also compatibility with the other components in the diesel fuel and other devices that are typically associated with diesel engines.

[0281] One prominent example of such a device is a catalytic converter, which contains one or more oxidation catalysts, NO_X storage catalysts, and/or NH_3 reduction catalysts (e.g., a combination of catalytic metals such as platinum, and metal oxides). Catalytic converters are installed in the exhaust systems, for example, the exhaust pipes of automobiles, to convert the toxic gases to nontoxic gases. The catalysts, however, can be poisoned and rendered less effective, if not useless, as a result of exposure to certain elements or compounds, especially phosphorus compounds and compounds that produces sulfated ash. Among the many ways phosphorus compounds may be introduced into the exhaust gas is the degradation of phosphorus-containing additives. Examples of phosphorus lubricating oil additives include zinc dialkyldithiophosphates, which are among the most effective and conventionally used antioxidants and antiwear agents. Examples of sulfur and sulfur containing compounds that

produces sulfated ash in the exhaust gas include various sulfur-containing additives such as, for instance, magnesium sulfonate and other sulfated or sulfonated detergents. Suitable types and amounts of fuel additives can be determined in order to insure a reasonable service life for the catalytic converters.

[0282] Particulate traps are usually installed in the exhaust system, especially in diesel engines, to prevent the carbon black particles or very fine condensate particles or agglomerates thereof (i. e., "diesel soot") from being released into the environment. These traps, however, can be blocked by metallic ash, which is the degradation product of metal-containing additives including common ash-producing detergent additives. Accordingly, low ash or preferably ashless additives should be chosen for compatibility with particulate traps.

[0283] Conventionally, fuel additives can be formulated into "additive packages," each comprising a major part (i.e., >50%) of one or more base oil and a minor part (i.e., <50%) of various additives. These additive packages can then be added to a blended fuel composition, such as, for example, a B20 biodiesel fuel, to enhance the overall performance of the fuel or engine. The additive packages are typically added to a fuel in an amount that is less than 10 wt. % , preferably less than 7 wt. %, more preferably less than 5 wt. % of the final fuel composition.

[0284] The preparation of additive packages for use with diesel fuels is within the knowledge of a person ordinarily skilled in the art. For example, one or more base oils can be used in a single additive package. The base oils are selected from a variety of oils of lubricating viscosity. The one or more base oils typically are present in the additive package in a major amount (i.e., an amount greater than about 50 wt. %), preferably in an amount greater than about 60 wt. %, or greater than about 70 wt. %, or greater than about 80 wt. % of the additive package. The sulfur content of the base oil is typically less than about 1.0 wt. %, preferably less than about 0.6 wt. %, more preferably less than about 0.4 wt. %, and particularly preferably less than about 0.3 wt. %.

[0285] Suitable base oils are those that have a viscosity of at least about 2.5 cSt. (i.e., mm^2/s), or at least about 3.0 cSt. at 40° C. Suitable base oils are ones that have pour points below about 20° C., or below about 10° C., or even below about 5° C., such as below about 0° C.

[0286] The base oil used in the additive package may be a natural oil, a synthetic oil, or a mixture thereof, provided that the sulfur content of such an oil does not exceed the above-indicated sulfur concentration limit such that the additive package does not contribute to the emission of sulfate and production of sulfated ash. Suitable natural oils include animal oils, vegetable oils (e.g., castor oil, lard oil), mineral oils, and solvent-treated or acid-treated mineral oils. Oils derived from coal or shale can also be used. Synthetic oils include hydrocarbon oils such as polymerized and interpolymerized olefins, poly(1-hexenes), poly-(1-octenes), poly(1-decenes), etc. and mixtures thereof; alkylbenzenes; polyphenyls; alkylated diphenyl ethers and the derivatives, analogs and homologs thereof; and the like. Synthetic lubricating oils also include oils prepared by Fischer-Tropsch gas-to-liquid synthetic procedure. Suitable synthetic lubricating oils also include, for example, alkylene oxide polymers and interpolymers and derivatives thereof where the terminal hydroxyl groups have been modified by a process such as esterification or etherification. Other suitable synthetic oils include esters of dicarboxylic acids with a variety of alcohols. The synthetic oil can also be a poly-alpha-olefin (PAO). Examples of useful PAOs include those derived from octane, decene, mixtures, and the like, which may have a viscosity from 2 to 15, or from 3 to 12, or from 4 to 8 mm^2/s (cSt.) at 100° C. Unrefined, refined and rerefined oils, either natural or synthetic (as well as mixtures of two or more) of the types of oils disclosed above can be used as the base oil.

[0287] Fuel additives can be blended into the additive package in a minor amount (i.e., <50 wt. % of the additive package). They can be used to alter the freezing/gelling point, cloud point, lubricity, viscosity, oxidative stability, ignition quality, cetane level, and flash point. Accordingly, fuel additives can include, for example, lubricants, dispersants, emulsifiers, corrosion inhibitors, oxidation inhibitors, friction modifiers, demulsifiers, anti-wear agents, anti-foam agents, detergents, rust inhibitors, and the like.

[0288] Engine performance additives can be added to improve diesel engine performance. They are often also referred to as diesel ignition improvers or cetane number improvers, which are added to reduce combustion noise and smoke. 2'-Ethylhexyl nitrate (EHN), also called octyl nitrate, is the most widely used cetane number improver. Cetane number improvers are typically used in the concentration range of about 0.05 wt. % to about 0.4 wt. % in the final fuel composition, giving rise to an about 3 to about 8 (e.g., about 3, 4, 5, 6, 7, or 8) cetane number benefit. Other alkyl nitrates, ether nitrates, some nitroso compounds, and di-tertiary butyl peroxide can also be used.

[0289] Various detergents or dispersants known to those skilled in the art can be used to remove deposits that form in the nozzle area of the fuel injectors and other diesel engine parts. They also serve as acid neutralizers or rust inhibitors, thereby reducing wear and corrosion and extending diesel engine life. Suitable detergents typically comprise a polar head comprising a metal salt of an acidic organic compound, and a long hydrophobic tail. The metal salts may be, for example, Group 1 and Group 2 metal salts, preferably, sodium, potassium, lithium, copper, or magnesium, calcium, barium or zinc, and particularly sodium and calcium salts. Exemplary detergents include borated carbonate salts (see, e.g., U.S. Pat. No. 4,744,920) and borated sulfonate salts (see, e.g., U.S. Pat. No. 4,965,003). To provide an increased acid-neutralization capacity, suitable detergents can be overbased, such that the detergent has a total base number (TBN) of 10 or higher, 60 or higher, 100 or higher, 200 or higher, 300 or higher, 400 or higher, or even 500 or higher. It is within the knowledge of an ordinarily skilled person in the art to overbase detergents and measure the TBN in accordance with well known methods, such as, for example, ASTM test D 2896 and other equivalent procedures.

[0290] The additive package of the present invention thus may suitably include ashless dispersants, such as nitrogen-containing detergents, which are basic, contribute to the TBN of a fuel to which they are added, without introducing additional ash. An ashless dispersant generally comprises an oil-soluble polymeric hydrocarbon backbone having functional groups that are capable of associating with particles to be dispersed. Many types of ashless dispersants are known in the art. They include, without limitation, carboxylic dispersants, succinimide dispersants, amine dispersants, Mannich dispersants. Carboxylic dispersants are imide, amide, or ester reaction products of carboxylic acylating agents, comprising at least 34 and preferably at least 54 carbon atoms, with nitrogen containing compounds, organic hydroxyl com-

pounds (e.g., aliphatic compounds), and/or basic inorganic materials. Succinimide dispersants are a type of carboxylic dispersants, produced by reacting hydrocarbyl-substituted succinic acylating agent with organic hydroxyl compounds, or with amine comprising at least one hydrogen attached to a nitrogen atom, or with a mixture of the hydroxyl compounds and amines (see, e.g., U.S. Pat. Nos. 3,172,892, 3,219,666, 3,272,746, 4,234,435, 6,440,905, and 6,165,235, the disclosures of which, to the extent they pertain to succinimide dispersants, are incorporated by reference). Amine dispersants are products of relatively high molecular weight aliphatic halides and amines, preferably polyalkelene polyamines (see, e.g., U.S. Pat. Nos. 3,275,544, 3.438,757, 3,565,804, the disclosures of which, to the extent they pertain to amine dispersants, are incorporated by reference herein). Mannich dispersants are reaction products of alkyl phenols in which the alkyl group contains at least 30 carbon atoms with aldehydes (especially formaldehyde) and amines (especially polyalkylene polyamines), as described in, for example, U.S. Pat. Nos. 3,036,003, 3,586,629, 3,591,598, 3,980,569, the disclosures of which, to the extent they pertain to Mannich dispersants, are incorporated by reference). Suitable dispersants may also include post-treated dispersants, which are obtained by reacting the above-mentioned dispersants with reagents such as dimercaptothioazoles, urea, thiourea, carbon disulfide, aldehydes, ketones, carboxylic acids, hydrocarbon-substituted succinic anhydrides, nitrile epoxides, boron compounds and the like. See, e.g., U.S. Pat. Nos. 3,329,658, 3,449,250, 3,666, 730, and the like, the disclosures of which, to the extent they pertains to post-treated dispersants, are incorporated by reference. Suitable ashless dispersants may be polymeric, such as, for example, interpolymers of oil-solublizing monomers such as decyl methacrylate, vinyl decyl ether and high molecular weight olefins with monomers containing polar substitutes. Suitable ashless dispersants can be present in an amount of about 0.025 wt. % to about 0.5 wt. % (e.g., about 0.025, 0.030, 0.035, 0.040, 0.045, 0.050, or 0.055 wt. %) of the overall fuel.

[0291] The additive package may further comprise one or more antiwear agents. Dihydrocarbyl dithiophosphate metal salts, and especially alkali or alkaline earth metal salts, such as zinc, aluminum, or copper salts, are often used to provide antiwear benefits as well as to serve as antioxidant agents. Methods of making these agents are well known in the art, and they may be included in the additive package in an amount of about 12 to about 24 mM (e.g., about 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 mM). See, e.g., U.S. Pat. No. 5,898,023, the content of which, to the extent it relates to antiwear agents, is incorporated by reference.

[0292] The additive package to be blended into a biodiesel fuel may further comprise one or more viscosity index modifiers, friction modifiers, antioxidants, and minor amounts of other additives, including, without limitation, rust inhibitors, antifoaming agents, and seal fixes.

[0293] Viscosity index improvers (VII's) are typically polymeric materials of number average molecular weights of from about 5,000 to about 250,000 (e.g., about 5,000, 7,500, 10,000, 15,000, 20,000, 30,000, 50,000, 75,000, 100,000, 150,000, 200,000, or 250,000).

[0294] Friction modifiers are typically sulfur-containing organo-molybdenum compounds that are known to also provide antiwear and antioxidant credits.

[0295] In addition to the other multi-purpose additives (e.g., those described herein) that impart antioxidation properties, the additive package may also suitably contain one or more dedicated antioxidant additives, which further reduces the tendency of deterioration of the fuels. They may be hindered phenols, alkaline earth metal salts of alkylphenolthioeters having C_5 to C_{12} alkyl side chains, calcium nonylphenol sulfides, oil soluble phenates and sulfurized phenates, phosphosulfurized or sulfurized hydrocarbons or esters, phosphorous esters, metal thiocarbamates, as well as oil soluble copper compounds as described in, for example, U.S. Pat. No. 4,867,892. Also suitable are aromatic amines with at least two aromatic groups attached directly to the nitrogen. They are typically used in a range of about 10 ppm to about 80 ppm (e.g., about 8, 10, 20, 30, 40, 50, 60, 70, 80, or 90 ppm).

[0296] Rust inhibitors or anticorrosion agents may be a non-ionic polyoxyethylene surface active agent. They can be included in the additive package and added to a biodiesel fuel composition in a concentration range of about 5 ppm to about 15 ppm (e.g., about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 ppm).

