



<a href="#">5646098</a>	July 1997	Brois
<a href="#">6043200</a>	March 2000	Carroll et al.
<a href="#">2006/0206957</a>	September 2006	Schalk

#### Foreign Patent Documents

2 849 052	Jun., 2004	FR
2 849 052	Jun., 2004	FR
2849052	Jun., 2004	FR
WO 2006/095219	Sep., 2006	WO

#### Other References

Air BP Handbook of Products, 2000. cited by examiner .

Liang et al., "The Organic Composition of Diesel Particulate Matter, Diesel Fuel and Engine Oil of a Non-Road Diesel Generator," Journal of Environmental Monitoring, 7(10):983-988 (2005). cited by other .

PCT Notification of Transmittal of the International Search Report and the Written Opinion of PCT//US07/21890, mailed on Mar. 28, 2008. cited by other .

International Search Report of PCT/US07/21890, mailed on Mar. 28, 2008. cited by other .

Written Opinion of PCT/US07/21890, mailed on Mar. 28, 2008. cited by other .

Material Safety Data Sheet of Jet B Aviation Turbine Fuel, pp. 1-5. (2001) Petro-Canada. cited by other .

Oil Properties of Jet B, pp. 1-4.

<http://www.etc.ed.gc.ca/databases/Oilproperties/Default.aspx>. (2001) Canada Environmental Technology Centre. cited by other .

Standard Specification for Aviation Turbine Fuels, Designation: D 1655-02, pp. 1-12.

.COPYRGT. 2002 ASTM International. cited by other .

"Aviation Fuels Technical Review," pp. 1-90. .COPYRGT. 2006 Chevron Corporation. cited by other .

U.S. Appl. No. 11/973,901, filed Oct. 9, 2007, Renninger et al. cited by other.

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#### *Parent Case Text*

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#### PRIOR RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. .sctn. 119(e) of U.S. Provisional Patent Application Nos. 60/850,881, filed Oct. 10, 2006; and 60/860,854, filed Nov. 21, 2006, all of which are incorporated herein by reference in their entirety.

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#### *Claims*

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What is claimed is:

1. A fuel composition comprising or obtainable from a mixture comprising: (a) an isoprenoid compound having the formula: ##STR00042## or a stereoisomer thereof, wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl; (b) a petroleum-based fuel; and (c) a fuel additive, wherein the amount of the isoprenoid compound is at least about 5 vol.% and the amount of the petroleum-based fuel is at least about 5 vol. %, both amounts based on the total volume of the fuel composition, and wherein the fuel composition has a flash point equal to or greater than 38.degree. C. and has an initial boiling point between about 100.degree. C. and about 200.degree. C.
2. The fuel composition of claim 1, wherein the fuel composition has a T90 distillation temperature from about 270.degree. C. to about 350.degree. C.
3. The fuel composition of claim 1, wherein the amount of the isoprenoid compound is less than about 75 vol. %, based on the total volume of the fuel composition.
4. The fuel composition of claim 1, wherein the amount of the isoprenoid compound is from about 5 vol. % to about 10 vol. %, based on the total volume of the fuel composition.
5. The fuel composition of claim 1, wherein the amount of the isoprenoid compound is from about 15 vol. % to about 25 vol. %, based on the total volume of the fuel composition.
6. The fuel composition of claim 1, wherein the amount of the isoprenoid compound is from about 45 vol. % to about 65 vol %, based on the total volume of the fuel composition.
7. The fuel composition of claim 1, wherein the petroleum-based fuel is petrodiesel.
8. The fuel composition of claim 1, wherein the fuel additive is at least one additive selected from the group consisting of an antioxidant, a cetane improver, a stabilizer, a lubricity improver, and combinations thereof.
9. The fuel composition of claim 1, wherein Z is H.
10. A method of making a fuel composition comprising obtaining a petroleum distillate and adding an isoprenoid compound having the formula ##STR00043## and a fuel additive thereto, wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl, wherein the amount of the petroleum distillate is at least about 5 vol. % and the amount of the isoprenoid compound is at least about 5 vol. %, both amounts based on the total volume of the fuel composition, and wherein the fuel composition has a flash point equal to or greater than 38.degree. C. and has an initial boiling point between about 100.degree. C. and about 200.degree. C.
11. The method of claim 10, wherein the amount of the isoprenoid compound is less than about 65 vol. % based on the total volume of the fuel composition.
12. The method of claim 10, wherein the fuel composition has a T90 distillation temperature from about 282.degree. C. and about 338.degree. C.
13. The method of claim 12, wherein the method further comprises chemically converting a C.sub.15 isoprenoid starting material from a biological source to an isoprenoid compound having the formula ##STR00044## wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl; wherein said converting is prior to adding the isoprenoid compound to the petroleum distillate.
14. The method of claim 13, wherein the C.sub.15 isoprenoid starting material is .alpha.-farnesene,

.beta.-farnesene or a combination thereof.

15. The method of claim 10, wherein the fuel additive is at least one additive selected from the group consisting of an antioxidant, a cetane improver, a corrosion inhibitor, a lubricity improver, and combinations thereof.

16. The method of claim 10, wherein the petroleum distillate is petrodiesel.

17. The method of claim 10, wherein Z is H.

18. A vehicle comprising an internal combustion engine, a fuel tank connected to the internal combustion engine, and a fuel composition in the fuel tank, wherein the fuel composition comprises: (a) isoprenoid compound having the formula ##STR00045## or a stereoisomer thereof, wherein Z is H, O--R, or O--C(.dbd.O)R,; and R is H, alkyl, cycloalkyl, aryl, alkaryl or aralkyl; (b) a petroleum-based fuel; and (c) a fuel additive, wherein the amount of the isoprenoid compound is at least about 5 vol. % and the amount of the petroleum-based fuel is at least about 5 vol. %, both amounts based on the total volume of the fuel composition, and wherein the fuel composition has a flash point equal to or greater than 38.degree. C. and has an initial boiling point from about 100.degree. C. to 200.degree. C., and wherein the fuel composition is used to power the internal combustion engine.

19. The vehicle of claim 18, wherein the internal combustion engine is a diesel engine.

20. The vehicle of claim 18, wherein Z is H.

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### *Description*

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#### FIELD OF THE INVENTION

This invention encompasses, among other things, fuel compositions such as diesel fuels and jet fuels. In particular, this invention encompasses fuel compositions comprising farnesane, and methods of making and using the fuel compositions. In certain embodiments, the invention encompasses a stable fuel composition comprising farnesane which is readily and efficiently produced, at least in part, from a microorganism. In certain embodiments, the present invention encompasses a fuel composition comprising a high concentration of a bioengineered farnesane.

#### BACKGROUND OF THE INVENTION

Biologically produced fuels ("biofuels") have received considerable attention over the past few decades due to concerns over rising oil prices, impending supply constraints, and increasing global carbon dioxide emissions. In contrast to non-renewable natural energy sources such as petroleum and coal, biofuels are derived from renewable naturally sources, typically living organisms and their metabolic byproducts.

To date, biofuels that are suitable for internal combustion engines such as diesel engines are generally derived from vegetable oils. The so called first generation "biodiesels" are typically C.sub.16-C.sub.18 fatty acid methyl esters formed from the transesterification of vegetable oil. More recently, a second generation "biodiesel" is being produced by new processes such as the NExBTL process, as disclosed in WO2006/075057, which hydrogenates vegetable oils or animal fat to yield the corresponding alkanes or paraffins. Because of the nature of the starting materials, both methods yield a complex and





"Biofuel" refers to any fuel that is derived from a biomass, i.e., recently living organisms or their metabolic byproducts, such as manure from cows. It is a renewable energy source, unlike other natural resources such as petroleum, coal, and nuclear fuels.

"C.sub.15 isoprenoid starting material" refers to farnesyl pyrophosphate ("FPP") or a compound that is capable of being derived from FPP.

"Cetane number" refers to a measure of how readily a fuel starts to burn (autoignite) under conditions described by ASTM D 613. A fuel with a high cetane number starts to burn shortly after it is injected into the cylinder; it has a short ignition delay period. Conversely, a fuel with a low cetane number resists autoignition and has a longer ignition delay period.

"Cloud point" refers to the temperature at which a cloud of wax crystals first appears in a fuel sample that is cooled under conditions described by ASTM D 2500.

"Cold filter plugging point" (CFPP) refers to an approximate indication of the temperature at which the fuel first fails to pass through a wire mesh in a set period of time. The ASTM D 6371 test simulates the flow of the cooled fuel through a filter in the fuel system. Therefore, the CFPP is a measure of the dynamic cold flow properties of the fuel.

