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**Renninger , et al.** **July 15, 2008**

# Fuel compositions comprising farnesane and farnesane derivatives and method of making and using same

## Abstract

A fuel composition comprises farnesane and/or farnesane derivatives and a conventional fuel component selected from diesel fuel, jet fuel, kerosene or gasoline. The farnesane or farnesane derivative can be used as a fuel component or as a fuel additive in the fuel composition. The fuel composition may further comprise a conventional fuel additive. Methods of making and using the fuel composition are also disclosed.

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## Related U.S. Patent Documents

| Application Number | Filing Date | Patent Number | Issue Date |
|---|---|---|---|
| 60850881 | Oct., 2006 | | |
| 60860854 | Nov., 2006 | | |

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**Current International Class:** C10L 1/18 (20060101)
**Field of Search:** 44/385,388,447,451

## References Cited [Referenced By]

### U.S. Patent Documents

| | | |
|---|---|---|
| 5646098 | July 1997 | Brois |
| 6043200 | March 2000 | Carroll et al. |
| 2006/0206957 | 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*

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.

## *Claims*

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

heterogeneous mixture of products that may vary from batch to batch. This product variability can complicate making a fuel with defined specifications or requirements. As a result, there are needs for fuel additives and fuel components for making fuel compositions and needs for fuel components which can be made reliably and reproducibly for use in internal combustion engines such as diesel engines and jet engines.

## SUMMARY OF THE INVENTION

Provided herein are fuel compositions, fuel components or fuel additives comprising isoprenoids or their derivatives and methods of making and using same. Embodiments of these compositions are believed to satisfy the above-mentioned needs. More specifically, isoprenoids and their derivatives can be used as fuel components in the fuel compositions. In certain embodiments, the isoprenoid or their derivatives can be used as the fuel composition itself, a major component of the fuel composition or a minor component of the fuel composition. Isoprenoids and their derivatives can be made from microorganisms, including bioengineered microorganisms. Fuel compositions disclosed herein can be used as a fuel for internal combustion engines such as gasoline engines, diesel engines, and jet engines.

In certain embodiments, the present invention encompasses a diesel fuel comprising one or more bioengineered fuel components. In certain embodiments, the present invention encompasses a jet fuel comprising one or more bioengineered fuel components. In these embodiments, the bioengineered fuel component can be produced by any microorganism capable of producing the bioengineered fuel component, such as a genetically engineered microorganism, a wild type microorganism, or a selected strain thereof. In certain embodiments, the bioengineered fuel component is an isoprenoid or a derivative thereof disclosed herein.

In certain embodiments, the bioengineered fuel component can be obtained from a readily available, renewable material. Remarkably, the present invention thus provides readily available, renewable sources of energy and methods of their use for the production of energy. In certain embodiments, the bioengineered fuel component can be obtained from a sugar such as a monosaccharide (simple sugar) or a disaccharide.

In certain other embodiments, the bioengineered fuel component can be obtained from a readily available non-fermentable carbon source such as acetate or glycerol.

## DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of the mevalonate ("MEV") pathway for the production of isopentenyl diphosphate ("IPP").

FIG. 2 is a schematic representation of the DXP pathway for the production of IPP and dimethylallyl pyrophosphate ("DMAPP"). Dxs is 1-deoxy-D-xylulose-5-phosphate synthase; Dxr is 1-deoxy-D-xylulose-5-phosphate reductoisomerase (also known as IspC); IspD is 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; IspE is 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; IspF is 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG is 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (IspG); and ispH is isopentenyl/dimethylallyl diphosphate synthase.

FIG. 3 shows a map of expression plasmid pAM97.

FIG. 4 shows a map of expression plasmid pAM408.

FIG. 5 shows a map of expression plasmid pAM424.

FIG. 6A-E show maps of the ERG20-P.sub.GAL-tHMGR insert of vector pAM489; the ERG13-P.sub.GAL-tHMGR insert of vector pAM491; the IDI1-P.sub.GAL-tHMGR insert of vector pAM493; the ERG10-P.sub.GAL-ERG12 insert of vector pAM495; and the ERG8-P.sub.GAL-ERG19 insert of vector pAM497.

FIG. 7 shows a map of expression plasmids pAM373 and pAM342.