[0297] Foam inhibitors typically include alkyl methacrylate polymers and dimethyl silicon polymers. They can be included in the additive package and added to a biodiesel fuel composition at a concentration of about 10 ppm or less.

[0298] Seal fixes, seal swelling agents, or seal pacifiers are often employed to insure proper elastomer sealing, and prevent premature seal failure and leakages, and these agents may also be a part of the additive package. They may be, for example, oil-soluble, saturated, aliphatic, or aromatic hydrocarbon esters such as di-2-ethylhexylphthalate, mineral oils with aliphatic alcohols such as tridecyl alcohol, triphosphite ester in combination with a hydrocarbonyl-substituted phenol, and di-2-ethylhexylsebacate.

[0299] Lubricity additives, which are typically fatty acids and/or fatty esters, for example, polyol esters of C_{12}-C_{28} acids, can be applied in the concentration range of about 10 ppm to about 50 ppm (e.g., about 10, 20, 30, 40, or 50 ppm) for the acids, and about 50 ppm to about 250 ppm (e.g., about 50, 75, 100, 125, 150, 175, 200, 225, 250 ppm) for the esters.

[0300] Some organometallic compounds, for example, barium or other metal (e.g., iron, cerium, platinum, etc.) organometallics, can act as combustion catalysts, and can be used as smoke suppressants, which reduce the black smoke emissions that result from incomplete combustion.

[0301] In addition, low molecular weight alcohols or glycerols can be added to diesel fuel to prevent ice formation in low temperature applications.

[0302] Other additives can be used to lower a diesel fuel's pour point or cloud point, or improve its cold flow properties. These additives are typically additives capable of interacting with the wax crystals that form in diesel fuels when they are cooled below the cloud points.

[0303] Drag reducing additives can also be added to increase the volume of the product that can be delivered. They may be included in the additive package and added to a biodiesel fuel at concentrations below about 15 ppm.

[0304] Metal deactivators can be used to chelate various metal impurities, neutralizing their catalytic effects on fuel performance. They can also be included in the additive package and added to a biodiesel fuel in the concentration range of about 1 ppm to about 15 ppm (e.g., about 1, 3, 5, 7, 9, 11, 13, or 15 ppm).

[0305] Biocides, which preferably dissolve in both the fuel and water, can be used when contamination by microorganisms reaches problem levels. They can be added to a biodiesel fuel at a concentration range of about 200 ppm to about 600 ppm (e.g., about 180, 200, 250, 300, 350, 400, 500, or 600 ppm).

[0306] Demulsifiers are surfactants that break the emulsions and allow fuel and water phases to separate. They are typically used in the concentration range of about 5 ppm to about 30 ppm.

[0307] Pour point depressants such as C_8-C_{18} dialkyl fumarate vinyl acetate copolymers, polymethacrylates and wax naphthalene are well known to those skilled in the art.

[0308] In the United States, all fuel additives must be registered with the Environmental Protection Agency (EPA). Companies that sell fuel additives and the name of the fuel additive are publicly available on the EPA's web site or also by contacting the EPA. One of ordinary skill in the art will appreciate that the fatty esters described herein can be mixed with one or more such additives to impart a desired quality.

[0309] The fuel additives described herein can be prepared by mixing between about 80% to 99.7% biodiesel fuel and between about 0.3 to about 20% of the additive package, each by volume. The components can be mixed in any suitable manner. Optimal selection of an appropriate ratio of fuel vs. fuel additive package will depend on a variety of factors, including the season (i.e., winter, summer, spring, or fall), altitude, in which the fuel composition is used. It also depends upon the types of fatty esters made according to the present invention, and the types of fuels that are blended. The amount of additives added may be determined by following the cetane value or other performance parameters of the diesel fuel composition as the additive package is gradually and continuously added and blended into the fuel. Means of mixing or blending the components are well known to those skilled in the art. During blending, it may be advantageous to remove aliquots of the blended fuel and measure various properties, such as vapor pressure and cetane values, to insure that the blend has the desired properties.

[0310] One of ordinary skill in the art will also appreciate that the fatty esters described herein can be mixed with other fuels, such as biodiesel derived from triglycerides, various alcohol, such as ethanol and butanol, and petroleum derived products, such as petroleum diesel. In some examples, fatty esters, such as those having C16:1 ethyl ester or C18:1 ethyl ester, can be mixed with petroleum derived diesel to provide a mixture that is at least and often greater than 5% biodiesel. In some examples, the mixture includes at least about 20% or greater of the fatty esters. In some examples, the mixture comprises about 95% (e.g., about 80% to about 95%) or less petroleum diesel. In this regard, the percentage of biodiesel and/or of petroleum diesel can be based on weight percent or volume percent.

[0311] As will be appreciated by one of ordinary skill in the art, any of the above fatty esters and fatty ester compositions can be converted into a biofuel, or more specifically biodiesel, if desired. Thus, the corresponding biofuels and biodiesels are also provided herein.

[0312] Embodiments of the invention are also described in WO 2009/042950 A1, WO 2009/009391 A2, WO 2008/147781 A2, WO 2008/119082 A2, WO 2008/113041 A2, WO 2008/100251 A1, WO 2007/136762 A2, which are incorporated herein by reference in their entirety.

[0313] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

Example 1

[0314] This example describes the construction of a genetically engineered microorganism wherein the expression of a fatty acid degradation enzyme is attenuated.

[0315] The fadE gene of *E. coli* MG1655 (an *E. coli* K strain) was deleted using the Lambda Red system described in Datsenko et al., *Proc. Natl. Acad. Sci. USA* 97: 6640-6645 (2000), with the following modifications.

[0316] Two primers were used to create the deletion:

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Del-fadE-F
                                                    (SEQ ID NO: 1)
5'-AAAAACAGCAACAATGTGAGCTTTGTTGTAATTATATTGTAAACATA

TTGATTCCGGGGATCCGTCGACC

Del-fadE-R
                                                    (SEQ ID NO: 2)
5'-AAACGGAGCCTTTCGGCTCCGTTATTCATTTACGCGGCTTCAACTTT