"Diesel fuel" refers to a fuel suitable for use in a diesel engine where the fuel is ignited by the heat of air under high compression. The class of diesel fuels includes hydrocarbons having a broad range of molecular weights. In some embodiments, the diesel fuels herein include hydrocarbons comprising at least 15 carbons. In other embodiments, the diesel fuels herein include hydrocarbons comprising at least 15 carbons, alcohols comprising at least 3 carbons, fatty esters comprising at least 10 carbons, and mixtures thereof. Types of diesel fuels include, but are not limited to, petrodiesel, biodiesel, bioengineered diesel, or mixtures thereof. Diesel fuels can also be obtained from synthetic fuels such as shale oil, or Fischer-Tropsch fuels such as those derived from synthetic gas and coal liquefaction.

"Farnesane" refers to a compound having formula (III):

##STR00001## or a stereoisomer thereof. In some embodiments, the farnesane comprises a substantially pure stereoisomer of farnesane. In other embodiments, the farnesane comprises a mixture of stereoisomers, such as enantiomers and diastereoisomers, of farnesane. In further embodiments, the amount of each of the stereoisomers in the farnesane mixture is independently from about 0.1 wt. % to about 99.9 wt. %, from about 0.5 wt. % to about 99.5 wt. %, from about 1 wt. % to about 99 wt. %, from about 5 wt. % to about 95 wt. %, from about 10 wt. % to about 90 wt. %, from about 20 wt. % to about 80 wt. %, based on the total weight of the farnesane mixture.

".alpha.-Farnesene" refers to a compound having the following formula:

##STR00002## or a stereoisomer thereof. In some embodiments, the .alpha.-farnesene comprises a substantially pure stereoisomer of .alpha.-farnesene. In other embodiments, the .alpha.-farnesene comprises a mixture of stereoisomers, such as cis-trans isomers. In further embodiments, the amount of each of the stereoisomers in the .alpha.-farnesene mixture is independently from about 0.1 wt. % to about 99.9 wt. %, from about 0.5 wt. % to about 99.5 wt. %, from about 1 wt. % to about 99 wt. %, from about 5 wt. % to about 95 wt. %, from about 10 wt. % to about 90 wt. %, from about 20 wt. % to about 80 wt. %, based on the total weight of the .alpha.-farnesene mixture.

".beta.-Farnesene" refers to a compound having the following formula:

##STR00003## or a stereoisomer thereof. In some embodiments, the .beta.-farnesene comprises a substantially pure stereoisomer of .beta.-farnesene. In other embodiments, the .beta.-farnesene comprises a mixture of stereoisomers, such as cis-trans isomers. In further embodiments, the amount of each of the stereoisomers in the .beta.-farnesene mixture is independently from about 0.1 wt. % to about 99.9 wt. %, from about 0.5 wt. % to about 99.5 wt. %, from about 1 wt. % to about 99 wt. %, from about 5 wt. % to about 95 wt. %, from about 10 wt. % to about 90 wt. %, from about 20 wt. % to about 80 wt. %, based on the total weight of the .beta.-farnesene mixture.

"Flash point" refers to the lowest temperature at which the application of an ignition source causes vapors above the diesel fuel to ignite under conditions described by ASTM D93.

"Fuel" refers to one or more hydrocarbons, one or more alcohols, one or more fatty esters, or a mixture thereof. Preferably, liquid hydrocarbons are used. Fuel can be used to power internal combustion engines such as reciprocating engines (e.g., gasoline engines and diesel engines), Wankel engines, jet engines, some rocket engines, missile engines, and gas turbine engines. In some embodiments, fuel typically comprises a mixture of hydrocarbons such as alkanes, cycloalkanes, and aromatic hydrocarbons. In some embodiments, fuel comprises one or more of the C.sub.15 isoprenoid compounds disclosed herein.

"Fuel additive" refers to a minor fuel component such as chemical components added to fuels to alter the properties of the fuel, e.g., to improve engine performance, fuel handling, fuel stability, or for contaminant control. Types of additives include, but are not limited to, antioxidants, thermal stability improvers, cetane improvers, stabilizers, cold flow improvers, combustion improvers, anti-foams, anti-haze additives, corrosion inhibitors, lubricity improvers, icing inhibitors, injector cleanliness additives, smoke suppressants, drag reducing additives, metal deactivators, dispersants, detergents, demulsifiers, dyes, markers, static dissipaters, biocides, and combinations thereof. The term "conventional additives" refers to fuel additives known to the skilled artisan, such as those described above, that are not the isoprenoid compounds of the invention.

"Fuel composition" refers to a fuel that comprises at least two fuel components.

"Fuel component" refers to any compound or a mixture of compounds that are used to formulate a fuel composition. There are "major fuel components" and "minor fuel components." A major fuel component is present in a fuel composition by at least 50% by volume; and a minor fuel component is present in a fuel composition by less than 50%. Fuel additives are minor fuel components. The isoprenoid compounds disclosed herein can be a major component or a minor component, by themselves or in a mixture with other fuel components.

"Isoprenoid" and "isoprenoid compound" are used interchangeably herein and refer to a compound derivable from isopentenyl diphosphate ("IPP").

"Initial boiling point" and "final boiling point" refer to points in a distillation curve that relate the fraction of a sample that is removed by heating the sample to progressively higher temperatures. The initial boiling point is the boiling temperature of the first drop of liquid leaving the condenser, and the final boiling point is the boiling temperature of the last drop of liquid leaving the condenser. When the sample is composed of a single component, the initial and final boiling points are identical and referred to as the "boiling point." The generally accepted procedure for determining the distillation curve for fuel is ASTM Standard D 86.

"Jet fuel" refers to a fuel suitable for use in a jet engine.

"Kerosene" refers to a specific fractional distillate of petroleum (also known as "crude oil"), generally between 150.degree. C. and 275.degree. C. at atmospheric pressure. Crude oils are composed primarily of hydrocarbons of the paraffinic, naphthenic, and aromatic classes.

"Lubricity" refers to a measure of the capacity of a diesel fuel to provide for more efficient wear protection to components of the engine during metal to metal contact under high pressure rolling point contact under conditions described by ASTM D 6079.

"Petrodiesel" refers to a specific fractional distillate of petroleum, generally from between 120.degree. C. and 380.degree. C. at atmospheric pressure. In other embodiments, petrodiesel is a fractional distillate of petroleum from between 150.degree. C. and 370.degree. C. at 1 atmospheric pressure.

"Pour point" refers to an approximate indication of the lowest temperature at which a fuel can be poured or removed from containers or can be caused to flow through tubing and piping, and is measured under conditions described by ASTM D 97. The pour point is one of the characteristics that determines a fuel's usefulness and serviceability in colder climates.

A composition that is a "substantially pure" compound refers to a composition that is substantially free of one or more other compounds, i.e., the composition contains greater than 80%, greater than 90%, greater than 95%, greater than 96%, greater than 97%, greater than 98%, greater than 99%, greater than 99.5%, greater than 99.6%, greater than 99.7%, greater than 99.8%, or greater than 99.9% of the compound; or less than 20%, less than 10%, less than 5%, less than 3%, less than 1%, less than 0.5%, less than 0.1%, or less than 0.01% of the one or more other compounds, based on the total volume or weight of the composition.

A composition that is "substantially free" of a compound refers to a composition containing less than 20%, less than 10%, less than 5%, less than 4%, less than 3%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, or less than 0.01% of the compound, based on the total volume or weight of the composition.

In addition to the definitions above, certain compounds described herein have one or more double bonds that can exist as one or more stereoisomers such as cis-isomers, trans-isomers, E-isomers and Z-isomers. In certain embodiments, these compounds as individual stereoisomers are substantially free of other stereoisomers. In certain other embodiments, these compounds are mixtures of various stereoisomers.

"Tx" refers to the distillation temperature at which x % of the original volume of the fuel composition has been distilled according to ASTM D-86, which is incorporated herein by reference. For example, "T10", "T50", and "T90" refer to the distillation temperatures at which 10%, 50%, and 90% respectively of the original volume of the fuel composition has been distilled according to ASTM D 86. "T10", "T50", and "T90" are also known as the 10 vol. % temperature, the 50 vol. % temperature, and the 90 vol. % temperature respectively.

In the following description, all numbers disclosed herein are approximate values, regardless whether the word "about" or "approximate" is used in connection therewith. Numbers may vary by 1 percent, 2 percent, 5 percent, or, sometimes, 10 to 20 percent. Whenever a numerical range with a lower limit, R<sub>sup.L</sub>, and an upper limit, R<sub>sup.U</sub>, is disclosed, any number falling within the range is specifically disclosed. In particular, the following numbers within the range are specifically disclosed:  $R = R_{sup.L} + k * (R_{sup.U} - R_{sup.L})$ , wherein k is a variable ranging from 1 percent to 100 percent with a 1 percent increment, i.e., k is 1 percent, 2 percent, 3 percent, 4 percent, 5 percent, . . . , 50 percent, 51 percent, 52 percent, . . . , 95 percent, 96 percent, 97 percent, 98 percent, 99 percent, or 100 percent. Moreover, any numerical range defined by two R numbers as defined in the above is also specifically



mixture of formulae (III), (IV), and (V).