FIG. 8 shows a map of expression plasmid pAM404.

FIG. 9 shows the ASTM D 975 testing data for No. 2 diesel from the BP Whiting Refinery and 5%, 20%, and 50% blends of farnesane (AMD-200) with this fuel.

FIG. 10 shows the ASTM D 975 testing data for a diesel fuel from the BP Carson Refinery that meets the California Air Resources Board requirements (CARB fuel) and 5%, 20%, 50%, and 65% blends of farnesane (AMD-200 with this fuel). This particular sample of CARB fuel does not contain lubricity enhancers that are typically found in CARB fuel.

FIG. 11A-B show the distillation profiles of No. 2 diesel and CARB diesel blended with various amounts of farnesane (AMD-200).

DEFINITIONS

The ASTM D 975 specifications, published by ASTM International, set certain minimum acceptance requirements for the different grades of diesel fuels used in the United States. For example, ultra low sulfur diesel fuel Grade No. 2-D is expected to have a maximum sulfur content of 0.05% by weight (under an ASTM D 2622 test), a maximum ash content of 0.01% by weight (under an ASTM D 482 test), a minimum cetane number of 40 (under an ASTM D 6079 test), a viscosity at 40.degree. C. of from 1.9 cSt to 2.4 cSt (under an ASTM D 445 test), and a minimum flash point of 52.degree. C. Japan and Europe have similar diesel fuel specifications to those of the United States for comparable grades of diesel fuels. For example, Japan's JIS K 2204, Grade No. 2 diesel fuel is expected to have a minimum viscosity at 40.degree. C. of 2.0 cSt, a maximum sulfur content of 0.05% by weight, and a minimum cetane number of 45. By comparison, Europe's CEN 590, Grade A-F diesel fuel is expected to have a viscosity at 40.degree. C. of from 2.0 cSt to 4.5 cSt, a maximum sulfur content of 0.05% by weight, and a minimum cetane number of 49. In some embodiments, the fuel composition disclosed herein meets at least one or all of the above properties.

The ASTM D 1655 specifications, published by ASTM International, set certain minimum acceptance requirements for Jet A.

"Ash content" refers to the amount of residue remaining after the diesel fuel is allowed to burn under conditions described by ASTM D 482.

"Biodiesel" refers to the variety of diesel fuels derived from biological sources, such as vegetable oils or animal fats. Biodiesel is mainly a mixture of alkyl esters, including fatty acid methyl esters, derived from the transesterification of a mixture of the oils and methanol. Although soybean oil is the largest source of biodiesel, oils from other plants or animal fats also can be the source materials.

"Bioengineered fuel component" refers to a fuel component made at least in part by a host cell, including any archae, bacterial, or eukaryotic cell.

"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.sup.L, and an upper limit, R.sup.U, 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

disclosed.

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

Embodiments of the invention provide fuel compositions comprising one or more C.sub.15 isoprenoid compounds as a major or minor fuel component. Any C.sub.15 isoprenoid compound can be used herein. In some embodiments, each of the C.sub.15 isoprenoid compounds can have one of the formulae:

##STR00004## wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl. In some embodiments, Z is O--R or O--C(.dbd.O)R; and R is C.sub.1-C.sub.6 alkyl. In other embodiments, Z is O--R or O--C(.dbd.O)R wherein R is methyl. In other embodiments, Z is O--R or O--C(.dbd.O)R wherein R is ethyl. In still other embodiments, the C.sub.15 isoprenoid compound is farnesane, i.e., Z of formula (I) or (II) is H.

In one set of embodiments, the isoprenoid compound is:

##STR00005## wherein Z is as defined above.

In another set of embodiments, the isoprenoid compound is:

##STR00006## wherein Z is as defined above.

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

##STR00007## wherein Z is as defined above. Formulae (I-a), (I-b), (I-c), and (I-d) are the four possible stereoisomers of formula (I), and Formulae (II-a), (II-b), (II-c), and (II-d) are the four possible stereoisomers of formula (II).

In another set of embodiments, the isoprenoid compound is

##STR00008## or a stereoisomer thereof.

In another set of embodiments, the isoprenoid compound is

##STR00009## or a stereoisomer thereof, wherein R is as previously defined. In another set of embodiments, R is C.sub.1-C.sub.3 alkyl. In another set of embodiment, R is methyl. In yet another set of embodiment, R is ethyl.