CCTGTAGGCTGGAGCTGCTTC
```

[0317] The Del-fadE-F and Del-fadE-R primers were used to amplify the Kanamycin resistance (Km^R) cassette from plasmid pKD13 (as described in Datsenko et al., supra) by PCR. The PCR product was then used to transform electrocompetent *E. coli* MG1655 cells containing pKD46 (described in Datsenko et al., supra). These cells had been previously induced with arabinose for 3-4 h. Following a 3-h outgrowth in a super optimal broth with catabolite repression (SOC) medium at 37° C., the cells were plated on Luria agar plates containing 50 μg/mL Kanamycin. Resistant colonies were identified and isolated after an overnight incubation at 37° C. Disruption of the fadE gene was confirmed in select colonies using PCR amplification with primers fadE-L2 and fadE-R1, which were designed to flank the fadE gene:

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fadE-L2 5'-CGGGCAGGTGCTATGACCAGGAC   (SEQ ID NO: 3)

fadE-R1 5'-CGCGGCGTTGACCGGCAGCCTGG   (SEQ ID NO: 4)
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[0318] After the fadE deletion was confirmed, a single colony was used to remove the Km^R marker, using the pCP20 plasmid as described in Datsenko et al., supra. The resulting MG1655 *E. coli* strain with the fadE gene deleted and the Km^R marker removed was named *E. coli* MG1655 4fadE , or *E. coli* MG1655 D1.

Example 2

[0319] This example describes the construction of a genetically engineered microorganism in which the expression of a fatty acid degradation enzyme and an outer membrane protein receptor are attenuated.

[0320] The fhuA (also known as tonA) gene of *E. coli* MG1655, which encodes a ferrichrome outer membrane transporter (GenBank Accession No. NP_414692), was deleted from strain *E. coli* MG1655 D1 of Example 1 using the Lambda Red system described in Datsenko et al., supra, but with the following modifications.

[0321] Two primers were used to create the deletion:

Del-fhuA-F (SEQ ID NO: 5)
5'-ATCATTCTCGTTTACGTTATCATTCACTTTACATCAGAGATATACCA
ATGATTCCGGGGATCCGTCGACC;

Del-fhuA-R (SEQ ID NO: 6)
5'-GCACGGAAATCCGTGCCCCAAAAGAGAAATTAGAAACGGAAGGTTGC
GGTTGTAGGCTGGAGCTGCTTC

[0322] The Del-fhuA-F and Del-fhuA-R primers were used to amplify the Km^R cassette from plasmid pKD13 by PCR. The PCR product obtained was used to transform the electrocompetent *E. coli* MG1655 D1 cells containing pKD46 (see Example 1). These cells had been previously induced with arabinose for 3-4 h. Following a 3-h outgrowth in SOC medium at 37° C., the cells were plated on Luria agar plates containing 50 μg/mL Kanamycin. Kanamycin resistant colonies were identified and isolated after an overnight incubation at 37° C. Disruption of the fhuA gene was confirmed in select colonies by PCR amplification with primers fhuA-verF and-fhuA-verR, which were designed to flank the fhuA gene.

[0323] Confirmation of the deletion was performed using the following primers:

fhuA-verF 5'-CAACAGCAACCTGCTCAGCAA (SEQ ID NO: 7)

fhuA-verR 5'-AAGCTGGAGCAGCAAAGCGTT (SEQ ID NO: 8)

[0324] After the fhuA deletion was confirmed, a single colony was used to remove the Km^R marker, using the pCP20 plasmid as described in Datsenko et al., supra. The resulting MG1655 *E. coli* strain having the fadE and fhuA gene deletions was named *E. coli* MG1655 AfadE AfhuA, or *E. coli* MG1655 DV2.

Example 3

[0325] This example describes the construction of a genetically engineered microorganism in which nucleotide sequences encoding a thioesterase, an acyl-CoA synthase, and an ester synthase are integrated into the microorganism's chromosome.

[0326] The following nucleotide sequences, 'tesA, fadD, and aftA1, were integrated into the chromosome of *E. coli* MG1655 ΔfadE ΔfhuA strain (or DV2 strain, see Example 2) at the lacZ locus. The sequences were integrated in the order of 'tesA, followed by fadD, and followed by aftA1.

[0327] 'tesA is a nucleotide sequence comprising a leaderless *E. coli* tesA (GenBank entry AAC73596, refseq accession U00096.2). 'tesA encodes an *E. coli* thioesterase (EC 3.1.1.5, 3.1.2.-) in which the first twenty-five amino acids were deleted and the amino acid in position 26, alanine, was replaced with methionine. That methionine then became the first amino acid of 'tesA. See Cho et al., *J. Biol. Chem.*, 270:4216-4219 (1995).

[0328] *E. coli* fadD (GenBank entry AAC74875; REFSEQ: accession U00096.2) encodes an acyl-CoA synthase.

[0329] Alcanivorax borkumensis strain SK2 atfA1 (GenBank entry YP_694462; REFSEQ: accession NC_008260.1) encodes an ester synthase.

[0330] 'tesA, fadD, and atfA1 were integrated into the chromosome of *E. coli* MG1655 DV2 at the lacZ locus, all under the control of a Trc promoter, as described below.

[0331] Design and Creation of a 'tesA, fadD, atfA1 Integreation Cassette

Construction of the 'tesA Plasmid

[0332] 'tesA was amplified from a pETDuet-1-'tesA plasmid constructed as described below. (see also, e.g., WO 2007/136762 A2, which is incorporated by reference). The 'tesA gene was cloned into an NdeI/AvrII digested pETDuet-1 plasmid (Novagen, Madison, Wis.).

Construction of the fadD Plasmid

[0333] fadD was amplified from a pHZ1.61 plasmid constructed as described below. A fadD gene was cloned into a pCDFDuet-1 plasmid (Novagen, Madison, Wis.) under the control of a T7 promoter, generating a pHZ1.61 plasmid containing the following nucleotide sequence:

(SEQ ID NO: 9)
GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAA
CTTTAATAAGGAGATATACCATGGTGAAGAAGGTTTGGCTTAACCGTTAT
CCCGCGGACGTTCCGACGGAGATCAACCCTGACCGTTATCAATCTCTGGT
AGATATGTTTGAGCAGTCGGTCGCGCGCTACGCCGATCAACCTGCGTTTG
TGAATATGGGGGAGGTAATGACCTTCCGCAAGCTGGAAGAACGCAGTCGC
GCGTTTGCCGCTTATTTGCAACAAGGGTTGGGGCTGAAGAAAGGCGATCG
CGTTGCGTTGATGATGCCTAATTTATTGCAATATCCGGTGGCGCTGTTTG
GCATTTTGCGTGCCGGGATGATCGTCGTAAACGTTAACCCGTTGTATACC
CCGCGTGAGCTTGAGCATCAGCTTAACGATAGCGGCGCATCGGCGATTGT
TATCGTGTCTAACTTTGCTCACACACTGGAAAAAGTGGTTGATAAAACCG
CCGTTCAGCACGTAATTCTGACCCGTATGGGCGATCAGCTATCTACGGCA
AAAGGCACGGTAGTCAATTTCGTTGTTAAATACATCAAGCGTTTGGTGCC
GAAATACCATCTGCCAGATGCCATTTCATTTCGTAGCGCACTGCATAACG
GCTACCGGATGCAGTACGTCAAACCCGAACTGGTGCCGGAAGATTTAGCT
TTTCTGCAATACACCGGCGGCACCACTGGTGTGGCGAAAGGCGCGATGCT
GACTCACCGCAATATGCTGGCGAACCTGGAACAGGTTAACGCGACCTATG
GTCCGCTGTTGCATCCGGGCAAAGAGCTGGTGGTGACGGCGCTGCCGCTG
TATCACATTTTTGCCCTGACCATTAACTGCCTGCTGTTTATCGAACTGGG
TGGGCAGAACCTGCTTATCACTAACCCGCGCGATATTCCAGGGTTGGTAA
AAGAGTTAGCGAAATATCCGTTTACCGCTATCACGGGCGTTAACACCTTG
TTCAATGCGTTGCTGAACAATAAAGAGTTCCAGCAGCTGGATTTCTCCAG
TCTGCATCTTTCCGCAGGCGGAGGGATGCCAGTGCAGCAAGTGGTGGCAG
AGCGTTGGGTGAAACTGACAGGACAGTATCTGCTGGAAGGCTATGGCCTT
ACCGAGTGTGCGCCGCTGGTCAGCGTTAACCCATATGATATTGATTATCA
TAGTGGTAGCATCGGTTTGCCGGTGCCGTCGACGGAAGCCAAACTGGTGG
ATGATGATGATAATGAAGTACCACCGGGTCAACCGGGTGAGCTTTGTGTC
AAAGGACCGCAGGTGATGCTGGGTTACTGGCAGCGTCCGGATGCTACAGA

-continued

TGAGATCATCAAAAATGGCTGGTTACACACCGGCGACATCGCGGTGATGG

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GCATCCTGGCGTACAGGAAGTCGCGGCTGTTGGCGTACCTTCCGGCTCCA

GTGGTGAAGCGGTGAAAATCTTCGTAGTGAAAAAAGATCCATCGCTTACC

GAAGAGTCACTGGTGACCTTTTGCCGCCGTCAGCTCACGGGCTACAAAGT

ACCGAAGCTGGTGGAGTTTCGTGATGAGTTACCGAAATCTAACGTCGGAA

AAATTTTGCGACGAGAATTACGTGACGAAGCGCGCGGCAAAGTGGACAAT

AAAGCCTGAAAGCTTGCGGCCGCATAATGCTTAAGTCGAACAGAAAGTAA

TCGTATTGTACACGGCCGCATAATCGAAATTAATACGACTCACTATAGGG

GAATTGTGAGCGGATAACAATTCCCCATCTTAGTATATTAGTTAAGTATA

AGAAGGAGATATACATATGCGCCCATTACATCCGATTGATTTTATATTCC

TGTCACTAGAAAAAAGACAACAGCCTATGCATGTAGGTGGTTTATTTTTG

TTTCAGATTCCTGATAACGCCCCAGACACCTTTATTCAAGATCTGGTGAA

TGATATCCGGATATCAAAATCAATCCCTGTTCCACCATTCAACAATAAAC

TGAATGGGCTTTTTTGGGATGAAGATGAAGAGTTTGATTTAGATCATCAT

TTTCGTCATATTGCACTGCCTCATCCTGGTCGTATTCGTGAATTGCTTAT

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AAAATTAAGAAAATCATGTCTGGTATTAAGAGTCAGCTTCAGGCGACACC

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CGTGTGAGCTCATCGCGACGTTTTGCAGCACAGTCTTTTGACCTAGATCG

TTTTCGTAATATTGCCAAATCGTTGAATGTGACCATTAATGATGTTGTAC

TAGCGGTATGTTCTGGTGCATTACGTGCGTATTTGATGAGTCATAATAGT

TTGCCTTCAAAACCATTAATTGCCATGGTTCCAGCCTCTATTCGCAATGA

CGATTCAGATGTCAGCAACCGTATTACGATGATTCTGGCAAATTTGGCAA

CCCACAAAGATGATCCTTTACAACGTCTTGAAATTATCCGCCGTAGTGTT

CAAAACTCAAAGCAACGCTTCAAACGTATGACCAGCGATCAGATTCTAAA

TTATAGTGCTGTCGTATATGGCCCTGCAGGACTCAACATAATTTCTGGCA

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ATTTAGATAAACTTGAAGTTGGTTTGATTGCATGCCGTAATGCATTGCCA

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GCGCTGTACCAAATGCGGGACAACGTAAGCACTACATTTCGCTCATCGCC

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CCTACCAAGGCAACGCTATGTTCTCTTGCTTTTGTCAGCAAGATAGCCAG

ATCAATGTCGATCGTGGCTGGCTCGAAGATACCTGCAAGAATGTCATTGC

GCTGCCATTCTCCAAATTGCAGTTCGCGCTTAGCTGGATAACGCCACGGA

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GCTCTCTCCAGGGGAAGCCGAAGTTTCCAAAAGGTCGTTGATCAAAGCTC

GCCGCGTTGTTTCATCAAGCCTTACGGTCACCGTAACCAGCAAATCAATA

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CGCTTTCTCATAGCTCACACACTGGTATCTCGGCTCGGTGTAGGTCGTTC

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GGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCA

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TACCGAGATGTCCGCACCAACGCGCAGCCCGGACTCGGTAATGGCGCGCA

TTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAGTGGGAACG

ATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGACATGGCACT

CCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCGAGTGAGAT

ATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAACTTAATGGG

CCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGATGCTCCAC

GCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTTGATGGGTG

TCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGCAGGCAGCT

TCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATGATCAGCCC

ACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACAGGCTTCGA

CGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCAGTTGATCG

GCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAGGGCCAG

ACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGCCAGTTGTT

GTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCGCTTCCACT

TTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACCACGCGGGA

AACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTATAACGTTA

CTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGCGCTATCAT

GCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGTCCGGGATCTCGAC

GCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAATACGACTCACTATA

Construction of the atfA1 Plasmid

[0334] atfA1 was amplified from a pHZ1.97-atfA1 plasmid constructed as described below. The atfA1 gene was synthesized by DNA2.0 (Menlo Park, Calif.) and cloned into an NdeI and AvrII digested pCOLA-Duet-1 plasmid (Novagen, Madison, Wis.), generating a pHZ1.97-atfA1plasmid having the following nucleotide sequence:

(SEQ ID NO: 10)

GGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAA

CTTTAATAAGGAGATATACCATGGGCAGCAGCCATCACCATCATCACCAC

AGCCAGGATCCGAATTCGAGCTCGGCGCGCCTGCAGGTCGACAAGCTTGC

-continued

GGCCGCATAATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACACGGCC

GCATAATCGAAATTAATACGACTCACTATAGGGGAATTGTGAGCGGATAA

CAATTCCCCATCTTAGTATATTAGTTAAGTATAAGAAGGAGATATACATA

TGAAAGCGCTTAGCCCAGTGGATCAACTGTTCCTGTGGCTGGAAAAACGA

CAGCAACCCATGCACGTAGGCGGTTTGCAGCTGTTTTCCTTCCCGGAAGG

TGCCGGCCCCAAGTATGTGAGTGAGCTGGCCCAGCAAATGCGGGATTACT

GCCACCCAGTGGCGCCATTCAACCAGCGCCTGACCCGTCGACTCGGCCAG

TATTACTGGACTAGAGACAAACAGTTCGATATCGACCACCACTTCCGCCA

CGAAGCACTCCCCAAACCCGGTCGCATTCGCGAACTGCTTTCTTTGGTCT

CCGCCGAACATTCCAACCTGCTGGACCGGGAGCGCCCCATGTGGGAAGCC

CATTTGATCGAAGGGATCCGCGGTCGCCAGTTCGCTCTCTATTATAAGAT

CCACCATTCGGTGATGGATGGCATATCCGCCATGCGTATCGCCTCCAAAA

CGCTTTCCACTGACCCCAGTGAACGTGAAATGGCTCCGGCTTGGGCGTTC

AACACCAAAAAACGCTCCCGCTCACTGCCCAGCAACCCGGTTGACATGGC

CTCCAGCATGGCGCGCCTAACCGCGAGCATAAGCAAACAAGCTGCCACAG

TGCCCGGTCTCGCGCGGGAGGTTTACAAAGTCACCCAAAAAGCCAAAAAA

GATGAAAACTATGTGTCTATTTTTCAGGCTCCCGACACGATTCTGAATAA

TACCATCACCGGTTCACGCCGCTTTGCCGCCCAGAGCTTTCCATTACCGC

GCCTGAAAGTTATCGCCAAGGCCTATAACTGCACCATTAACACCGTGGTG