In another set of embodiments, the isoprenoid compound comprises at least two different compounds having formula (III), (IV) or (V)

##STR00012## or a stereoisomer thereof, wherein R is C.sub.1-C.sub.5 alkyl and the two compounds are each present in an amount at least about 5%, based on the total weight or volume of the fuel composition.

In another set of embodiments, the isoprenoid compound is one or more of:

##STR00013## wherein R is as defined above. Formulae (III-a), (III-b), (III-c), and (III-d) are the four possible stereoisomers of formula (III). Formulae (IV-a), (IV-b), (IV-c), and (IV-d) are the four possible stereoisomers of formula (IV). Formulae (V-a), (V-b), (V-c), and (V-d) are the four possible stereoisomers of formula (V).

Each of the isoprenoid compounds in the fuel compositions can function as a fuel component which can release energy when it chemically reacts with an oxidant such as oxygen; or a fuel additive which can alter the performance or properties of the fuel component. In some embodiments, the isoprenoid compound is present in an amount of at least about 2%, at least about 3%, at least about 5%, at least about 10%, at least about 15%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90%, based on the total weight or volume of the fuel composition. In other embodiments, the isoprenoid compound is present in an amount of at most about 5%, at most about 10%, at most about 15%, at most about 20%, at most about 25%, at most about 30%, at most about 35%, at most about 40%, at most about 45%, at most about 50%, at most about 60%, at most about 70%, at most about 80%, or at most about 90%, based on the total weight or volume of the fuel composition. In further embodiments, the isoprenoid compound is present in an amount from about 2% to about 99%, from about 2.5% to about 95%, from about 5% to about 90%, from about 7.5% to about 85%, from about 10% to about 80%, from about 15% to about 80%, from about 20% to about 75%, or from about 25% to about 75%, based on the total weight or volume of the fuel composition.

In some embodiments, the C.sub.15 isoprenoid compound is derived from a bioengineered C.sub.15 isoprenoid starting material. In certain embodiments, the bioengineered C.sub.15 isoprenoid starting material is made by host cells by converting a carbon source into the C.sub.15 isoprenoid starting material.

In other embodiments, the carbon source is a sugar such as a monosaccharide (simple sugar), a disaccharide, or one or more combinations thereof. In certain embodiments, the sugar is a simple sugar capable of supporting the growth of one or more of the cells provided herein. The simple sugar can be any simple sugar known to those of skill in the art. Some non-limiting examples of suitable simple sugars or monosaccharides include glucose, galactose, mannose, fructose, ribose, and combinations thereof. Some non-limiting examples of suitable disaccharides include sucrose, lactose, maltose, trehalose, cellobiose, and combinations thereof.

In other embodiments, the carbon source is a polysaccharide. Some non-limiting examples of suitable polysaccharides include starch, glycogen, cellulose, chitin, and combinations thereof.

In still other embodiments, the carbon source is a non-fermentable carbon source. Some non-limiting examples of suitable non-fermentable carbon source include acetate and glycerol.



heptamethylnonane, an isomer of cetane, has the assigned cetane number of 0. The cetane number of a diesel fuel is determined by comparison with blends of cetane and heptamethylnonane. It corresponds to the number of parts by volume of cetane in a cetane-heptamethylnonane blend which has the same ignition quality as the fuel.

Generally, regular diesel fuels have an aromatic content above 20 wt. % and a sulfur content of several hundred parts per million or more. They may further include additional oxygen and/or nitrogen impurities. To obtain a desired diesel fuel, a regular diesel fuel typically undergoes a conversion step in which the aromatic hydrocarbons present in the regular diesel fuel are converted to non-aromatic hydrocarbons, such as cycloparaffins. This is typically achieved by hydrogenating the regular diesel fuel in the presence of a hydrogenation catalyst. Other conversion processes may also be used.

Ordinarily, "straight run" diesel fuel produced by simple distillation of crude oil is fairly low in aromatic hydrocarbons. Catalytic cracking of residual oil to increase gasoline and diesel production, however, results in increased aromatic content. A typical straight run diesel might contain from 20 to 25% aromatics by volume, whereas a diesel blended from catalytically cracked stocks could have from 40 to 50% aromatics. The aromatic hydrocarbon content of the fuel composition disclosed herein may be less than about 50 vol. %, about 45 vol. %, about 40 vol. %, about 35 vol. %, about 30 vol. %, about 25 vol. %, or about 20 vol. %, based on the total volume of the fuel composition. In some embodiments, the aromatic hydrocarbon content of the fuel composition is less than 15 vol. %, less than 10 vol. %, less than 5 vol. %, less than 2.5 vol. % or less than 1 vol. %, based on the total volume of the fuel composition. In other embodiments, the fuel composition is substantially free of aromatic hydrocarbon content.

Aromatic hydrocarbons have poor self-ignition qualities, so that diesel fuels containing a high fraction of aromatics tend to have low cetane numbers. Typical cetane values of straight run diesel are in the range of from 50 to 55; those of highly aromatic diesel fuels are typically in the range of from 40 to 45, and may be even lower. This may cause more difficulty in cold starting and increased combustion noise due to the increased ignition delay.

To reduce the sulfur content of the fuel composition disclosed herein, a desulfurization process can be used to reduce the diesel fuel component in the fuel composition and/or a higher amount of the isoprenoid compounds can be used. Any desulfurization method can be used in embodiments of the invention. Additional steps which remove oxygen and/or nitrogen can also be employed to obtain the desired diesel fuel. U.S. Pat. Nos. 5,611,912, 5,068,025, 4,746,420, and 4,675,102 disclose hydrogenation and/or desulfurization processes which may be used in embodiments of the invention. The disclosures of all of the preceding patents are incorporated by reference herein in their entireties. The sulfur content of the fuel composition disclosed herein can have or can be made to have less than about 500 ppm, about 100 ppm, about 50 ppm, about 30 ppm, about 20 ppm, or about 15 ppm, based on the total weight of the fuel composition. In other embodiments, the sulfur content of the fuel composition is less than 10 ppm. In further embodiments, the fuel composition is substantially free of sulfur content.

In certain embodiments, the fuel composition is intended for use in jet engines. The most common jet fuel is a kerosene/paraffin oil-based fuel classified as Jet A-1, which is produced to an internationally standardized set of specifications. In the United States only, a version of Jet A-1 known as Jet A is also used. Another jet fuel that is commonly used in civilian aviation is called Jet B. Jet B is a lighter fuel in the naphtha-kerosene region that is used for its enhanced cold-weather performance. The distillation range for Jet B is generally 140 to 460.degree. F. (from 50 to 250.degree. C.). Jet A, Jet A-1, and Jet B are specified in ASTM Specification D. 1655-68. Alternatively, jet fuels are classified by militaries around the world with a system of JP numbers. Some are almost identical to their civilian counterparts





compound is at least about 5 vol. % and the amount of the conventional fuel component is at least about 5 vol. %, both amounts based on the total volume of the fuel composition; and wherein the fuel composition has a flash point equal to or greater than 38.degree. C. and an initial boiling point between about 100.degree. C. and about 200.degree. C.

In some embodiments, the amount of the isoprenoid compound in the fuel compositions disclosed herein is from about 5 vol. % to about 90 vol. %, based on the total volume of the fuel composition. In other embodiments, the amount of the isoprenoid compound is less than about 75 vol. %, is less than about 65 vol. %, is less than about 50 vol. %, or is less than about 45 vol. %, based on the total volume of the fuel composition. In other embodiments, the amount of the isoprenoid compound is from about 5 vol. % to about 10 vol. %. In other embodiments, the amount of the isoprenoid compound is from about 15 vol. % to about 25 vol. %. In still other embodiments, the amount of the isoprenoid compound is from about 45 vol. % to about 55 vol. %.

In other embodiments, the amount of conventional fuel component in the fuel compositions disclosed herein is at least about 20% and the amount of isoprenoid compound is from about 5% to about 75%, based on the total volume of the fuel composition. In certain embodiments, the amount of conventional fuel component is at least 30% and the amount of the isoprenoid compound is from about 5% to about 65%, based on the total volume of the fuel composition. In certain other embodiments, the amount of conventional fuel is at least 40% and the amount of isoprenoid is from about 5% to about 50%, based on the total volume of the fuel composition. In certain other embodiments, the amount of conventional fuel is at least 50% and the amount of isoprenoid is from about 5% to about 45%, based on the total volume of the fuel composition.

In some embodiments, the conventional fuel component is a coal-based fuel. In other embodiments, the conventional fuel component is petrodiesel. In still other embodiments, the conventional fuel component is kerosene.