In another set of embodiments, the isoprenoid compound is

##STR00010## or a stereoisomer thereof, wherein R is as previously defined. In another set of embodiments, R is C.sub.1-C.sub.3 alkyl. In another set of embodiments, R is methyl. In yet another set of embodiments, R is ethyl.

In another set of embodiments, the isoprenoid compound has a formula:

##STR00011## wherein R is alkyl such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, tert-butyl, and linear or branched pentyl, hexyl, heptyl, octyl, nonyl, decyl, dodecyl, tetradecyl, hexadecyl, octadecyl, eicosyl, docosyl and the like. In other embodiments, the isoprenoid compound comprises a

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.

In other embodiments, the fuel compositions may further comprise a conventional fuel component derived from petroleum, coal, wood, or any other hydrocarbon source. Illustrative examples of conventional fuel components include diesel fuels, jet fuels, kerosene, gasoline, and Fischer-Tropsch derived fuels. In some embodiments, the conventional fuel component is derived from petroleum or coal. In certain embodiments, the fuel component is or comprises a diesel fuel, jet fuel, kerosene, gasoline, or a combination thereof. In other embodiments, the fuel component is or comprises a distillate diesel fuel. In further embodiments, the amount of the fuel component is at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90%, based on the total weight or volume of the fuel composition. In still further embodiments, the amount of the fuel component is at most 10%, at most 20%, at most 30%, at most 40%, at most 50%, at most 60%, at most 70%, at most 80%, or at most 90%, based on the total weight or volume of the fuel composition.

In some embodiments, the fuel compositions may further comprise a conventional fuel additive. The nature and amount of the one or more additives depend on the desired use of the final fuel composition.

In certain embodiments, the fuel composition is intended for use in diesel engines. The American Society for Testing and Materials (ASTM) categorizes diesel fuels into three general groups. The need to categorize these fuels results from the varied uses of diesel engines, which are designed to operate efficiently on one of the standard diesel fuels.

No. 1-D is a light distillate, similar to kerosine, for engines where frequent load changes and speed changes (e.g., truck, tractor engines) are essential. This fuel has a flash point greater than 38.degree. C. and a minimum cetane number of 40. This fuel is particularly suitable for cold-weather operation.

No. 2-D is a medium distillate fuel with a lower volatility and higher density than No. 1-D. This fuel finds use in heavier-duty engines, for example, railroad engines, which operate at uniform speeds but with heavier loads than encountered during the use of No. 1-D. The flash point is greater than 52.degree. C. and the minimum cetane number is 40.

No. 4-D is a heavy distillate fuel with the highest density and lowest volatility of the three diesel fuels. It finds use in low- and medium-speed engines such as marine engines and electric power generation engines, which operate under sustained loads. The flash point is greater than 55.degree. C. and the minimum cetane rating is 30.

The premium grade diesel fuels are those that meet or exceed either the National Conference on Weights and Measures (NCWM) or the Engine Manufacturers Association (EMA) premium diesel definition.

Generally, a diesel fuel is a complex mixture of thousands of individual compounds. Most of these compounds are C.sub.10-C.sub.22 hydrocarbons and are generally parrafins, naphthenes (cycloparaffins) and aromatics. Normal paraffins refer to alkanes (which are composed of hydrogen and carbon) with a straight carbon chain.

Diesel fuel generally has a distillation range from 390 to 715.degree. F. (from 200 to 380.degree. C.) at 1 atmospheric pressure and a specific gravity range from 0.760 to 0.935. In addition to these properties, diesel fuel should have <1 wt. % of sulfur, <0.1 wt. % of ash, <0.5 vol. % of water and sediment, and a flash point greater than 55.degree. C.

Diesel fuel quality can be characterized by the cetane number, which usually falls into the range from 30 to 60. A high cetane number indicates the potential for easy starting and smooth operation of the engine. The cetane number is the analog of the automobile engine octane number, with cetane (n-hexadecane, C.sub.16H.sub.34) having the arbitrarily assigned number of 100. At the other end of the scale,

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 naptha-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

and differ only by the amounts of a few additives. For example, Jet A-1 is similar to JP-8 and Jet B is similar to JP-4. Alternatively, jet fuels can also be classified as kerosene or naphtha-type. Some non-limiting examples of kerosene-type jet fuels include Jet A, Jet A1, JP-5, and JP-8. Some non-limiting examples of naphtha-type jets fuels include Jet B and JP4. In other embodiments, the fuel composition does not comprise Jet B according to ASTM Specification D 1655-68 when the fuel composition comprises formula (III) or formula (I) or (II) wherein Z is H.