CTCTCCATGTGTGGCCACGCTCTGCGCGAATACTTGATTAGCCAACACGC

GCTGCCCGATGAGCCACTGATTGCAATGGTGCCCATGAGCCTGCGGCAGG

ACGACAGCACTGGCGGCAACCAGATCGGTATGATCTTGGCTAACCTGGGC

ACCCACATCTGTGATCCAGCTAATCGCCTGCGCGTCATCCACGATTCCGT

CGAGGAAGCCAAATCCCGCTTCTCGCAGATGAGCCCGGAAGAAATTCTCA

ATTTCACCGCCCTCACTATGGCTCCCACCGGCTTGAACTTACTGACCGGC

CTAGCGCCAAAATGGCGGGCCTTCAACGTGGTGATTTCCAACATACCCGG

GCCGAAAGAGCCGCTGTACTGGAATGGTGCACAGCTGCAAGGAGTGTATC

CAGTATCCATTGCCTTGGATCGCATCGCCCTAAATATCACCCTCACCAGT

TATGTAGACCAGATGGAATTTGGGCTTATCGCCTGCCGCCGTACTCTGCC

TTCCATGCAGCGACTACTGGATTACCTGGAACAGTCCATCCGCGAATTGG

AAATCGGTGCAGGAATTAAATAGTAACCTAGGCTGCTGCCACCGCTGAGC

AATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTT

TTGCTGAAACCTCAGGCATTTGAGAAGCACACGGTCACACTGCTTCCGGT

AGTCAATAAACCGGTAAACCAGCAATAGACATAAGCGGCTATTTAACGAC

CCTGCCCTGAACCGACGACAAGCTGACGACCGGGTCTCCGCAAGTGGCAC

TTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATAC

ATTCAAATATGTATCCGCTCATGAATTAATTCTTAGAAAAACTCATCGAG

CATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATT

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CATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTCCGACTCGTCCAAC

ATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTG

AGAAATCACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTA

TGCATTTCTTTCCAGACTTGTTCAACAGGCCAGCCATTACGCTCGTCATC

AAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCTGAG

CGAGACGAAATACGCGGTCGCTGTTAAAAGGACAATTACAAACAGGAATC

GAATGCAACCGGCGCAGGAACACTGCCAGCGCATCAACAATATTTTCACC

TGAATCAGGATATTCTTCTAATACCTGGAATGCTGTTTTCCCGGGGATCG

CAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATG

GTCGGAAGAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATC

TGTAACATCATTGGCAACGCTACCTTTGCCATGTTTCAGAAACAACTCTG

GCGCATCGGGCTTCCCATACAATCGATAGATTGTCGCACCTGATTGCCCG

ACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGA

ATTTAATCGCGGCCTAGAGCAAGACGTTTCCCGTTGAATATGGCTCATAC

TCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATG

AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGCATGCT

AGCGCAGAAACGTCCTAGAAGATGCCAGGAGGATACTTAGCAGAGAGACA

ATAAGGCCGGAGCGAAGCCGTTTTTCCATAGGCTCCGCCCCCCIGACGAA

CATCACGAAATCTGACGCTCAAATCAGTGGTGGCGAAACCCGACAGGACT

ATAAAGATACCAGGCGTTTCCCCCTGATGGCTCCCTCTTGCGCTCTCCTG

TTCCCGTCCTGCGGCGTCCGTGTTGTGGTGGAGGCTTTACCCAAATCACC

ACGTCCCGTTCCGTGTAGACAGTTCGCTCCAAGCTGGGCTGTGTGCAAGA

ACCCCCCGTTCAGCCCGACTGCTGCGCCTTATCCGGTAACTATCATCTTG

AGTCCAACCCGGAAAGACACGACAAAACGCCACTGGCAGCAGCCATTGGT

AACTGAGAATTAGTGGATTTAGATATCGAGAGTCTTGAAGTGGTGGCCTA

ACAGAGGCTACACTGAAAGGACAGTATTTGGTATCTGCGCTCCACTAAAG

CCAGTTACCAGGTTAAGCAGTTCCCCAACTGACTTAACCTTCGATCAAAC

CGCCTCCCCAGGCGGTTTTTTCGTTTACAGAGCAGGAGATTACGACGATC

GTAAAAGGATCTCAAGAAGATCCTTTACGGATTCCCGACACCATCACTCT

AGATTTCAGTGCAATTTATCTCTTCAAATGTAGCACCTGAAGTCAGCCCC

ATACGATATAAGTTGTAATTCTCATGTTAGTCATGCCCCGCGCCCACCGG

AAGGAGCTGACTGGGTTGAAGGCTCTCAAGGGCATCGGTCGAGATCCCGG

TGCCTAATGAGTGAGCTAACTTACATTAATTGCGTTGCGCTCACTGCCCG

CTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAA

CGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTT

TCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCTGA

GAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATC

CTGTTTGATGGTGGTTAACGGCGGGATATAACATGAGCTGTCTTCGGTAT

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CGTCGTATCCCACTACCGAGATGTCCGCACCAACGCGCAGCCCGGACTCG

GTAATGGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCAT

CGCAGTGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAAC

CGGACATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGA

TTGCGAGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGAC

AGAACTTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGA

CCAGATGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATA

CTGTTGATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATT

AGTGCAGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGT

TAATGATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCT

TTACAGGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGC

ACCCAGTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCG

CGTGCAGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTG

CCCGCCAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCAT

CGCCGCTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGT

TCACCACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACA

TCGTATAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTC

CGGGCGCTATCATGCCATACCGCGAAAGGTTTTGCGCCATTCGATGGTGT

CCGGGATCTCGACGCTCTCCCTTATGCGACTCCTGCATTAGGAAATTAAT

ACGACTCACTATA

Construction of pACYC-PTrc Plasmid Containing 'tesA, fadD, and atfA1

[0335] A pACYC-PTrc vector having the following sequence was used to construct a pACYC-PTrc-'tesA-fadD-atfA1 plasmid. The nucleotide sequence of the pACYC-PTrc vector is as follows:

(SEQ ID NO: 11)

ACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAA

TTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACT

TCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACA

TGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAA

GCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAAC

AACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGC

AACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTG

CGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGG

TGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGC

CCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGAT

GAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTG

GTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAAC

TTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTC

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ATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCC

CTTAATAAGATGATCTTCTTGAGATCGTTTTGGTCTGCGCGTAATCTCTT

GCTCTGAAAACGAAAAAACCGCCTTGCAGGGCGGTTTTTCGAAGGTTCTC

TGAGCTACCAACTCTTTGAACCGAGGTAACTGGCTTGGAGGAGCGCAGTC

ACCAAAACTTGTCCTTTCAGTTTAGCCTTAACCGGCGCATGACTTCAAGA

CTAACTCCTCTAAATCAATTACCAGTGGCTGCTGCCAGTGGTGCTTTTGC

ATGTCTTTCCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGC

GGTCGGACTGAACGGGGGGTTCGTGCATACAGTCCAGCTTGGAGCGAACT

GCCTACCCGGAACTGAGTGTCAGGCGTGGAATGAGACAAACGCGGCCATA

ACAGCGGAATGACACCGGTAAACCGAAAGGCAGGAACAGGAGAGCGCACG

AGGGAGCCGCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTT

TCGCCACCACTGATTTGAGCGTCAGATTTCGTGATGCTTGTCAGGGGGGC

GGAGCCTATGGAAAAACGGCTTTGCCGCGGCCCTCTCACTTCCCTGTTAA

GTATCTTCCTGGCATCTTCCAGGAAATCTCCGCCCCGTTCGTAAGCCATT

TCCGCTCGCCGCAGTCGAACGACCGAGCGTAGCGAGTCAGTGAGCGAGGA

AGCGGAATATATCCTGTATCACATATTCTGCTGACGCACCGGTGCAGCCT

TTTTTCTCCTGCCACATGAAGCACTTCACTGACACCCTCATCAGTGCCAA

CATAGTAAGCCAGTATACACTCCGCTAGCGCTGAGGTCTGCCTCGTGAAG

AAGGTGTTGCTGACTCATACCAGGCCTGAATCGCCCCATCATCCAGCCAG

AAAGTGAGGGAGCCACGGTTGATGAGAGCTTTGTTGTAGGTGGACCAGTT

GGTGATTTTGAACTTTTGCTTTGCCACGGAACGGTCTGCGTTGTCGGGAA

GATGCGTGATCTGATCCTTCAACTCAGCAAAAGTTCGATTTATTCAACAA

AGCCACGTTGTGTCTCAAAATCTCTGATGTTACATTGCACAAGATAAAAA

TATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAA

GGGGTGTTATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGA

TTAAATTCCAACATGGATGCTGATTTATATGGGTATAAATGGGCTCGCGA

TAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGCCCG

ATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGAT

GTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCT

TCCGACCATCAAGCATTTTATCCGTACTCCTGATGATGCATGGTTACTCA

CCACTGCGATCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCT

GATTCAGGTGAAAATATTGTTGATGCGCTGGCAGTGTTCCTGCGCCGGTT

GCATTCGATTCCTGTTTGTAATTGTCCTTTTAACAGCGATCGCGTATTTC

GTCTCGCTCAGGCGCAATCACGAATGAATAACGGTTTGGTTGATGCGAGT

GATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGA

AATGCATAAGCTTTTGCCATTCTCACCGGATTCAGTCGTCACTCATGGTG

ATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAAATTAATAGGTTGT

ATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCAT

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CAGACCGTTCCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACC

TACAACAAAGCTCTCATCAACCGTGGCTCCCTCACTTTCTGGCTGGATGA

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GACCTCAGCGCTCAAAGATGCAGGGGTAAAAGCTAACCGCATCTTTACCG

ACAAGGCATCCGGCAGTTCAACAGATCGGGAAGGGCTGGATTTGCTGAGG

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AAGGAATCAAATTTGGCCGCAGGCGTACCGTGGACAGGAACGTCGTGCTG

ACGCTTCATCAGAAGGGCACTGGTGCAACGGAAATTGCTCATCAGCTCAG

TATTGCCCGCTCCACGGTTTATAAAATTCTTGAAGACGAAAGGGCCTCGT

GATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGA

CGTCTTAATTAATCAGGAGAGCGTTCACCGACAAACAACAGATAAAACGA

AAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGCAGT

TCCCTACTCTCGCATGGGGAGACCCCACACTACCATCGGCGCTACGGCGT

TTCACTTCTGAGTTCGGCATGGGGTCAGGTGGGACCACCGCGCTACTGCC

GCCAGGCAAATTCTGTTTTATCAGACCGCTTCTGCGTTCTGATTTAATCT

GTATCAGGCTGAAAATCTTCTCTCATCCGCCAAAACAGCCAAGCTGGAGA

CCGTTTAAACTCAATGATGATGATGATGATGGTCGACGGCGCTATTCAGA

TCCTCTTCTGAGATGAGTTTTTGTTCGGGCCCAAGCTTCGAATTCCCATA

TGGTACCAGCTGCAGATCTCGAGCTCGGATCCATGGTTTATTCCTCCTTA

TTTAATCGATACATTAATATATACCTCTTTAATTTTTAATAATAAAGTTA

ATCGATAATTCCGGTCGAGTGCCCACACAGATTGTCTGATAAATTGTTAA

AGAGCAGTGCCGCTTCGCTTTTTCTCAGCGGCGCTGTTTCCTGTGTGAAA

TTGTTATCCGCTCACAATTCCACACATTATACGAGCCGGATGATTAATTG

TCAACAGCTCATTTCAGAATATTTGCCAGAACCGTTATGATGTCGGCGCA

AAAAACATTATCCAGAACGGGAGTGCGCCTTGAGCGACACGAATTATGCA

GTGATTTACGACCTGCACAGCCATACCACAGCTTCCGATGGCTGCCTGAC

GCCAGAAGCATTGGTGCACCGTGCAGTCGATGATAAGCTGTCAAACCAGA

TCAATTCGCGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTC

CAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGC

GGGGAGAGGCGGTTTGCGTATTGGGCGCCAGGGTGGTTTTTCTTTTCACC

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TGATGGTGGTTGACGGCGGGATATAACATGAGCTGTCTTCGGTATCGTCG

TATCCCACTACCGAGATATCCGCACCAACGCGCAGCCCGGACTCGGTAAT

GGCGCGCATTGCGCCCAGCGCCATCTGATCGTTGGCAACCAGCATCGCAG

TGGGAACGATGCCCTCATTCAGCATTTGCATGGTTTGTTGAAAACCGGAC

ATGGCACTCCAGTCGCCTTCCCGTTCCGCTATCGGCTGAATTTGATTGCG

AGTGAGATATTTATGCCAGCCAGCCAGACGCAGACGCGCCGAGACAGAAC

TTAATGGGCCCGCTAACAGCGCGATTTGCTGGTGACCCAATGCGACCAGA

TGCTCCACGCCCAGTCGCGTACCGTCTTCATGGGAGAAAATAATACTGTT

GATGGGTGTCTGGTCAGAGACATCAAGAAATAACGCCGGAACATTAGTGC

AGGCAGCTTCCACAGCAATGGCATCCTGGTCATCCAGCGGATAGTTAATG

ATCAGCCCACTGACGCGTTGCGCGAGAAGATTGTGCACCGCCGCTTTACA

GGCTTCGACGCCGCTTCGTTCTACCATCGACACCACCACGCTGGCACCCA

GTTGATCGGCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGC

AGGGCCAGACTGGAGGTGGCAACGCCAATCAGCAACGACTGTTTGCCCGC

CAGTTGTTGTGCCACGCGGTTGGGAATGTAATTCAGCTCCGCCATCGCCG

CTTCCACTTTTTCCCGCGTTTTCGCAGAAACGTGGCTGGCCTGGTTCACC

ACGCGGGAAACGGTCTGATAAGAGACACCGGCATACTCTGCGACATCGTA

TAACGTTACTGGTTTCACATTCACCACCCTGAATTGACTCTCTTCCGGGC

GCTATCATGCCATACCGCGAAAGGTTTTGCACCATTCGATGGTGTCAACG

TAAATGCATGCCGCTTCGCCTTCGCGCGCGAATTGATCTGCTGCCTCGCG

CGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGAC

GGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGG

GCGCGTCAGCGGGTGTTGGCGGGGCCGGCCTCG

[0336] The 'tesA, fadD, and atfA1 genes were amplified using high fidelity Phusion™ polymerase (New England Biolabs, Inc., Ipswich, Mass.), with the following primers from their respective plasmids, pETDuet-1-'tesA, pHZ1.61, and pHZ1.97

'tesAForward-

(SEQ ID NO: 12)

5'-CTCTAGAAATAATTTAACTTTAAGTAGGAGAUAGGTACCCATGGCGG

ACACGTTATTGAT

'tesAReverse-

(SEQ ID NO: 13)

5'-CTTCGAATTCCATTTAAATTATTTCTAGAGTCATTATGAGTCATGAT

TTACTAAAGGC

fadDForward-

(SEQ ID NO: 14)

5'-CTCTAGAAATAATTTTAGTTAAGTATAAGAAGGAGATATACCATGGT

GAAGAAGGTTTGGCTTAA

-continued

fadDReverse-

(SEQ ID NO: 15)

5'-CTTCGAATTCCATTTAAATTATTTCTAGAGTTATCAGGCTTTATTGT

CCAC

atfA1Forward-

(SEQ ID NO: 16)

5'-CTCTAGAAATAATTTAGTTAAGTATAAGAAGGAGATATACAT

atfA1Reverse-

(SEQ ID NO: 17)

5'-CTTCGAATTCCATTTAAATTATTTCTAGAGTTACTATTTAATTCCTG

CACCGATTTCC

Insertion of 'tesA into pACYC-Ptrc Plasmid

[0337] Using NcoI and EcoRI sites on both the insert and vector, the 'tesA PCR product amplified from pETDuet-1-'tesA was cloned into the initial position of pACYC-PTrc vector (SEQ ID NO:11). A T4 DNA ligase (New England Biolabs, Ipswich, Mass.) was then used to ligate the pACYC-PTrc vector and 'tesA, producing a pACYC-PTrc-'tesA plasmid. Following overnight ligation, the DNA product was transformed into Top 10 One Shot® cells (Invitrogen, Carlsbad, Calif.). The 'tesA insertion into the pACYC-PTrc vector was confirmed by restriction digestion. An SwaI restriction site as well as overlapping fragments for In-Fusion™ cloning (Clontech, Mountain View, Calif.) was also created at the 3' end of the 'tesA insert.