In some embodiments, a fuel composition disclosed herein has an initial boiling point greater than about 100.degree. C., greater than about 110.degree. C., greater than about 120.degree. C., greater than about 130.degree. C., or greater than about 140.degree. C. In other embodiments, the initial boiling point is from about 100.degree. C. to about 150.degree. C.

In some embodiments, a fuel composition disclosed herein has a final boiling point greater than about 200.degree. C. In other embodiments, the final boiling point is greater than about 225.degree. C., greater than about 250.degree. C., greater than about 275.degree. C., greater than about 300.degree. C., or greater than about 325.degree. C. In further embodiments, the final boiling point is greater than about 350.degree. C. In certain embodiments, the final boiling point is greater than about 375.degree. C.

In other embodiments, a fuel composition disclosed herein has an initial boiling point of from about 100.degree. C. to about 200.degree. C. and a final boiling point greater than about 300.degree. C. In another embodiment, the fuel composition has an initial boiling point from about 110.degree. C. to about 140.degree. C. and a final boiling point greater than about 350.degree. C. In another embodiment, the fuel composition has an initial boiling point from about 110.degree. C. to about 140.degree. C. and a final boiling point greater than about 375.degree. C.

In some embodiments, a fuel composition disclosed herein has a T90 distillation temperature from about 270.degree. C. to about 350.degree. C. In other embodiments, the T90 distillation temperature is from about 282.degree. C. to about 338.degree. C.

In other embodiments, a fuel composition disclosed herein has a T50 distillation temperature from about



In some embodiments, the fuel composition further comprises C.sub.11-C.sub.19 hydrocarbons wherein each set of C.sub.1, C.sub.12, C.sub.13, C.sub.14, C.sub.15, C.sub.16, C.sub.17, C.sub.18, and C.sub.19 hydrocarbons is present in an amount at least about 1 vol %, based on the total volume of the fuel composition.

The fuel compositions disclosed herein can be used to power any equipment such as an emergency generator or internal combustion engine, which requires a fuel such as diesel fuels or jet fuels. In certain embodiments, provided are emergency fuels comprising one or more of the above fuel compositions. In certain embodiments, provided herein are uses of the above fuel compositions as emergency fuels. The term "emergency fuel" refers to a fuel which is generally stored in a container other than the gas tank of a vehicle. The fuel should be stable over an extended period of time, for example, six to twelve months. When the vehicle runs out of fuel, the emergency fuel is added to the gas tank of the vehicle and provides fuel to the vehicle. Because the flash point of the diesel fuel made in accordance with embodiments of the invention generally exceeds 140.degree. F., it can be safely stored in the trunk of a diesel vehicle. The fuel compositions can also be used as an alternative fuel as described in U.S. Pat. No. 6,096,103, which is incorporated by reference herein in its entirety.

In another aspect, a fuel system is provided comprising a fuel tank containing the fuel composition disclosed herein. Optionally, the fuel system may further comprise an engine cooling system having a recirculating engine coolant, a fuel line connecting the fuel tank with the internal combustion engine, and/or a fuel filter arranged on the fuel line. Some non-limiting examples of internal combustion engines include reciprocating engines (e.g., gasoline engines and diesel engines), Wankel engines, jet engines, some rocket engines, and gas turbine engines.

In some embodiments, the fuel tank is arranged with said cooling system so as to allow heat transfer from the recirculating engine coolant to the fuel composition contained in the fuel tank. In other embodiments, the fuel system further comprises a second fuel tank containing a second fuel for a diesel engine and a second fuel line connecting the second fuel tank with the internal combustion engine. Optionally, the first and second fuel lines can be provided with electromagnetically operated valves that can be opened or closed independently of each other or simultaneously. In further embodiments, the second fuel is a petrodiesel.

In another aspect, an engine arrangement is provided comprising an internal combustion engine, a fuel tank containing the fuel composition disclosed herein, a fuel line connecting the fuel tank with the internal combustion engine. Optionally, the engine arrangement may further comprise a fuel filter and/or an engine cooling system comprising a recirculating engine coolant. In some embodiments, the internal combustion engine is a diesel engine. In other embodiments, the internal combustion engine is a jet engine.

When using a fuel composition disclosed herein, it is desirable to remove particulate matter originating from the fuel composition before injecting it into the engine. Therefore, it is desirable to select a suitable fuel filter for use in a fuel system disclosed herein. Water in fuels used in an internal combustion engine, even in small amounts, can be very harmful to the engine. Therefore, it is desirable that water present in fuel composition be removed prior to injection into the engine. In some embodiments, water and particulate matter can be removed by the use of a fuel filter utilizing a turbine centrifuge, in which water and particulate matter are separated from the fuel composition to an extent allowing injection of the filtrated fuel composition into the engine, without risk of damage to the engine. Other types of fuel filters that can remove water and/or particulate matter also may be used.

In another aspect, a vehicle is provided comprising an internal combustion engine, a fuel tank containing the fuel composition disclosed herein, and a fuel line connecting the fuel tank with the internal

combustion engine. Optionally, the vehicle may further comprise a fuel filter and/or an engine cooling system comprising a recirculating engine coolant. Some non-limiting examples of vehicles include cars, motorcycles, trains, ships, and aircrafts.

In another aspect, a method of making an isoprenoid compound of the formula

##STR00018## is provided wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl. The method comprises a) obtaining a C.sub.15 isoprenoid starting material from a biological source and b) converting the C.sub.15 isoprenoid starting material into the compound using chemical synthesis.

In another aspect, an isoprenoid compound is provided

##STR00019## wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl wherein the compound is made by a) obtaining a C.sub.15 isoprenoid starting material from a biological source and b) converting the C.sub.15 isoprenoid starting material into the compound using chemical synthesis.

In another aspect, a biofuel is provided produced from a) obtaining a C.sub.15 isoprenoid starting material from a biological source and b) converting the C.sub.15 isoprenoid starting material using chemical synthesis to make an isoprenoid compound of the formula

##STR00020## wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl.

In one set of embodiments, the C.sub.15 isoprenoid starting material is

##STR00021## which is hydrogenated to produce

##STR00022## or a stereoisomer thereof.

In another set of embodiments, the C.sub.15 isoprenoid starting material is

##STR00023## which is hydrogenated and esterified to produce

##STR00024## or a stereoisomer thereof, wherein R is alkyl.

In another set of embodiments, the C.sub.15 isoprenoid starting material is

##STR00025## which is hydrogenated and esterified to produce

##STR00026## or a stereoisomer thereof, wherein R is alkyl.

In another aspect, a method of making a fuel composition is provided comprising: a) contacting a cell capable of making a C.sub.15 isoprenoid starting material with a simple sugar under conditions suitable for making the C.sub.15 isoprenoid starting material; b) hydrogenating the C.sub.15 isoprenoid starting material to form a hydrogenated C.sub.15 isoprenoid compound; and c) mixing the hydrogenated C.sub.15 isoprenoid compound with one or more fuel components or fuel additives to make the fuel composition.

In another aspect, a method of making a fuel composition is provided comprising: a) contacting a cell

capable of making a C.sub.15 isoprenoid starting material with a non-fermentable carbon source under conditions suitable for making the C.sub.15 isoprenoid starting material; b) hydrogenating the C.sub.15 isoprenoid starting material to form a hydrogenated C.sub.15 isoprenoid compound; and c) mixing the hydrogenated C.sub.15 isoprenoid compound with one or more fuel components or fuel additives to make the fuel composition.

In another aspect, a facility is provided for manufacture of a fuel, bioengineered fuel component, or bioengineered fuel additive of the invention. In certain embodiments, the facility is capable of biological manufacture of the C.sub.15 starting materials. In certain embodiments, the facility is further capable of preparing an isoprenoid fuel additive or fuel component from the starting material.

The facility can comprise any structure useful for preparing the C.sub.15 starting material using a microorganism. In some embodiments, the biological facility comprises one or more of the cells disclosed herein. In some embodiments, the biological facility comprises a cell culture comprising at least a C.sub.15 starting material in an amount of at least about 1 wt. %, at least about 5 wt. %, at least about 10 wt. %, at least about 20 wt. %, or at least about 30 wt. %, based on the total weight of the cell culture. In further embodiments, the biological facility comprises a fermentor comprising one or more cells described herein.

Any fermentor that can provide cells or bacteria a stable and optimal environment in which they can grow or reproduce can be used herein. In some embodiments, the fermentor comprises a culture comprising one or more of the cells disclosed herein. In other embodiments, the fermentor comprises a cell culture capable of biologically manufacturing farnesyl pyrophosphate (FPP). In further embodiments, the fermentor comprises a cell culture capable of biologically manufacturing isopentenyl diphosphate (IPP). In certain embodiments, the fermentor comprises a cell culture comprising at least a C.sub.15 starting material in an amount of at least about 1 wt. %, at least about 5 wt. %, at least about 10 wt. %, at least about 20 wt. %, or at least about 30 wt. %, based on the total weight of the cell culture.