Jet A is the standard jet fuel type in the U.S. used since the 1950s. Jet A is similar to Jet-A1, except for its higher freezing point of -40.degree. C. Like Jet A-1, Jet A has a fairly high flash point of minimum 38.degree. C., with an autoignition temperature of 210.degree. C.

In certain embodiments, the fuel composition comprises at least a conventional fuel additive. Some non-limiting examples of conventional fuel additives include 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 total amount of the fuel additives in the fuel composition may range from 0.001 to 10 wt %, based on the total weight of the fuel composition, and in one embodiment from 0.01 to 5 wt %.

Some conventional fuel additives have been described in Chunsham Song et al., "Chemistry of Diesel Fuel," Taylor & Francis, London, Chapter 1, pp. 32-36 (2000), which is incorporated herein by reference. Further, the following U.S. patents disclose various additives that can be employed in embodiments of the invention as additives: U.S. Pat. Nos. 6,054,420; 6,051,039; 5,997,593; 5,997,592; 5,993,498; 5,968,211; 5,958,089; 5,931,977; 5,891,203; 5,882,364; 5,880,075; 5,880,072; 5,855,629; 5,853,436; 5,743,922; 5,630,852; 5,529,706; 5,505,867; 5,492,544; 5,490,864; 5,484,462; 5,321,172; and 5,284,492. The disclosures of all of the preceding U.S. patents are incorporated by reference herein in their entirety.

In certain other embodiments, the fuel composition includes a fuel additive that is a lubricity improver or enhancer. In some embodiments, one or more lubricity improvers are mixed with the diesel fuel. Typically, the concentration of the lubricity improver in the fuel falls in the range of from about 1 ppm to about 50,000 ppm, from about 10 ppm to about 20,000 ppm, from about 25 ppm to 10,000 ppm, or from about 50 ppm and 1000 ppm, based on the total weight of the fuel composition. Some non-limiting examples of suitable lubricity improvers include esters of fatty acids such as glycerol monooleate and di-isodecyl adipate; amide-based additives such as those available from the Lubrizol Chemical Company (e.g., LZ 539 C); dimerised linoleic acid; aminoalkylmorpholines; dithiophosphoric diester-dialcohols; and alkyl aromatic compounds having at least one carboxyl group. Some suitable lubricity improvers or enhancers are described in patent literature such as WO 95/33805; WO 94/17160; WO 98/01516; and U.S. Pat. Nos. 5,484,462 and 5,490,864; and in the paper by Danping Wei and H. A. Spikes, "The Lubricity of Diesel Fuels", Wear, III (1986) 217 235, all of which are incorporated herein by reference. Some non-limiting examples of commercially available lubricity improvers include OLI 9000 (from Octel Corporation, Manchester, UK), PARADYNE.TM. 655 and VEKTRON.TM. 6010 (from Infineum, Linden, N.J.), and HITEC.TM. E580 (from Ethyl Corporation, Richmond, Va.).

In certain other embodiments, the fuel composition includes a fuel additive that is a detergent. Generally, the amount of the detergent additive is less than 10,000 ppm, less than 1000 ppm, less than 100 ppm, or less than 10 ppm, based on the total weight of the fuel composition. Some non-limiting examples of suitable detergents include polyolefin substituted succinimides or succinamides of polyamines, for instance polyisobutylene succinimides or polyisobutylene amine succinamides, aliphatic amines, Mannich bases or amines, and polyolefin (e.g. polyisobutylene) maleic anhydrides. Some

suitable succinimide detergents are described in GB960493, EP0147240, EP0482253, EP0613938, EP0557561, and WO 98/42808, all of which are incorporated herein by reference. In some embodiments, the detergent is a polyolefin substituted succinimide such as polyisobutylene succinimide. Some non-limiting examples of commercially available detergent additives include F7661 and F7685 (from Infineum, Linden, N.J.) and OMA 4130D (from Octel Corporation, Manchester, UK).