Construction of pACYC-PTrc-'tesA-fadD-atfA1

[0338] The pACYC-PTrc-'tesA plasmid was then subject to an overnight digestion by SwaI. fadD amplified from pHZ1.61was cloned after the 'tesA gene using In-Fusion™ cloning. This insertion of fadD was verified with restriction digestion. The insertion of fadD destroys the SwaI site following the 'tesA gene, but recreates a new SwaI site at the 3' end of fadD.

[0339] The pACYC-PTrc-'tesA fadD plasmid was again linearized by SwaI, and OM amplified from pHZ1.97-atfA1 was cloned after the fadD gene using In-Fusion™ cloning. The proper insertion of atfAlwas verified by restriction digestion.

Construction of the pOP-80 (pCL) Plasmid

[0340] A low copy plasmid pCL1920 (in accordance with Lerner et al., *Nucleic Acids Res.* 18:4631 (1990)) carrying a strong transcriptional promoter was digested with restriction enzymes AflII and SfoI (New England BioLabs Inc. Ipswich, Mass.). Three DNA sequence fragments were produced by this digestion, among which a 3737 by fragment was gel-purified using a gel-purification kit (Qiagen, Inc. Valencia, Calif.).

[0341] In parallel, a fragment containing the Trc-promoter and lacI region from the commercial plasmid pTrcHis2 (Invitrogen, Carlsbad, Calif.) was amplified by PCR using the following primers:

(SEQ ID NO: 18)

LF302: 5'-ATATGACGTCGGCATCCGCTTACAGACA-3'

(SEQ ID NO: 19)

LF303 (5'-AATTCTTAAGTCAGGAGAGCGTTCACCGACAA-3'.

[0342] These two primers also introduced recognition sites for ZraI (gacgtc) and AflII (cttaag), at the end of the PCR product. The PCR product was purified using a PCR-purifi-

cation kit (Qiagen, Inc. Valencia, Calif.) and digested with ZraI and AflII following the recommendations of the supplier (New England BioLabs Inc., Ipswich, Mass.). The digested PCR product was then gel-purified and ligated with the 3737 by DNA sequence fragment derived from pCL1920. The ligation mixture was transformed in TOP10 chemically competent cells (Invitrogen, Carlsbad, Calif.), and the transformants were plated on Luria agar plates containing 100 μg/mL spectinomycin. After overnight incubation at 37° C., a number of colonies were visible. A select number of these colonies were purified, analyzed with restriction enzymes, and sequenced. One of the plasmids was retained and given the name pOP-80.

Construction of pCL-TFW-atfA1

[0343] The operon 'tesA-fadD-atfA1 was removed from pACYC-'tesA-fadD-atfA1 using restriction digestion with MluI and EcoRI (New England Biolabs, Inc., Ipswich, Mass.). It was then cloned into complementary sites on pOP-80 to create the plasmid pCL-TFW-atfA].

Integration of the PTrc-'tesA-fadD-atfA1 Operon into the *E. coli* MG1655 ΔfadE ΔfhuA Chromosome at the LacI-LacZ Locus

[0344] Plasmid pCL-TFW-atfA1 was digested with restriction enzyme HindIII (New England Biolabs, Inc., Ipswich). In parallel, a chloramphenicol gene cassette was obtained from plasmid pLoxPcat2 (Genbank Accession No. AJ401047) by digestion with restriction enzymes BamHI and AvrII (New England Biolabs, Inc., Ipswich, Mass.). Both DNA fragments were blunt-ended using the DNA polymerase Klenow fragment. The resulting fragments were ligated and transformed to generate plasmid pCLTFWcat (see, FIG. **1**).

[0345] Plasmid placZ was designed and synthesized by DNA2.0 (Menlo Park, Calif.) in accordance with SEQ ID NO:28. This plasmid was used as a template for PCR amplification of the region shown in FIG. **2**. PCR primers LacZFnotI and pKDRspeI were designed to create restriction sites for the NotI and SpeI, respectively:

(SEQ ID NO: 20)
LacZFnotI 5'-CAACCAGCGGCCGCGCAGACGATGGTGCAGGATATC

(SEQ ID NO: 21)
pKDRspeI 5'-CCACACACTAGTCAGATCTGCAGAATTCAGGCTGTC

[0346] The resulting DNA fragment was ligated with a DNA fragment from plasmid pCLTFWcat digested with SpeI and Nod enzymes.

[0347] The ligation mixture was used as a template for another PCR reaction using primers lacIF and lacZR located on the lad and lacZ regions.

lacIF 5'-GGCTGGCTGGCATAAATATCTC (SEQ ID NO: 22)

lacZR 5'-CATCGCGTGGGCGTATTCG (SEQ ID NO: 23)

[0348] The resulting PCR product ("Integration Cassette") contains approximately 500 bases of homology to lacI or lacZ at each end. This PCR product was used to transform *E. coli* MG1655 ΔfadE ΔfhuA (DV2) cells that were made hypercompetent with plasmid pKD46 (see, Example 2).

[0349] This example demonstrate the construction of *E. coli* MG1655 ΔfadE, ΔfhuA, lacZ::'tesΔfadD atfA1, which is a genetically engineered microorganism in which a fatty acid degradation enzyme and an outer membrane protein receptor for ferrichrome are attenuated and nucleotide sequences encoding a thioesterase, an acyl-CoA synthase, and an ester synthase are integrated into the microorganism's chromosome. This strain was given the name "IDV2."

Example 4

[0350] This example describes processes that can be used to produce a fatty ester composition using the genetically modified microorganisms described herein.

[0351] The fatty ester composition produced by the processes described herein may produce a fatty ester composition comprising fatty acid methyl esters (FAME) and/or fatty acid ethyl esters (FAEE). This fatty ester composition may then be used as biodiesel.

Fermentation

[0352] The fermentation process described herein can be carried out by using methods well known to those of ordinary skill in the art. For example, a fermentation process can be carried out in a 2 to 5 L lab-scale fermentor. Alternatively, a fermentation process can be scaled up using the methods described herein or alternative methods known in the art.

[0353] In one embodiment, various fermentation steps were carried out in 2 L fermentor. *E. coli* cells from a frozen stock were grown overnight in a defined medium consisting of: 1.5 g/L of KH_2PO_4, 4.54 g/L of K_2HPO_4 trihydrate, 4 g/L of $(NH_4)_2SO_4$, 0.15 g/L of $MgSO_4$ heptahydrate, 20 g/L of glucose, 200 mM of Bis-Tris buffer (pH 7.2), and 1.25 mL/L of a vitamin solution. The vitamin solution comprised 0.42 g/L of riboflavin, 5.4 g/L of pantothenic acid, 6 g/L of niacin, 1.4 g/L of pyridoxine, 0.06 g/L of biotin, and 0.04 g/L of folic acid.

[0354] 50 mL of the culture grown overnight from the frozen stock was then used to inoculate 1 L of medium in a fermentor with controlled temperature, pH, agitation, aeration and dissolved oxygen concentration. The medium was similar to the one described above except that it contained 5 g/L of glucose. In a preferred embodiment, the fermentation conditions were: 32° C., pH 6.8, and dissolved oxygen (DO) equal to 30% of saturation. pH was maintained by addition of NH_4OH, which also acted as a nitrogen source for cell growth.

[0355] When the initial supply of glucose is almost exhausted, a feed consisting of 60% glucose, 3.9 g/L $MgSO_4$ heptahydrate, and 10 mL/L of the trace metals solution described above is supplied to the fermentor. The trace metals solution comprises 27 g/L of $FeCl_3.6H_2O$, 2 g/L of $ZnCl_2.4H_2O$, 2 g/L of $CaCl_2.6H_2O$, 2 g/L of $Na_2MoO_4.2H_2O$, 1.9 g/L of $CuSO_4.5H_2O$, 0.5 g/L of H_3BO_3, and 100 mL/L of concentrated HCl.

[0356] The feed rate is set up to match the cells' growth rate, and to avoid accumulation of glucose in the fermentor. By avoiding glucose accumulation, it is possible to reduce or eliminate the formation of by-products that are otherwise commonly produced by *E. coli,* such as, for example, acetate, formate, and/or ethanol. In the early phases of cell growth, the production of esters, such as FAME, is induced by the addition of 1 mM IPTG and 20 mL/L of pure methanol. The fermentation step is carried out for about 3 days. Methanol is added several times during the fermentation step to replenish both the methanol consumed by the cells during the production of FAME and the methanol lost by evaporation in the off-gas. Additional methanol is provided to the fermentation

broth to maintain the concentration of methanol at between about 10 and about 30 mL/L Maintaining the concentration of methanol assists in the efficient production of FAME while avoiding inhibition of cell growth.

[0357] In one embodiment, this fermentation protocol was scaled up to a 700 L fermentor using methods known in the art.

Analysis of Fermentation

[0358] The analytical methods utilized to monitor the fermentation performance are described below.

[0359] The progress of the fermentation was monitored by measuring $_{OD600}$ (optical density at 600 nm), glucose consumption, and fatty ester production.

[0360] OD_{600} was measured by methods well known in art.

[0361] Glucose consumption throughout the fermentation process was analyzed by High Pressure Liquid Chromatography (HPLC). The HPLC analysis was performed according to methods well known in the art for measuring the contents of sugars (e.g., glucose) and organic acids. For example, HPLC analysis was conducted under the following conditions:

[0362] a. Instrument: Agilent HPLC 1200 Series with Refractive Index detector;

[0363] b. Column: Aminex HPX-87H, 300 mm×7.8 mm;

[0364] c. Column temperature: 350° C.;

[0365] d. Mobile phase: 0.01M H_2SO_4 (aqueous);

[0366] e. Flow rate: 0.6 mL/min;

[0367] f. Injection volume: 20 μL.

[0368] The production of FAME and FAEE was followed and analyzed by gas chromatography with a flame ionization detector (GC-FID). Samples from the fermentation broth were extracted with ethyl acetate in a ratio of 1:1 vol/vol. After vigorous vortexing, the samples were centrifuged. Next, the organic phase was analyzed by GC-FID. The analysis conditions were as follows:

[0369] a. Instrument: Trace GC Ultra, Thermo Electron Corporation with Flame ionization detector (FID) detector;

[0370] b. Column: DB-1 (1% diphenyl siloxane; 99% dimethyl siloxane) CO1 UFM 1/0.1/5 01 DET from Thermo Electron Corporation, phase pH 5, FT: 0.4 μm, length 5 m, id: 0.1 mm;

[0371] c. Inlet conditions: 250° C. splitless, 3.8 min 1/25 split method was used depending on the sample concentration with split flow of 75 mL/min;

[0372] d. Carrier gas & flow rate: helium, at 3.0 mL/min;

[0373] e. Block temperature: 330° C.;

[0374] f. Oven temperature: 0.5 minute hold at 50° C.; 100° C/min to 330° C.; 0.5 min hold at 330° C.;

[0375] g. Detector temperature: 300° C.;

[0376] h. Injection volume: 2 μL;

[0377] i. Run time & flow rate: 6.3 min & 3.0 mL/min (using the splitless method); 3.8 min & 1.5 mL/min (using the split 1/25 method); 3.04 min & 1.2 mL/min (using the split 1/50 method).

Recovery

[0378] After fermentation, the fatty ester composition may be suitably separated from the fermentation broth using various methods well known in the art.

[0379] In one embodiment, the fermentation broth is centrifuged to separate a first light phase comprising the esters from a first heavy phase comprising water, salt(s), and microbial biomass.

[0380] The first light phase is centrifuged a second time to separate a second light phase from a second heavy phase. The second light phase comprises a mixture of fatty esters. In one embodiment, the second light phase comprises a mixture of esters which can be used as biodiesel. In an alternate embodiment, the second light phase is subject to one or more polishing steps before it can be used as biodiesel.