The facility can further comprise any structure capable of manufacturing the fuel component or fuel additive from the C.sub.15 starting material. The structure may comprise a hydrogenator for the hydrogenation of the C.sub.15 starting materials. Any hydrogenator that can be used to reduce C.dbd.C double bonds to C--C single bonds under conditions known to skilled artisans may be used herein. The hydrogenator may comprise a hydrogenation catalyst disclosed herein. In some embodiments, the structure further comprises a mixer, a container, and a mixture of the hydrogenation products from the hydrogenation step and a conventional fuel additive in the container.

### Host Cell

A C.sub.15 isoprenoid starting material can be made by any method known in the art including biological methods, chemical syntheses (without the use of biologically derived materials), and hybrid methods where both biological and chemical means are used. When the C.sub.15 isoprenoid starting material is made biologically, one method comprises the use of a host cell that has been modified to produce the desired product. Like all isoprenoids, a C.sub.15 isoprenoid starting material is made biochemically through a common intermediate, isopentenyl diphosphate ("IPP").

The host cell can be grown according to any technique known to those of skill in the art. In particular, the host cell can be grown in culture medium appropriate for the host cell. In advantageous embodiments, the culture medium comprises readily available, renewable components. The present invention thus provides readily available, renewable sources of energy methods of their use to produce fuel compositions. In certain embodiments, the host cell is grown or cultured by contact with a simple sugar under conditions suitable for their growth and production of a C.sub.15 isoprenoid. In certain

embodiments, the host cell can be grown or cultured by contact with glucose, galactose, mannose, fructose, ribose, or a combination thereof. The present invention thus provides fuel compositions derived from simple sugars, e.g. glucose, galactose, mannose, fructose, ribose, and combinations thereof, and methods of their production from the simple sugars.

Any suitable host cell may be used in the practice of the present invention. In one embodiment, the host cell is a genetically modified host microorganism in which nucleic acid molecules have been inserted, deleted or modified (i.e., mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), to either produce the desired isoprenoid or isoprenoid derivative, or to increase yields of the desired isoprenoid or isoprenoid derivative. In another embodiment, the host cell is capable of being grown in liquid growth medium.

Illustrative examples of suitable host cells include archae cells, bacterial cells, and eukaryotic cells. Some non-limiting examples of archae cells include those belong to the genera: *Aeropyrum*, *Archaeoglobus*, *Halobacterium*, *Methanococcus*, *Methanobacterium*, *Pyrococcus*, *Sulfolobus*, and *Thermoplasma*. Some non-limiting examples of archae strains include *Aeropyrum pernix*, *Archaeoglobus fulgidus*, *Methanococcus jannaschii*, *Methanobacterium thermoautotrophicum*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Thermoplasma acidophilum*, and *Thermoplasma volcanium*, and the like.

Some non-limiting examples of bacterial cells include those belonging to the genera: *Agrobacterium*, *Alicyclobacillus*, *Anabaena*, *Anacystis*, *Arthrobacter*, *Azobacter*, *Bacillus*, *Brevibacterium*, *Chromatium*, *Clostridium*, *Corynebacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Lactobacillus*, *Lactococcus*, *Mesorhizobium*, *Methylobacterium*, *Microbacterium*, *Phormidium*, *Pseudomonas*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*, *Rhodococcus*, *Salmonella*, *Scenedesmun*, *Serratia*, *Shigella*, *Staphlococcus*, *Streptomyces*, *Synnecoccus*, and *Zymomonas*.

Some non-limiting examples of bacterial strains include *Bacillus subtilis*, *Bacillus amyloliquefacines*, *Brevibacterium ammoniagenes*, *Brevibacterium immariophilum*, *Clostridium beigerinckii*, *Enterobacter sakazakii*, *Escherichia coli*, *Lactococcus lactis*, *Mesorhizobium loti*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Pseudomonas pudica*, *Rhodobacter capsulatus*, *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Salmonella enterica*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Staphylococcus aureus*, and the like.

In general, if a bacterial host cell is used, a non-pathogenic strain is preferred. Some non-limiting examples of non-pathogenic strains include *Bacillus subtilis*, *Escherichia coli*, *Lactibacillus acidophilus*, *Lactobacillus helveticus*, *Pseudomonas aeruginosa*, *Pseudomonas mevalonii*, *Pseudomonas pudita*, *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum*, and the like.

Some non-limiting examples of eukaryotic cells include fungal cells. Some non-limiting examples of fungal cells include those belonging to the genera: *Aspergillus*, *Candida*, *Chrysosporium*, *Cryptococcus*, *Fusarium*, *Kluyveromyces*, *Neotyphodium*, *Neurospora*, *Penicillium*, *Pichia*, *Saccharomyces*, and *Trichoderma*.

Some non-limiting examples of eukaryotic strains include *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Candida albicans*, *Chrysosporium lucknowense*, *Fusarium graminearum*, *Fusarium venenatum*, *Kluyveromyces lactis*, *Neurospora crassa*, *Pichia angusta*, *Pichia finlandica*, *Pichia kodamae*, *Pichia membranaefaciens*, *Pichia methanolica*, *Pichia opuntiae*, *Pichia pastoris*, *Pichia piperi*, *Pichia quercuum*, *Pichia salictaria*, *Pichia thermotolerans*, *Pichia trehalophila*, *Pichia stipitis*, *Streptomyces ambofaciens*, *Streptomyces aureofaciens*, *Streptomyces aureus*, *Saccaromyces bayanus*, *Saccaromyces boulardi*, *Saccharomyces cerevisiae*, *Streptomyces fungicidicus*, *Streptomyces*



*Saccharomyces cerevisiae*).

In the fifth step, a second phosphate group is enzymatically added to mevalonate 5-phosphate to form mevalonate 5-pyrophosphate. An enzyme known to catalyze this step is, for example, phosphomevalonate kinase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include (AF429385; *Hevea brasiliensis*), (NM.sub.--006556; *Homo sapiens*), and (NC.sub.--001145. complement 712315 . . . 713670; *Saccharomyces cerevisiae*).

In the sixth step, mevalonate 5-pyrophosphate is enzymatically converted into IPP. An enzyme known to catalyze this step is, for example, mevalonate pyrophosphate decarboxylase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include (X97557; *Saccharomyces cerevisiae*), (AF290095; *Enterococcus faecium*), and (U49260; *Homo sapiens*).

If IPP is to be converted to DMAPP, then a seventh step is required. An enzyme known to catalyze this step is, for example, IPP isomerase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include (NC.sub.--000913, 3031087 . . . 3031635; *Escherichia coli*) and (AF082326; *Haematococcus pluvialis*).

### DXP Pathway

A schematic representation of the DXP pathway is shown in FIG. 2. In general, the DXP pathway comprises seven steps. In the first step, pyruvate is condensed with D-glyceraldehyde 3-phosphate to make 1-deoxy-D-xylulose-5-phosphate. An enzyme known to catalyze this step is, for example, 1-deoxy-D-xylulose-5-phosphate synthase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AF035440; *Escherichia coli*), (NC.sub.--002947, locus tag PP0527; *Pseudomonas putida* KT2440), (CP000026, locus tag SPA2301; *Salmonella enterica* Paratyphi, see ATCC 9150), (NC.sub.--007493, locus tag RSP.sub.--0254; *Rhodobacter sphaeroides* 2.4.1), (NC.sub.--005296, locus tag RPA0952; *Rhodopseudomonas palustris* CGA009), (NC.sub.--004556, locus tag PD1293; *Xylella fastidiosa* Temecula), and (NC.sub.--003076, locus tag AT5G11380; *Arabidopsis thaliana*).

In the second step, 1-deoxy-D-xylulose-5-phosphate is converted to 2C-methyl-D-erythritol-4-phosphate. An enzyme known to catalyze this step is, for example, 1-deoxy-D-xylulose-5-phosphate reductoisomerase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AB013300; *Escherichia coli*), (AF148852; *Arabidopsis thaliana*), (NC.sub.--002947, locus tag PP1597; *Pseudomonas putida* KT2440), (AL939124, locus tag SCO5694; *Streptomyces coelicolor* A3 (2)), (NC.sub.--007493, locus tag RSP.sub.--2709; *Rhodobacter sphaeroides* 2.4.1), and (NC.sub.--007492, locus tag Pfl.sub.--1107; *Pseudomonas fluorescens* PfO-1).

In the third step, 2C-methyl-D-erythritol-4-phosphate is converted to 4-diphosphocytidyl-2C-methyl-D-erythritol. An enzyme known to catalyze this step is, for example, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AF230736; *Escherichia coli*), (NC.sub.--007493, locus\_tag RSP.sub.--2835; *Rhodobacter sphaeroides* 2.4.1), (NC.sub.--003071, locus\_tag AT2G02500; *Arabidopsis thaliana*), and (NC.sub.--002947, locus\_tag PP1614; *Pseudomonas putida* KT2440).