In certain other embodiments, the fuel composition includes a fuel additive that is a cetane improver. Some non-limiting examples of cetane improvers include peroxides, nitrates, nitrites, azo compounds and the like. Alkyl nitrates such as amyl nitrate, hexyl nitrate and mixed octyl nitrates, 2-methyl-2-nitropropyl nitrate, and 2-ethylhexyl nitrate can be used. In some embodiments, the cetane improver is 2-ethylhexyl nitrate which is commercially available from the Associated Octel Company Limited under the brand name Cl-0801. The cetane improver may be present in the fuel composition at a concentration of about 0.001 to 5 wt %, based on the total weight of the fuel composition, and in one embodiment from 0.01 to 2.5 wt %.

In certain other embodiments, the fuel composition includes a fuel additive that is a stabilizer. Some non-limiting examples of stabilizers include tertiary alkyl primary amines. Many stabilizers also act as corrosion inhibitors. The stabilizer may be present in the fuel composition at a concentration of about 0.001 to 2 wt %, based on the total weight of the fuel composition, and in one embodiment from 0.01 to 1% by weight.

In certain other embodiments, the fuel composition includes a fuel additive that is a combustion improver. Some non-limiting examples of combustion improvers include ferrocene (dicyclopentadienyl iron), iron-based combustion improvers (e.g., TURBOTEC.TM. ER-18 from Turbotect (USA) Inc., Tomball, Tex.), ***barium***-based combustion improvers, cerium-based combustion improvers, and iron and magnesium-based combustion improvers (e.g., TURBOTECT.TM. 703 from Turbotect (USA) Inc., Tomball, Tex.). The combustion improver may be present in the fuel composition at a concentration of about 0.001 to 1 wt %, based on the total weight of the fuel composition, and in one embodiment from 0.01 to 1% by weight.

In another aspect, a fuel composition is provided comprising: (a) an isoprenoid compound having the formula

##STR00014## (b) a conventional fuel component; and, (c) a fuel additive wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl; the amount of the isoprenoid compound is at least about 1 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 has 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 at least 2 vol. %, 3 vol. %, or 4 vol. %, based on the total volume of the fuel composition. In other embodiments, the amount of the isoprenoid compound is from about 1 vol. % to about 90 vol. %, from about 2 vol. % to about 90 vol. %, from about 3 vol. % to about 90 vol. %, or from about 4 vol. % to about 90 vol. %, based on the total volume of the fuel composition.

In another aspect, a fuel composition is provided comprising: (a) an isoprenoid compound having the formula

##STR00015## (b) a conventional fuel component; and, (c) a fuel additive wherein Z is H, O--R, or O--C(.dbd.O)R; and R is H, alkyl, cycloalkyl, aryl, alkaryl, or aralkyl; the amount of the isoprenoid

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

175.degree. C. to about 375.degree. C., from about 200.degree. C. to about 350.degree. C., from about 225.degree. C. to about 325.degree. C., or from about 250.degree. C. to about 300.degree. C.

In other embodiments, a fuel composition disclosed herein has a T10 distillation temperature from about 150.degree. C. to about 350.degree. C., from about 175.degree. C. to about 325.degree. C., from about 200.degree. C. to about 300.degree. C., or from about 225.degree. C. to about 275.degree. C.

In some embodiments, a fuel composition disclosed herein has a cetane number of at least about 40, at least about 45, at least about 50, at least about 55, at least about 60, or at least about 65. In further embodiments, the fuel composition has a cetane number of at least about 70. In certain embodiments, the fuel composition has a cetane number from 40 to 90, from 45 to 80, or from 50 to 70.

In some embodiments, a fuel composition disclosed herein has a cloud point that is equal to or less than 0.degree. C. In another set of embodiments, the fuel composition has a cloud point that is equal to or less than -5.degree. C. In another set of embodiments, the fuel composition has a cloud point that is equal to or less than -10.degree. C. In another set of embodiments, the fuel composition has a cloud point that is equal to or less than -15.degree. C. In another set of embodiments, the fuel composition has a cloud point that is equal to or less than -20.degree. C. In another set of embodiments, the fuel composition has a cloud point that is equal to or less than -25.degree. C.