[0381] In one embodiment, the centrifugation step is performed in disk-stacked continuous centrifuges of pilot scale capacity (e.g., fixed centrifugal force ~10,000 g, etc.) with flows from about 1 to about 5 L/min. The same centrifuge can be used for the first and second centrifugation steps. Normal adjustments to centrifugation configurations and conditions (e.g., gravity ring size, back pressure in outlets, flow rate, etc.), which are well known to those of ordinary skill in the art, can be performed in each case to achieve the most favorable separation conditions with respect to recovery efficiency and purity of the product. For the first centrifugation step, the fermentation broth is sent directly from the fermentor to the centrifuge without any physical or chemical adjustments.

[0382] In alternate embodiments, depending on the fermentation broth characteristics, it is more difficult to break the emulsion to obtain the second light phase. In these embodiments, the first light phase is pretreated to help separate the second light phase from the second heavy phase during the second centrifugation step. The pretreatments consisted of one or more of the following: heating to about 60 to about 80° C., adjusting pH to about 2.0 to about 2.5 with acid (e.g., sulfuric acid), and/or addition of demulsifiers (e.g., ARB-8285 (Baker Hughes, Tex.), less than 1% of the emulsion/light phase volume). The temperature was held for about 1 to about 2 h before the second centrifugation step is performed.

[0383] The fatty esters separated from the fermentation broth can also be separated by other methods well known in the art, including steps such as decanting, distillation, and/or filtration. In alternate embodiments, a single-step centrifugation can be employed.

Polishing

[0384] In some instances, the recovered ester composition is further subjected to optional polishing step(s). These polishing step(s) are well known in the art.

[0385] In certain instances, the second light phase obtained from the second centrifugation step has characteristics close to a commercial-grade biodiesel, such as a biodiesel conforming to the ASTM D 6751 standard, having low levels of trace elements, or meeting the requirements of the emission standards set by various environmental regulatory agencies. For example, the second light phase may meet or exceed the following ASTM D 6751 standards: cetane number, kinematic viscosity, flash point, oxidation stability, copper corrosion, free and total glycerin content, methanol content, phosphorous content, sulfate content, K^+ content, and/or Na^+ content.

[0386] In certain instances, only minor additional purification or polishing steps are required to eliminate a few other impurities. The optional polishing step(s) that were performed on the second light phase in order to eliminate any remaining impurities include: lime wash or acid methylation to remove free fatty acids, dilute acid wash to remove excess calcium, tangential filtration to remove remaining free acid introduced during the acid methylation or dilute acid wash, water wash, final drying, and/or absorption/adsorption with

resin to remove other minor impurities. These step(s) are optional and thus are not necessarily performed each time depending on the result of analysis obtained from the second light phase prior to polishing.

[0387] To comply with ASTM D 6751 or with the EPA trace element and emission standards additional polishing step(s) were sometimes performed. For example, ASTM D 6751 requires a low calcium and magnesium content in biodiesel. In some embodiments, the calcium and/or magnesium content may be minimal in the first or second light phase, but the calcium and/or magnesium content may increase during polishing (e.g., during the lime wash). Thus, in some embodiments, a dilute acid wash is carried out to remove excess calcium and/or magnesium.

[0388] In other embodiments, small quantities of free fatty acids are produced during the fermentation and contained in either the first or second light phase. ASTM D 6751 establishes a low limit for acid content in biodiesel, which is termed the Acid Number and measured using the standard procedure described in ASTM D 664. Thus, even in instances where the free fatty acid level in the second light phase are as low as 1 to 2%, the above mentioned polishing step(s), such as, for example, acid methylation, is required to produce a biodiesel meeting ASTM D 6571.

[0389] In some embodiments, the dilute acid wash may result in an excess amount of free acid (e.g., sulfuric acid, phosphoric acid, or lactic acid). In alternate embodiments, the content of free acid may increase when acid methylation is used as a means to reduce the level of free fatty acids. In other embodiments, the removal of this excess free acid may require washing with water.

[0390] In some embodiments, a final treatment step using absorbent/adsorbent resins such as Magnesol™ (the Dallas Group of America, Inc., Whitehouse, N.J.), Amberlist™ BD20 (Dow Chemicals, Philadelphia, Pa.), Biosil™ (Polymer Technology Group, Berkeley, Calif.), or other similar adsorbent/absorption resins well known in the art are employed to remove excess water, methanol, sulfur, and/or other minor impurities present. In other embodiments, some of the potential impurities are reduced by modifications to the fermentation process to avoid their presence in the first place.

Fatty Ester Composition

[0391] In certain instances, the genetically modified strains of *E. coli* described herein when fermented, recovered, and/or polished as described herein produced a mixture of FAME with the following composition profile:

[0392] Methyl dodecanoate (C12:0): 5-25%

[0393] Methyl dodecenoate (C12:1): 0-10%

[0394] Methyl tetradecanoate (C14:0): 30-50%

[0395] Methyl 7-tetradecenoate (C14:1): 0-10%

[0396] Methyl hexadecanoate (C16:0): 0-15%

[0397] Methyl 9-hexadecenoate (C16:1): 10-40%

[0398] Methyl 11-octadecenoate (C18:1): 0-15%

[0399] The actual composition of the FAME mixture was dependent on the specific *E. coli* strain used for production, but not on the conditions of the fermentation process or recovery. Accordingly, the lots of biodiesel produced from a given *E. coli* strain were consistent from batch to batch.

Example 5

[0400] This example illustrates the impurity profile of the fatty ester composition produced using the genetically modified microorganism described in Example 3.

[0401] A fatty ester composition was produced as described herein. After isolation of the fatty ester composition after two centrifugations, the fatty ester composition was subjected to analysis. The results of the analysis are set forth in Table E5. The test methods followed the protocols set out in the ASTM D 6571 biodiesel standard.

TABLE E5

Component	Test Method	Results
Sulfur	D 5453	23 ppm
Sulfated Ash	D 874	<0.001
Microcarbon Residue	D 4530	0.07 wt. %
Water and Sediment	D 2709	0.01 vol. %
Sodium	EN 14538	2.3 ppm
Potassium	EN 14538	<0.1 ppm
Magnesium	EN 14538	<0.1 ppm
Calcium	EN 14538	0.8 ppm
Methanol content	EN 14110	0.03 vol. %
Phosphorous	D 4951	<0.0001 wt. %

Example 6

[0402] This example illustrates the performance profile of the fatty ester composition produced using the genetically modified microorganism described in Example 3.

[0403] A fatty ester composition was produced as described herein. The fatty ester composition was obtained by centrifuging the fermentation broth a first time to obtain a first light phase. The first light phase was then pretreated by adjusting the pH of the first light phase to about 2.0 and heating the first light phase to about 80° C. for 2 h. After pretreatment, the first light phase was centrifuged a second time to obtain a second light phase. The second light phase was subjected to two lime washes. The fatty ester composition obtained was analyzed using the methods described herein.

[0404] The results of the analysis are set forth in Table E6. The test methods followed the protocols set out in the ASTM D 6571 biodiesel standard.

TABLE E6

Component	Test Method	Results
Flash Point	D 93A	>320
Kinematic Viscosity @ 40.0° C.	D 445	3.181
Cloud Point	D 2500	+1
Copper Corrosion	D 130	1b
Derived Cetane Number	D 6890	61.8
Sulfur	D 5453	18 ppm
Acid Number	D 664	0.04 mg KOH/g
Sulfated Ash	D 874	0.012 wt. %
Microcarbon Residue	D 4530	0.07 wt. %
Water and Sediment	D 2709	0.02 vol. %
Sodium	EN 14538	0.5 ppm
Potassium	EN 14538	<0.1 ppm
Magnesium	EN 14538	0.3 ppm
Calcium	EN 14538	65 ppm
Oxidation Stability	EN 14112	6+
Methanol Content	EN 14110	<0.01 vol. %
Phosphorous	D 4951	0.5 ppm

Example 7

[0405] The fatty ester composition of Example 6 was subjected to a further dilute acid wash. Following isolation of the fatty ester composition, the calcium content of the fatty ester composition, as determined by test method EN 14538, was 7.4 mg/kg.

Example 8

[0406] A fatty ester composition was produced using the genetically engineered microorganism of Example 3. The fatty ester composition was sequentially treated with (1) a lime wash, (2) a dilute acid wash, (3) a water wash, (4) treatment with Magnesol™ D60 (The Dallas Group, Whitehouse, N.J.) one or more times, and (5) filtration. The processed fatty ester composition was subjected to analysis. The results of the analysis are set forth in Table E8. The test methods followed the protocols set out in the ASTM D 6571 biodiesel standard.

TABLE E8

Component or Property	Test Method	Results
Flash Point	D 93A	142° C.
Calcium and Magnesium Combined	EN 14538	<1
Water and Sediment	D 2709	<0.05 vol. %
Sulfur	D 5453	12 ppm
Kinematic Viscosity @ 40.0° C.	D 445	3.326
Acid Number	D 664	0.08 mg KOH/g
Sulfated Ash	D 874	0.001 wt. %
Copper Corrosion	D 130	1a
Derived Cetane Number	D 6890	69.9
Cloud Point	D 2500	+2° C.
Microcarbon Residue	D 4530	<0.01 wt. %
Free Glycerin	D 6584	0.002 wt. %
Total Glycerin	D 6584	0.007 wt. %
Phosphorous	D 4951	<0.001 wt. %
Vacuum Distillation 90% (AET)	D 1160	323° C.
Sodium and Potassium Combined	EN 14538	<1 ppm
Oxidation Stability	EN 14112	6.1 h
Annex A1 Cold Soak Filtration (time for 300 mL)	D 6751-08	86 seconds

Example 9

[0407] A fatty ester composition was produced using the genetically engineered microorganism of Example 3.

[0408] A fatty ester composition was produced as described herein. The fatty ester composition was obtained by centrifuging the fermentation broth a first time to obtain a first light phase. The first light phase was then pretreated by heating the first light phase to about 80° C. for 2 h. After pretreatment, the first light phase was centrifuged a second time to obtain a second light phase. The second light phase was sequentially treated with (1) a lime wash, (2) a dilute acid wash, (3) a water wash, and (4) a Magnesol™ D60 (The Dallas Group, Whitehouse, N.J.) treatment. The resulting fatty ester composition was subjected to analysis. The results of the analysis are set forth in Table E9. The test methods followed the protocols set out in the Brazilian ANP 7 biodiesel standard.

TABLE E9

Test	Method	Result
Density of Liquids by Digital Density Meter Density @ 20° C.	ASTM D 4052	0.8728 g/cm^3
Kinematic Viscosity @ 104° F./40° C.	ASTM D 445	3.465 mm^2/s
Water and Sediment in Middle Distillate Fuels (Centrifuge Method)		
Pensky-Martens Closed Cup Flash Point	ASTM D 93	147° C.
Micro Carbon Residue	ASTM D 4530	0.00 Wt %
Sulfated Ash from Lubricating Oils and Additives	ASTM D 874	0.001 Wt %
Cold Filter Plugging Point of Diesel and Heating Fuels	ASTM D 6371	−4° C.
Acid Number of Petroleum Products by Potentiometric Titration	ASTM D 664	0.15 mg KOH/g
Determination of Free and Total Glycerin in B-100	ASTM D 6584	
Biodiesel Methyl Esters By Gas Chromatography		
Free Glycerin		<0.005 Wt %
Total Glycerin		<0.050 Wt %
Determination of Oxidation Stability (Accelerated Oxidation Test)	EN 14112	7.2 h
Determination of Na Content by Atomic Absorption	EN 14108	<1.0 mg/kg
Determination of K Content by AA Spectrometry	EN 14109	<0.5 mg/kg
Determination of Total Contamination in Middle Distillates	EN 12662: 2008	19.3 mg/kg
Determination of Ester Content	EN 14103	96.7% (m/m)