In the fourth step, 4-diphosphocytidyl-2C-methyl-D-erythritol is converted to 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate. An enzyme known to catalyze this step is, for example, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AF216300; *Escherichia coli*) and (NC.sub.--007493, locus\_tag RSP.sub.--1779; *Rhodobacter sphaeroides* 2.4.1).





occurring terpenes that can be produced by a wide variety of plants, such as *Copaifera langsdorfii*, conifers, and sporges; insects, such as swallowtail butterflies, leaf beetles, termites, and pine sawflies; and marine organisms, such as algae, sponges, corals, mollusks, and fish.

*Copaifera langsdorfii* or *Copaifera* tree is also known as the diesel tree and kerosene tree. It has many names in local languages, including kupa'y, cabismo, and copa va. *Copaifera* tree may produce a large amount of terpene hydrocarbons in its wood and leaves. Generally, one *Copaifera* tree can produce from about 30 to about 40 liters of terpene oil per year.

Terpene oils can also be obtained from conifers and sporges. Conifers belong to the plant division Pinophyta or Coniferae and are generally cone-bearing seed plants with vascular tissue. The majority of conifers are trees, but some conifers can be shrubs. Some non-limiting examples of suitable conifers include cedars, cypresses, douglas-firs, firs, junipers, kauris, larches, pines, redwoods, spruces, and yews. Sporges, also known as Euphorbia, are a very diverse worldwide genus of plants, belonging to the spurge family (Euphorbiaceae). Consisting of about 2160 species, sporges are one of the largest genera in the plant kingdom.

The C.sub.15 isoprenoid starting materials are sesquiterpenes which are part of a larger class of compound called terpenes. A large and varied class of hydrocarbons, terpenes include hemiterpenes, monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes, tetraterpenes, and polyterpenes. As a result, suitable C.sub.15 isoprenoid starting materials can be isolated from terpene oils for use in the present invention.

#### Chemical Conversion

The fuel components of the fuel compositions disclosed herein may comprise,

##STR00032## wherein Z is as previously defined. Formula (I) or (II) can be prepared by any method known in the art including biological methods or chemical syntheses (without the use of biologically derived materials). In one embodiment, the C.sub.15 isoprenoid starting material is isolated from naturally occurring sources. For example, farnesol can be isolated from citronella, enoli, cyclamen, lemon grass, tuberose, and rose. In another embodiment, the C.sub.15 isoprenoid starting material is made by a host cell that has been modified either to produce the compound or to increase the yields of the naturally occurring compound.

Irrespective of its source, each of the C.sub.15 isoprenoid starting materials can be chemically converted into a fuel component or fuel additive disclosed herein by any known reduction reaction such as hydrogenation or a combination of reduction reaction and esterification. In some embodiments, the C.sub.15 isoprenoid starting material can be reduced by hydrogen with a catalyst such as Pd, Pd/C, Pt, PtO.sub.2, Ru(PPh.sub.3).sub.2Cl.sub.2, Raney nickel, or combinations thereof. In one embodiment, the catalyst is a Pd catalyst. In another embodiment, the catalyst is 5% Pd/C. In a further embodiment, the catalyst is 10% Pd/C in a high pressure reaction vessel and the reaction is allowed to proceed until completion. Generally, after completion, the reaction mixture can be washed, concentrated, and dried to yield the corresponding hydrogenated product. Alternatively, any reducing agent that can reduce a C.dbd.C bond to a C-C bond can also be used. For example, the C.sub.15 isoprenoid starting material can be hydrogenated by treatment with hydrazine in the presence of a catalyst, such as 5-ethyl-3-methylumiflavinium perchlorate, under O.sub.2 atmosphere to give the corresponding hydrogenated products. The reduction reaction with hydrazine is disclosed in Imada et al., J. Am. Chem. Soc., 127, 14544-14545 (2005), which is incorporated herein by reference.

In some embodiments, the C.dbd.C bonds in the C.sub.15 isoprenoid starting material are reduced to the





As demonstrated above, embodiments of the invention provide various fuel compositions which are particularly useful as diesel or jet fuels. As compared to currently available diesel and fatty acid methyl ester derived biodiesel fuels, the fuel compositions disclosed herein can be more resistant to oxidative degradation and thus have an increased shelf life. Consequently, in some embodiments, the fuel composition has a shelf life of at least about one year, at least about two years, at least about three years, at least about four years, at least about five years, at least about ten years, at least about fifteen years, at least about twenty years, or at least about twenty five years. In other embodiments, the fuel composition has a shelf life of at least about fifty years. In further embodiments, the fuel composition has a shelf life of more than fifty years.

While the invention has been described with respect to a limited number of embodiments, the specific features of one embodiment should not be attributed to other embodiments of the invention. No single embodiment is representative of all aspects of the invention. In some embodiments, the compositions or methods may include numerous compounds or steps not mentioned herein. In other embodiments, the compositions or methods do not include, or are substantially free of, any compounds or steps not enumerated herein. Variations and modifications from the described embodiments exist. For example, the diesel fuel need not be a mixture of normal paraffins and branched paraffins. It can comprise any type of hydrocarbons, so long as the aromatic content in the diesel fuel is less than 10% by weight and the sulfur content is less than 100 ppm. While it is preferred that the diesel fuel have an aromatic content of less than 10% by weight and a sulfur content of less than 100 ppm, a diesel fuel with an aromatic content greater than 10% by weight and/or a sulfur content higher than 100 ppm is also acceptable for some purposes. It should be noted that the application of the diesel fuel is not limited to diesel engines; it can be used in any equipment which requires a diesel fuel, such as an emergency generator. Although it is a regulatory requirement that all diesel fuels have a cetane number of at least 40, not all diesel fuels in accordance with embodiments of the invention need to meet this regulatory requirement. In other words, diesel fuels with a cetane number of less than 40 are also acceptable. It is noted that the methods for making and using the diesel fuel are described with reference to a number of steps. In some embodiments, these steps can be practiced in any sequence. In some embodiments, one or more steps may be omitted or combined but still achieve substantially the same results. The appended claims intend to cover all such variations and modifications as falling within the scope of the invention.

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

## EXAMPLES

The practice of the present invention can employ, unless otherwise indicated, conventional techniques of the biosynthetic industry and the like, which are within the skill of the art. To the extent such techniques are not described fully herein, one can find ample reference to them in the scientific literature.

In the following examples, efforts have been made to ensure accuracy with respect to numbers used (for example, amounts, temperature, and so on), but variation and deviation can be accommodated, and in the event a clerical error in the numbers reported herein exists, one of ordinary skill in the arts to which this invention pertains can deduce the correct amount in view of the remaining disclosure herein. Unless indicated otherwise, temperature is reported in degrees Celsius, and pressure is at or near atmospheric

pressure at sea level. All reagents, unless otherwise indicated, were obtained commercially. The following examples are intended for illustrative purposes only and do not limit in any way the scope of the present invention.

#### Example 1

This example describes methods for making expression plasmids that encode enzymes including enzymes of the MEV pathway from *Saccharomyces cerevisiae* organized in operons.

Expression plasmid pMevT was generated by inserting the MevT operon into the pBAD33 vector. The MevT operon encodes the set of MEV pathway enzymes that together transform the ubiquitous precursor acetyl-CoA to (R)-mevalonate, namely acetoacetyl-CoA thiolase, HMG-CoA synthase, and HMG-CoA reductase. The MevT operon was generated by PCR amplifying from *Escherichia coli* genomic DNA the coding sequence of the *atoB* gene (GenBank accession number NC.sub.--000913 REGION: 2324131 . . . 2325315) (encodes an acetoacetyl-CoA thiolase), from *Saccharomyces cerevisiae* genomic DNA the coding sequence of the *ERG13* gene (GenBank accession number X96617, REGION: 220 . . . 1695) (encodes a HMG-CoA synthase), and from *Saccharomyces cerevisiae* genomic DNA a segment of the coding region of the *HMG1* gene (GenBank accession number M22002, REGION: 1660 . . . 3165) (encodes a truncated HMG-CoA reductase (tHMGR)). The upstream PCR primer used for the amplification of the *HMG1* gene fragment included an artificial start codon. The amplified fragments were spliced together using overlap extensions (SOEing), during which process ribosome binding sites were introduced after the *atoB* and the *ERG13* coding sequences. After the addition of 3' A overhangs, the MevT operon was ligated into the TA cloning vector pCR4 (Invitrogen, Carlsbad, Calif.). The MevT operon was subsequently ligated into the XmaI PstI restriction site of vector pBAD33 (Guzman et al. (1995) *J. Bacteriol.* 177(14): 4121-4130). To place the operon under the control of the P.sub.Lac promoter, the *araC*-P.sub.BADNsiI-XmaI fragment of pBAD33 was replaced with the NsiI-XmaI fragment of pBBRIMCS, yielding expression plasmid pMevT (see U.S. Pat. No. 7,192,751).