Determination of Ca and Mg Content by ICP OES	EN 14538	<2.0000000000 mg/kg
Determination of Phosphorus Content by (ICP) Emission Spectrometry	EN 14107	<4.0 mg/kg
Determination of Methanol Content	EN 14110	0.01% (m/m)

TABLE E9-continued

Test	Method	Result
Determination of Iodine Value	EN 14111	52 g I2/100 g
Determination of Ca and Mg Content by ICP OES	EN 14538	<2.0000000000 mg/kg
Sulfur Content by UV Fluorescence	ASTM D 5453	15 mg/kg

Example 10

[0409] This example illustrates the amounts of various trace elements that were present in the fatty ester composition produced by the genetically modified microorganism of Examples 3.

[0410] A fatty ester composition was produced as described herein. The composition was obtained by centrifuging the fermentation broth a first time to obtain a first light phase. The first light phase was then pretreated by adjusting the pH of the first light phase to about 2.0 and heating the first light phase to about 80° C. for 2 h. After pretreatment, the first light phase was centrifuged a second time to obtain a second light phase. The second light phase was subjected to a four-step process: (1) a lime wash, (2) a dilute acid wash, (3) a water wash, and (4) a Magnesol™ D60 (The Dallas Group, Whitehouse, N.J.) treatment.

[0411] The fatty ester composition thus obtained was sent to Galbraith Laboratories, Inc. (Knoxville, Tenn.), an EPA approved testing laboratory, for quantitative elemental analysis of trace elements, including, for example, boron, chromium, iron, molybdenum, nitrogen, total halogens, zinc, and copper. Preparatory and analytical methods are described below. Results are show in Table E10-6. Boron, chromium, iron, molybdenum, total halogens, and zinc, if existed in the sample at all, were below the level of quantitation (LOQ). The amount of nitrogen was below the LOQ of standard testing method ME-2, but was detected using a dramatically more sensitive method. Thus, the fatty ester compositions prepared in accordance with the present disclosures contain low levels of trace elements.

Method ME-2, Rev. 20: Carbon, Hydrogen, and Nitrogen Determination Using PerkinElmer 240 Elemental Analyzer

[0412] A PerkinElmer 240 Elemental Analyzer was used to burn samples in pure oxygen at 950° C. under static conditions to produce combustion products of CO_2, H_2O and N_2. The instrument automatically analyzed these products in a self-integrating, steady-state thermal conductivity analyzer. In certain instances, Tungstic anhydride was added as combustion aid.

TABLE E10-1

Sample Introduction	Weighed 1.0-2.5 mg into AI capsule; crimped for liquids; washed with solvent prior to weighing upon request.					
Decomposition	Combustion at >950° C., reduction at >675° C. = CO_2, H_2O, N_2					
Calibration	Acetanilide (1-2.5 mg)					
Control	s-1409, s-1410: cyclohexanone-2,4-dinitrophenyl-hydrazone (51.79% C, 5.07% H, 20.14% N)					
Determination	CO_2; H_2O; N_2 by thermal conductivity analyzer					
LOQ	0.5% C, 0.5% H, 0.5% N					
	Instrument 1			Instrument 2		
Precision/Accuracy	C	H	N	C	H	N
RSD (%)	0.28	1.26	0.39	0.35	1.12	0.41
Mean Recovery (%)	99.94	101.25	99.86	100.13	100.40	100.04
Interference	Metals and some halogens cause incomplete combustion. Combustion aids and/or an extended combustion time can be used to alleviate this problem.					

Method: ME-13, Rev. 3: Total Halogens Measurement by MCC-TOX-100 Analyzer

[0413] A MCC-TOX-100 Analyzer was used to determine the total halogen content (including any halides). The results were expressed as chlorine or chloride. The sample was heated in a quartz combustion tube to 950° C. in an oxygen atmosphere. The combustion process converted the halogens to halides and oxyhalides, which were directed into a coulometric titration cell where they react quantitatively with silver irons. Total organic halogens in aqueous samples were determined by first passing the sample through a carbon column followed by washing with nitrate solution to desorb the inorganic halide ions. The LOQ of this method is 31 ppm.

TABLE E10-2

Preparation	Direction injections were made by microsyringe or difference weighing into quartz carrier boat
Decomposition	Performed using O_2 combustion train at 900 to 950° C.
Calibration	Cell calibration by sodium chloride solution injection (into cell)
Determination	For total halogens: microcoulometric cell trapping and titration of combustion gases

TABLE E10-2-continued

Precision/Accuracy		RSD	RE
p-1702	Total halogens	7.76%	0.64%
p-1703	Total halides	5.80%	0.46%
Interferences	Extremely high levels of S		

Method ME-30, Rev. 0: Method for Testing Elements in Digestates by Inductively Coupled Plasma Mass Spectrometry

[0414] Samples were introduced into a PerkinElmer Sciex Elan 6100 ICP Mass Spectrometer by pneumatic nebulization into a radio frequency plasma where energy transfer processes caused desolvation, atomization, and ionization. The ions were extracted from the plasma through a differentially pumped vacuum interface and separated on the basis of their mass-to-charge ratio by a quadrupole mass spectrometer.

TABLE E10-3

Decomposition	Performed with an appropriate solubilizer and digestion method
Calibration	10-20-100 ppb
Sample introduction	Pesistaltic pump, cross flow II nebulizer
Determination	Quadrupole mass spectrometer
LOQ limit	1.04 μg/l, mass 120
Precision/accuracy	RE 1.21%; RSD 5.64%
Interference	Te

Method ME-70, Rev. 5: Inductively Coupled Plasma Atomic Emission Spectrometry

[0415] Multi-elemental determinations were carried out by ICP-AES using simultaneous optical systems and axial or radial viewing of the plasma. The instrument measured the characteristic emission spectra by optical spectrometry. Samples were nebulized and the resulting aerosols were transported to the plasma torch. Element-specific emission spectra were produced by radio-frequency inductively coupled plasma. The spectra were dispersed by a grating spectrometer, and the intensities of the emission lines were monitored by photosensitive devices. Background corrections were required for trace element detection, which was measured adjacent to analyte lines on the samples during the analyses. The LOQ limit of this method is 0.01-2 ppm, but the upper limit is extendable by sample dilution.

TABLE E10-4

Decomposition	Prior to analysis, samples were acidified or digested using appropriate sample preparation methods.
Calibration	0.01 ppm-100 ppm plus matrix specific calibrations
Sample Introduction	Pesistaltic pump, cross flow nebulizer, gemcone nebulizer, scott ryton spray chamber and quartz cylonic spray chamber
Determination	Atomic emission by radio frequency inductively coupled plasma of element-specific emission spectra through a grating spectrometer monitored by photo-sensitive devices
LOQ limit	Element and calibration specific ranging from 0.01 to 2 ppm
Precision/Accuracy	±10% RSD
Interferences	Spectral, chemical, physical, memory

Method E7-6, Rev. 2: Determination of Trace Nitrogen by Kjeldahl Digestion and Ion-Selective Electrode

[0416] This method, which involved Kjeldahl digestion, was employed to determine the trace amount of organic nitrogen in the samples. This method was used because the standard ME-2 method for nitrogen detection was insufficiently sensitive because the low levels of trace nitrogen in the samples were below the detection limit. The LOQ limit of this method is 0.7 mg/L nitrogen.

TABLE E10-5

Instrument	Ammonium electrode, Orion Model 95-12 or equivalent; pH meter, Fischer Accumet 950, or equivalent		
Decomposition	The sample was digested in a mixture of concentrated sulfuric acid, sodium sulfate, and copper sulfate. The organic material was oxidized and the nitrogen converted to ammonium sulfate. Excess sodium hydroxide was added, and the ammonia was distilled and absorbed in a boric acid solution		
Determination	The pH of the sample was adjusted to be greater than 11. After rinsing the ammonium electrode, the electrode was immersed in the sample. The concentration of ammonium was read from the electrode.		
LOQ limit	0.7 mg/L nitrogen		
Calibration	0.1-20.0 mg/L nitrogen		
Precision/Accuracy		RSD	RE
Kjeldahl Nitrogen (E7-6)	k-0702	1.26%	N/A
	k-0703	3.21%	
	k-0704	4.69%	
Interference	Hg & Ag interfere by complexing with NH_4; excess NaOH eliminates the interference		

[0417] Results of the trace element analysis according to the methods listed above are shown in the Table E10-6 below.

TABLE E10-6

Element	Method	Result
Boron	ME-70	<1.6 ppm
Chromium	ME-70	<1.5 ppm
Iron	ME-70	<3.3 ppm
Molybdenum	ME-70	<1.5 ppm
Nitrogen	ME-2	<0.5%
Nitrogen, Kjeldahl	E7-6	29 ppm
Copper	ME-30	0.086 ppm
Total Halogens	ME-13	<31 ppm
Zinc	ME-70	<2.1 ppm

Results in the above table, when indicated with "<" before the numbers, were below the detection limit (or LOQ) of the specified methods used to make the measurement.

Example 11

[0418] This example illustrates the amount of benzene that was present in the fatty ester composition produced by the genetically modified microorganism of Examples 3.

[0419] A fatty ester composition was produced as described. The composition was obtained by centrifuging the fermentation broth a first time to obtain a first light phase. The first light phase was then pretreated by adjusting the pH of the first light phase to about 2.0 and heating the first light phase to about 80° C. for 2 h. After pretreatment, the first light phase was centrifuged a second time to obtain a second light phase. The second light phase was then subjected to a four-step

process: (1) a lime wash, (2) a dilute acid wash, (3) a water wash, and (4) a Magnesol™ D60 (The Dallas Group, Whitehouse, N.J.) treatment.

[0420] The fatty ester composition was sent to Galbraith Laboratories, Inc. (Knoxville, Tenn.), an EPA approved testing laboratory, for quantitative analysis of the presence of benzene using the protocol in (Method GC-100H) Table E11-1 below:

TABLE E11-1

Instrument	Hewlett-Packard Model 5890/6890 Gas Chromatograph
Analytical column	J&W DB-624, 30 m/0.53 mm/5 μM)
Detection	Flame ionization (FID)
Preparation	Samples were mixed well, weighed into crimped vials and dissolved in solvent.
Sample introduction	Headspace analysis, HP 7694 Sampler
Determination	Quantitation was performed by comparison to an external linear regression calibration curve. The instrument signal output was processed by HP ChemStation software.
Limit of quantitation	The practical limit of quantitation is equal to the concentration of the lowest point of calibration divided by the amount of sample used in grams.
Quality control standard	A reference standard, independent from the calibration standard, was analyzed under the same condition as the sample. Blanks and calibration verifications were analyzed at appropriate intervals.
Interferences	Potential interferences from coeluting volatile compounds could not be ruled out.
Calculations	External standard: ppm = mass of analyte (mg/μL × dilution factor)/mass of sample (g)

[0421] The LOQ in this case was 15 ppm. The analysis indicated that the amount of benzene present in the fatty acid composition produced by the genetically modified microorganism of Example 3 was less than 15 ppm.

Example 12

[0422] This example illustrates an emissions profile of the fatty ester composition produced by the genetically modified microorganism of Example 3.

[0423] A fatty ester composition was produced as described herein. The composition was obtained by centrifuging the fermentation broth a first time to obtain a first light phase. The first light phase was then pretreated by adjusting the pH of the first light phase to about 2.0 and heating the first light phase to about 80° C. for 2 h. After pretreatment, the first light phase was centrifuged a second time to obtain a second light phase. The second light phase was subjected to a four-step process: (1) a lime wash, (2) a dilute acid wash, (3) a water wash, and (4) a Magnesol™ D60 (The Dallas Group, Whitehouse, N.J.) treatment.

[0424] A sample of the resulting composition was submitted to the ReFUEL Laboratory of the National Renewable Energy Laboratory (Denver, Colo.) for engine testing. Regulated emissions measurements were performed using procedures consistent with the Code of Federal Regulations Title 40, Section 86, Subpart N. The test engine used was a 2008 model year 9.3L 330 horsepower International MaxxForce 10, with properties shown below in Table E1.2-1.

TABLE E12-1

Specifications	International MaxxForce 10
Serial Number	570HM2U3058670
Displacement L	9.3
Cylinders	6
Rated Power, kW	246 at 2000 rpm
Rated Torque	1560 N-m at 1160 rpm
Bore × Stroke	11.7 × 14.6 cm
Compression Ratio	17.2:1
Fuel System	Common Rail

[0425] The engine employs cooled high pressure exhaust gas recirculation (EGR), a variable geometry turbocharger, electronic control, and high-pressure common rail direct fuel injection. The engine, designed and calibrated to meet the 2007 U.S. heavy-duty emissions standards, also uses an actively regenerated diesel particulate filter (DPF) for reduction of particulate matter (PM), which captures and stores diesel soot under low exhaust temperature conditions. On occasions, the DPF may reach a high soot loading, and with the exhaust temperatures elevated to sufficiently high, the stored soot in the DPF may be oxidized. This is referred to as a DPF regenerating event. For the purpose of the present example, the state of the DPF was managed by the engine controller, which used a late in-cylinder fuel injection as the primary means for active DPF regeneration. The state of the DPF and occurrences of regeneration events have caused variations in engine emissions measurements.

[0426] Testing was performed with three fuels. The baseline fuel was a 2007 Certification Ultra Low Sulfur Diesel (ULSD) (Haltermann Products, Channelview, Tex.). This fuel was used for two purposes: (1) for baseline comparison; and (2) as diesel blend stock for the biodiesel blends. Two B20 biodiesel fuel samples were prepared. In the first sample, a soy-based diesel fuel (referred to herein as "SOY fuel") was blended into the baseline ULSD at a 20% blend by volume. In the second sample, and a fatty ester composition obtained from the microorganism of Example 3 in accordance with the description herein (referred to herein as "FAE fuel"), was blended into the baseline ULSD at a 20% blend by volume.

[0427] Testing was conducted over a heavy duty Federal Testing Procedure (FTP) transient cycle. The cycle engine speed and torque are shown in FIG. **4**. A minimum of 3 consecutive hot start repeats were conducted for each fuel on the first day of testing. On the second day of testing, three additional hot start repeats were conducted for each fuel, but in reverse order. A thorough fuel swap procedure was performed between experiments with each test fuel, including flushing 3x the volumetric capacity of the entire fueling system, which included the fuel lines, the fuel meter and the engine. Measurements of NO_X, PM, THC, CO and CO_2 emissions were collected. In addition measurements of fuel consumption was collected. NO_R emissions were determined by chemiluminescence detection (CLD), THC by flame ionization detection (FID) and CO and CO_2 by non-dispersive infrared (NDIR). Mass emissions levels were determined through dilute Constant Volume Sampling (CVS) with Critical Flow Venturis. Background and humidity corrections were applied to all emissions data. PM was collected on Pall 47 mm and 2.0 pm filters. Particle filter handling and weighing were conducted in an environmental chamber/clean room with constant humidity, barometric pressure and temperature control. Filter weighing was conducted on a Sartorious microbalance

with a readability of 0.1 μg. Fuel consumption was measured with a Pierburg fuel metering system, which measured volumetric fuel flow and density with an accuracy of +/−0.5% of reading.

[0428] A lack of consistency in emissions performance was observed with the data before the DPF regeneration event on the first day. This was determined by the ReFUEL Laboratory to be an inherent characteristic of the test engine as well as most modern diesel engines. Thus, emissions performance data collected after the DPF regeneration event on the first day is reported below in Table E12-2 and the levels of NO_X and CO emissions as well as the levels of fuel consumption were indicated in FIG. **5**.

TABLE E12-2

Fuel (g/bhp-hr)	NO_x (g/bhp-hr)	THC (g/bhp-hr)	CO (g/bhp-hr)	CO_2 (g/bhp-hr)	PM (g/bhp-hr)	Fuel Cons (g/bhp-hr)
ULSD	2.32	−0.01	0.29	647.23	0.0024	197.44
Soy	2.32	0.00	0.28	644.54	0.0041	202.11
FAE	2.24	0.00	0.35	646.96	0.0047	203.60

[0429] When compared to the baseline certification fuel ULSD, the SOY fuel resulted in an about 0.2% reduction of NO_X emissions, an about 69.3% increase of PM emission, and an about 164% or about 11% reduction in THC or CO emissions, respectively,. When compared to the certification fuel ULSD, the FAE fuel prepared in accordance with the description herein resulted in about a 3.34% or about a 121.9% reduction in NO_X or THC, respectively, and about a 98.8% or about 22.6% increase in PM or CO emissions, respectively,. Both the SOY and the FAE fuels resulted in somewhat higher fuel consumption than that of the ULSD: about 2.37% increase for the SOY fuel, and about 3.12% increase for the FAE fuel.

[0430] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0431] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

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<210> SEQ ID NO 11
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: pACYC-PTrc vector

<400> SEQUENCE: 11

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1. A composition produced by a microorganism, wherein the composition comprises one or more fatty esters.

2. The composition of claim **1**, wherein the composition comprises less than or equal to about 10 mg/kg of calcium and magnesium combined.

3. The composition of claim **1**, wherein the composition comprises less than or equal to about 500 ppm of sulfur.

4. The composition of claim **3**, wherein the composition comprises less than or equal to about 15 ppm of sulfur.

5. The composition of claim **1**, wherein the composition comprises less than or equal to about 0.02 wt. % of sulfated ash.

6. The composition of claim **1**, wherein the composition comprises less than or equal to about 0.05 vol. % of water and sediment.

7. The composition of claim **1**, wherein the composition comprises less than or equal to about 0.02 wt. % of free glycerin.

8. The composition of claim **1**, wherein the composition comprises less than or equal to about 0.38 wt. % of total glycerin.

9. The composition of claim **1**, wherein the composition has a kinematic viscosity of about 1.9 mm^2/s or more.

10. The composition of claim **1**, wherein the composition has a kinematic viscosity of about 6 mm^2/s or less.

11. The composition of claim **1**, wherein the composition has a kinematic viscosity of about 1.9 mm^2/s to about 6 mm^2/s.

12. The composition of claim **1**, wherein the composition has an acid number of less than or equal to about 0.8 mg KOH/g.

13. The composition of claim **1**, wherein the composition comprises less than or equal to about 10 mg/kg of phosphorous.

14. The composition of claim **1**, wherein the composition comprises less than or equal to about 10 mg/kg sodium and potassium combined.

15. The composition of claim **1**, wherein the composition has a cetane number of about 47 or more.

16. The composition of claim **1**, wherein the composition has an oxidation stability of about 3 hours or more.

17. The composition of claim **1**, wherein the composition has a cloud point of about 10° C. or less.

18. The composition of claim **1**, wherein the composition comprises less than or equal to about 24 mg/kg of contaminants in the middle distillates.

19. The composition of claim **1**, wherein the composition comprises less than or equal to about 0.1 wt. % of carbon residue.

20. The composition of claim **1**, wherein the composition has a density at 15° C. of about 860 kg/m^3 or more.

21. The composition of claim **1**, wherein the composition has a density at 20° C. of about 865 kg/m^3 or more.

22. The composition of claim **1**, wherein the composition has a flash point of about 100° C. or more.

23. The composition of claim **1**, wherein the composition comprises a total ester content of about 96.5 wt. % or more.

24. The composition of claim **1**, wherein the composition has a cold filter plugging point of about 5° C. or less.

25. The composition of claim **1**, wherein the composition has a copper strip corrosion rating of class 3 or lower.

26. The composition of claim **1**, wherein the composition has a methanol/ethanol content of equal to or less than about 0.5 wt. %.

27. The composition of claim **1**, wherein the composition has an iodine value of equal to or less than about 120 g/100 g.

28. The composition of claim **1**, wherein the composition comprises less than or equal to about 0.02 ppm of copper.

29. The composition of claim **1**, wherein the composition comprises less than or equal to about 2 ppm of boron.

30. The composition of claim **1**, wherein the composition comprises less than or equal to about 2.0 ppm of chromium.

31. The composition of claim **1**, wherein the composition comprises less than or equal to about 5 ppm of iron.

32. The composition of claim **1**, wherein the composition comprises less than or equal to about 2 ppm of molybdenum.

33. The composition of claim **1**, wherein the composition comprises less than or equal to about 35 ppm of nitrogen.

34. The composition of claim **1**, wherein the composition comprises less than or equal to about 35 ppm of total halogens.

35. The composition of claim **1**, wherein the composition comprises less than or equal to about 2.5 ppm of zinc.

36. The composition of claim **1**, wherein the composition emits about 2.3 g/bph-hr or less of NO_X gases.

37. The composition of claim **1**, wherein the composition emits equal to or less than 2 g/bhp-hr of total hydrocarbon.

38. The composition of claim **1**, wherein the composition emits about 0.007 g/bhp-hr or less of particulate matter.

39. The composition of claim **1**, wherein the composition emits about 0.001 to about 0.007 g/bhp-hr of particulate matter.

40. The composition of claim **1**, wherein the composition emits about 0.4 g/bhp-hr or less of CO.

41. The composition of claim **1**, wherein the composition emits 0.25 to about 0.4 g/bhp-hr of CO.

42. The composition of claim **1**, wherein the composition comprises less than or equal to about 15 ppm of benzene.

43. A biofuel composition comprising the composition of any one of claims **1-42**.

44. The biofuel composition of claim **43** further comprising petroleum diesel.

45. The biofuel composition of claim **43**, further comprising one or more fuel additives selected from: engine performance additives, detergents, dispersants, antiwear agents, viscosity index modifiers, friction modifiers, antioxidants, rust inhibitors, antifoaming agents, seal fixes, lubricity additives, pour point depressants, cloud point reducers, smoke suppressants, drag reducing additives, metal deactivators, biocides and demulsifiers.

46. The biofuel composition of claim **45**, wherein the one or more fuel additives are first blended into a fuel additive package, wherein the additive package comprises a major amount of one or more base oils and a minor amount of one or more additives.

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