Expression plasmid pAM36-MevT66 was generated by inserting the MevT66 operon into the pAM36 vector. The pAM36 vector was generated by inserting an oligonucleotide cassette containing AscI-SfiI-AsiSI-XhoI-PacI-FsII-PmeI restriction sites into the pACYC184 vector (GenBank accession number XO6403), and by removing the tetracycline resistance conferring gene in pACYC184. The MevT66 operon was synthetically generated using SEQ ID NO: 1 as a template, which comprises the *atoB* gene from *Escherichia coli* (GenBank accession number NC.sub.--000913 REGION: 2324131 . . . 2325315), the *ERG13* gene from *Saccharomyces cerevisiae* (GenBank accession number X96617, REGION: 220 . . . 1695), and a truncated version of the *HMG1* gene from *Saccharomyces cerevisiae* (GenBank accession number M22002, REGION: 1777 . . . 3285), all three sequences being codon-optimized for expression in *Escherichia coli*. The synthetically generated MevT66 operon was flanked by a 5' EcoRI restriction site and a 3' Hind III restriction site, and could thus be cloned into compatible restriction sites of a cloning vector such as a standard pUC or pACYC origin vector. The MevT66 operon was PCR amplified with flanking SfiI and AsiSI restriction sites, the amplified DNA fragment was digested to completion using SfiI and AsiSI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 4.2 kb DNA fragment was gel extracted using a gel purification kit (Qiagen, Valencia, Calif.), and the isolated DNA fragment was ligated into the SfiI AsiSI restriction site of the pAM36 vector, yielding expression plasmid pAM36-MevT66.

Expression plasmid pAM25 was generated by inserting the MevT66 operon into the pAM29 vector. The pAM29 vector was created by assembling the p15A origin of replication and kanamycin resistance conferring gene from pZS24-MCS1 (Lutz and Bujard (1997) *Nucl Acids Res.* 25:1203-1210) with an oligonucleotide-generated lacUV5 promoter. The DNA synthesis construct comprising the MevT66



MevT66, yielding expression plasmid pAM43. A DNA fragment comprising a nucleotide sequence encoding the lacUV5 promoter was synthesized from oligonucleotides, and sub-cloned into the AscI SfiI and AsiSI XhoI restriction sites of pAM43, yielding expression plasmid pAM45.

### Example 2

This example describes methods for making expression vectors encoding enzymes including enzymes of the MEV pathway from *Staphylococcus aureus* organized in operons.

Expression plasmid pAM41 was derived from expression plasmid pAM25 by replacing the coding sequence of the HMG1 gene, which encodes a truncated *Saccharomyces cerevisiae* HMG-CoA reductase, with the coding sequence of the *mvaA* gene, which encodes the *Staphylococcus aureus* HMG-CoA reductase (GenBank accession number BA000017, REGION: 2688925 . . . 2687648). The coding sequence of the *mvaA* gene was PCR amplified from *Staphylococcus aureus* subsp. *aureus* (ATCC 70069) genomic DNA using primers 4-49 *mvaA* SpeI (SEQ ID NO: 13) and 449 *mvaA*R XbaI (SEQ ID NO: 14), the amplified DNA fragment was digested to completion using SpeI restriction enzyme, the reaction mixture was resolved by gel electrophoresis, and the approximately 1.3 kb DNA fragment was gel extracted. The HMG1 coding sequence was removed from pAM25 by digesting the plasmid to completion using HindIII restriction enzyme. The terminal overhangs of the resulting linear DNA fragment were blunted using T4 DNA polymerase. The DNA fragment was then partially digested using SpeI restriction enzyme, the reaction mixture was resolved by gel electrophoresis, and the approximately 4.8 kb DNA fragment was gel extracted. The isolated DNA fragment was ligated with the SpeI-digested *mvaA* PCR product, yielding expression plasmid pAM41.

Expression plasmid pAM52 was derived from expression plasmid pAM41 by replacing the coding sequence of the ERG13 gene, which encodes the *Saccharomyces cerevisiae* HMG-CoA synthase, with the coding sequence of the *mvaS* gene, which encodes the *Staphylococcus aureus* HMG-CoA synthase (GenBank accession number BA000017, REGION: 2689180 . . . 2690346). The coding sequence of the *mvaS* gene was PCR amplified from *Staphylococcus aureus* subsp. *aureus* (ATCC 70069) genomic DNA using primers HMGS 5' Sa *mvaS*-S (SEQ ID NO: 15) and HMGS 3' Sa *mvaS*-AS (SEQ ID NO: 16), and the amplified DNA fragment was used as a PCR primer to replace the coding sequence of the HMG1 gene in pAM41 according to the method of Geiser et al. (BioTechniques 31:88-92 (2001)), yielding expression plasmid pAM52. The nucleotide sequence of the *atoB(opt):mvaS:mvaA* operon contained in pAM52 is SEQ ID NO: 2.

Expression plasmid pAM97 was derived from expression plasmid pAM45 by replacing the MevT66 operon with the *(atoB(opt):mvaS:mvaA)* operon of expression plasmid pAM52. Expression plasmid pAM45 was digested to completion using AsiSI and SfiI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, and the approximately 8.3 kb DNA fragment lacking the MevT66 operon was gel extracted. The *(atoB(opt):mvaS:mvaA)* operon of pAM52 was PCR amplified using primers 19-25 *atoB* SfiI-S (SEQ ID NO: 17) and 19-25 *mvaA*-AsiSI-AS (SEQ ID NO: 18), the PCR product was digested to completion using SfiI and AsiSI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 3.8 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the AsiSI SfiI restriction site of expression plasmid pAM45, yielding expression plasmid pAM97 (see FIG. 3 for a plasmid map).

### Example 3

This example describes methods for making expression plasmids that encode enzymes including enzymes of the DXP pathway from *Escherichia coli* organized in operons.













Expression plasmid pAM373 was generated by inserting a nucleotide sequence encoding the .beta.-farnesene synthase of *Artemisia annua* (GenBank accession number AY835398), codon-optimized for expression in *Escherichia coli*, into the pTrc99A vector. The nucleotide sequence encoding the .beta.-farnesene synthase was generated synthetically using as a template SEQ ID NO: 8, and was amplified by PCR from its DNA synthesis construct using primers Primer A (SEQ ID NO: 86) and Primer B (SEQ ID NO: 87). To create a leader NcoI restriction site in the PCR product comprising the .beta.-farnesene synthase coding sequence, the codon encoding the second amino acid in the original polypeptide sequence (TCG coding for serine) was replaced by a codon encoding aspartic acid (GAC) in the 5' PCR primer. The resulting PCR product was partially digested using NcoI restriction enzyme, and digested to completion using SacI restriction enzyme, the reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the .beta.-farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the NcoI SacI restriction site of the pTrc99A vector, yielding expression plasmid pAM373 (see FIG. 7 for a plasmid map).

Expression plasmid pAM342 was generated by inserting a nucleotide sequence encoding the .alpha.-farnesene synthase of *Picea abies* (GenBank accession number AY473627, REGION: 24 . . . 1766), codon-optimized for expression in *Escherichia coli*, into the pTrc99A vector. The nucleotide sequence encoding .alpha.-farnesene was generated synthetically, using as a template SEQ ID NO: 9, and was amplified by PCR from its DNA synthesis construct using primers Primer C (SEQ ID NO: 88) and Primer D (SEQ ID NO: 89). The resulting PCR product was digested to completion using NcoI and SacI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the .alpha.-farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the NcoI SacI restriction site of the pTrc99A vector, yielding expression plasmid pAM342 (see FIG. 7 for a plasmid map).

Expression plasmids pAM341 and pAM353 were generated by inserting a nucleotide sequence encoding an .alpha.-farnesene synthase or a .beta.-farnesene synthase, respectively, into the pRS425-Gal1 vector (Mumberg et. al. (1994) Nucl. Acids. Res. 22(25): 5767-5768). The nucleotide sequence inserts were generated synthetically, using as a template the coding sequence of the .alpha.-farnesene synthase gene of *Picea abies* (GenBank accession number AY473627, REGION: 24 . . . 1766) or of the .beta.-farnesene synthase gene of *Artemisia annua* (GenBank accession number AY835398), both sequences being codon-optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NOS: 11 and 10, respectively). The synthetically generated nucleotide sequences were flanked by 5' BamHI and 3' XhoI restriction sites, and could thus be cloned into compatible restriction sites of a cloning vector such as a standard pUC or pACYC origin vector. Each synthetically generated nucleotide sequence was isolated by digesting to completion the DNA synthesis construct using BamHI and XhoI restriction enzymes. The reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the .alpha.-farnesene synthase or .beta.-farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the BamHI XhoI restriction site of the pRS425-Gal1 vector, yielding expression plasmid pAM341 or pAM353, respectively.

Expression plasmid pAM404 was generated by inserting a nucleotide sequence encoding the .beta.-farnesene synthase of *Artemisia annua* (GenBank accession number AY835398), codon-optimized for expression in *Saccharomyces cerevisiae*, into vector pAM178. The nucleotide sequence encoding the .beta.-farnesene synthase was PCR amplified from pAM353 using primers GW-52-84 pAM326 BamHI (SEQ ID NO: 90) and GW-52-84 pAM326 NheI (SEQ ID NO: 91). The resulting PCR product was digested to completion using BamHI and NheI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 1.7 kb DNA fragment comprising the .beta.-farnesene synthase coding sequence was gel extracted, and the isolated DNA fragment was ligated into the BamHI NheI restriction site of vector pAM178, yielding expression plasmid pAM404 (see FIG. 8 for a plasmid map).

## Example 6

This example describes the generation of *Escherichia coli* host strains useful in the invention.

As detailed in Table 6, host strains were created by transforming chemically competent *Escherichia coli* parent cells with one or more expression plasmids of Examples 1 through 3 and Example 5.

TABLE-US-00006 TABLE 6 *Escherichia coli* host strains

Host	E. coli	Expression	Strain	Parent	Strain
Plasmids	Antibiotic	Selection	B526	DH1	pAM97
			100 ug/mL	carbenicillin	pAM373
			34 ug/mL	chloramphenicol	B552
			pMevT	100 ug/mL	carbenicillin
			pMBIS	34 ug/mL	chloramphenicol
			pAM373	5 ug/mL	tetracycline
			B592	pMevT	pMBIS
			pAM342	B650	DH10B
			pAM373	100 .mu.g/mL	carbenicillin
			B651	pAM408	100 .mu.g/mL
			carbenicillin	pAM373	50 .mu.g/mL
			kanamycin	B652	pAM424
			100 .mu.g/mL	carbenicillin	pAM373
			35 .mu.g/mL	chloramphenicol	B653
			pAM408	100 .mu.g/mL	carbenicillin
			pAM424	50 .mu.g/mL	kanamycin
			pAM373	35 .mu.g/mL	chloramphenicol

Host cell transformants were selected on Luria Bertoni (LB) agar containing antibiotics. Single colonies were transferred from LB agar to culture tubes containing 5 mL of LB liquid medium and antibiotics. B526, B552, and B592 host cell transformants were incubated at 37.degree. C. on a rotary shaker at 250 rpm until growth reached stationary phase. B650, B651, B652, and B653 host cell transformants were incubated at 30.degree. C. on a rotary shaker at 250 rpm for 30 hours. The cells were adapted to minimal media by passaging them through 4 to 5 successive rounds of M9-MOPS media containing 0.8% glucose and antibiotics (see Table 7 for the composition of the M9-MOPS medium). The cells were stored at -80.degree. C. in cryo-vials in 1 mL stock aliquots made up of 400 uL sterile 50% glycerol and 600 uL liquid culture.

TABLE-US-00007 TABLE 7 Composition of M9-MOPS Culture Medium

Component	Quantity (per L)
Na.sub.2HPO.sub.4	12.8 g
KH.sub.2PO.sub.4	3 g
NaCl	0.5 g
NH.sub.4Cl	1 g
MgSO.sub.4	2 mmol
CaCl.sub.2	0.1 mmol
Thiamine	0.1 ug
MOPS buffer	pH 7.4 100 mmol
(NH.sub.3).sub.6Mo7O.sub.24	3.7 ug
H.sub.3BO.sub.3	25 ug
CoCl.sub.2	7.1 ug
CuSO.sub.4	2.4 ug
MnCl.sub.2	16 ug
ZnSO.sub.4	2.9 ug
FeSO.sub.4	0.28 mg

## Example 7

This example describes the generation of *Saccharomyces cerevisiae* strains useful in the invention.

To prepare *Saccharomyces cerevisiae* strain Y141 and Y140, the expression plasmid from *Saccharomyces cerevisiae* strain EPY224 (Ro et al. (2006) *Nature* 440: 940-943; PCT Patent Publication WO2007/005604) was removed by culturing in rich medium, yielding strain EPY300. Strain EPY300 was then transformed with expression plasmids pAM341 or pAM353, yielding host strains Y141 or Y140, respectively. Host cell transformants were selected on synthetic defined media, containing 2% glucose and all amino acids except leucine (SM-glu). Single colonies were transferred to culture vials containing 5 mL of liquid SM-glu lacking leucine, and the cultures were incubated by shaking at 30.degree. C. until growth reached stationary phase. The cells were stored at -80.degree. C. in cryo-vials in 1 mL frozen aliquots made up of 400 uL 50% sterile glycerol and 600 uL liquid culture.

To prepare *Saccharomyces cerevisiae* strain Y258, *Saccharomyces cerevisiae* strains CEN.PK2-1C (Y002) (MATA; *ura3-52*; *trp1-289*; *leu2-3,112*; *his3.DELTA.1*; MAL2-8C; SUC2) and CEN.PK2-1D (Y003) (MATalpha; *ura3-52*; *trp1-289*; *leu2-3,112*; *his3.DELTA.1*; MAL2-8C; SUC2) (van Dijken et al. (2000) *Enzyme Microb. Technol* 26(9-10):706-714) were prepared for introduction of inducible MEV pathway genes by replacing the ERG9 promoter with the *Saccharomyces cerevisiae* MET3

promoter, and the ADE1 ORF with the *Candida glabrata* LEU2 gene (CgLEU2). This was done by PCR amplifying the KanMX-PMET3 region of vector pAM328 (SEQ ID NO: 12) using primers 50-56-pw100-G (SEQ ID NO: 93) and 50-56-pw101-G (SEQ ID NO: 94), which include 45 base pairs of homology to the native ERG9 promoter, transforming 10 ug of the resulting PCR product into exponentially growing Y002 and Y003 cells using 40% w/w Polyethelene Glycol 3350 (Sigma-Aldrich, St. Louis, Mo.), 100 mM Lithium Acetate (Sigma-Aldrich, St. Louis, Mo.), and 10 ug Salmon Sperm DNA (Invitrogen Corp., Carlsbad, Calif.), and incubating the cells at 30.degree. C. for 30 minutes followed by heat shocking them at 42.degree. C. for 30 minutes (Schiestl and Gietz. (1989) Curr. Genet. 16, 339-346). Positive recombinants were identified by their ability to grow on rich medium containing 0.5 ug/mL Geneticin (Invitrogen Corp., Carlsbad, Calif.), and selected colonies were confirmed by diagnostic PCR. The resultant clones were given the designation Y93 (MAT A) and Y94 (MAT alpha). The 3.5 kb CgLEU2 genomic locus was then amplified from *Candida glabrata* genomic DNA (ATCC, Manassas, Va.) using primers 61-67-CPK066-G (SEQ ID NO: 95) and 61-67-CPK067-G (SEQ ID NO: 96), which contain 50 base pairs of flanking homology to the ADE1 ORF, and 10 ug of the resulting PCR product were transformed into exponentially growing Y93 and Y94 cells, positive recombinants were selected for growth in the absence of leucine supplementation, and selected clones were confirmed by diagnostic PCR. The resultant clones were given the designation Y176 (MAT A) and Y177 (MAT alpha).

Strain Y188 was then generated by digesting 2 ug of pAM491 and pAM495 plasmid DNA to completion using PmeI restriction enzyme (New England Biolabs, Beverly, Mass.), and introducing the purified DNA inserts into exponentially growing Y176 cells. Positive recombinants were selected for by growth on medium lacking uracil and histidine, and integration into the correct genomic locus was confirmed by diagnostic PCR.

Strain Y189 was next generated by digesting 2 ug of pAM489 and pAM497 plasmid DNA to completion using PmeI restriction enzyme, and introducing the purified DNA inserts into exponentially growing Y177 cells. Positive recombinants were selected for by growth on medium lacking tryptophan and histidine, and integration into the correct genomic locus was confirmed by diagnostic PCR.

Strain Y238 was then generated by mixing approximately 1.times.10.sup.7 cells from strains Y188 and Y189 on a YPD medium plate for 6 hours at room temperature to allow for mating, and then plating the mixed cell culture to medium lacking histidine, uracil, and tryptophan to select for growth of diploid cells. The diploid cells were then transformed using 2 ug of pAM493 plasmid DNA that had been digested to completion using PmeI restriction enzyme, and introducing the purified DNA insert into exponentially growing diploid cells. Positive recombinants were selected for by growth on medium lacking adenine, and integration into the correct genomic locus was confirmed by diagnostic PCR.

Haploid strain Y211 (MAT alpha) was generated by sporulating strain Y238 in 2% Potassium Acetate and 0.02% Raffinose liquid medium, isolating approximately 200 geneti