In some embodiments, a fuel composition disclosed herein has a low sulfur content. In other embodiments, the sulfur content of the fuel composition is less than 500 ppm, based on the total weight of the fuel composition. In further embodiments, the sulfur content is less than 250 ppm, less than 150 ppm, less than 100 ppm, less than 50 ppm, less than 25 ppm, less than 20 ppm, less than 10 ppm, or less than 5 ppm, based on the total weight of the fuel composition. In certain embodiments, the fuel composition has no measurable sulfur content.

In some embodiments, the fuel compositions disclosed herein meet the ASTM D 975 specification for No. 2 Diesel.

In another aspect, a fuel composition is provided comprising: (a) C.sub.20 hydrocarbons in an amount at least about 1 vol. %; and (b) an isoprenoid compound of the formula

##STR00016## in an amount at least about 1 vol. % wherein each amount is based on the total volume of the fuel composition and Z is H, O--R, or O--C(.dbd.O)R; and R is H or C.sub.1-C.sub.6 alkyl. In some embodiments, the isoprenoid compound is in an amount at least about 2 vol. %, 3 vol. %, or 4 vol. %. In some embodiments, the fuel composition further comprises (c) C.sub.10 hydrocarbons in an amount at least about 1 vol. % based on the total volume of the fuel composition.

In another aspect, a fuel composition is provided comprising: (a) C.sub.20 hydrocarbons in an amount at least about 1 vol. %; and (b) an isoprenoid compound of the formula

##STR00017## in an amount at least about 5 vol. % wherein each amount is based on the total volume of the fuel composition and Z is H, O--R, or O--C(.dbd.O)R; and R is H or C.sub.1-C.sub.6 alkyl. In some embodiments, the fuel composition further comprises (c) C.sub.10 hydrocarbons in an amount at least about 1 vol. % based on the total volume of the fuel composition.

In some embodiments, the amount of the C.sub.10 hydrocarbons is at least about 2 vol. %, 3 vol. %, 4 vol. %, or 5 vol. %. In other embodiments, the amount of the C.sub.20 hydrocarbons is at least about 2 vol. %, 3 vol. %, 4 vol. %, or 5 vol. %.

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, Archaeglobus, 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, Strepromyces, 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, Rodobacter 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, Cryotococcus, 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

griseochromogenes, Streptomyces griseus, Streptomyces lividans, Streptomyces olivogriseus, Streptomyces rameus, Streptomyces tanashiensis, Streptomyces vinaceus, and Trichoderma reesei.

In general, if a eukaryotic cell is used, a non-pathogenic strain is preferred. Some non-limiting examples of non-pathogenic strains include Fusarium graminearum, Fusarium venenatum, Pichia pastoris, Saccaromyces boulardi, and Saccaromyces cerevisiae.

In addition, certain strains have been designated by the Food and Drug Administration as GRAS or Generally Regarded As Safe. Some non-limiting examples of these strains include Bacillus subtilis, Lactibacillus acidophilus, Lactobacillus helveticus, and Saccharomyces cerevisiae.

IPP Pathways

There are two known biosynthetic pathways that synthesize IPP and its isomer, dimethylallyl pyrophosphate ("DMAPP"). Eukaryotes other than plants use the mevalonate-dependent ("MEV") isoprenoid pathway exclusively to convert acetyl-coenzyme A ("acetyl-CoA") to IPP, which is subsequently isomerized to DMAPP. Prokaryotes, with some exceptions, use the mevalonate-independent or deoxyxylulose 5-phosphate ("DXP") pathway to produce IPP and DMAPP separately through a branch point. In general, plants use both the MEV and DXP pathways for IPP synthesis.

MEV Pathway

A schematic representation of the MEV pathway is shown in FIG. 1. In general, the pathway comprises six steps.

In the first step, two molecules of acetyl-coenzyme A are enzymatically combined to form acetoacetyl-CoA. An enzyme known to catalyze this step is, for example, acetyl-CoA thiolase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include the following GenBank accession numbers and the organism from which the sequences are derived: (NC.sub.--000913 REGION: 2324131 . . . 2325315; Escherichia coli), (D49362; Paracoccus denitrificans), and (L20428; Saccharomyces cerevisiae).

In the second step of the MEV pathway, acetoacetyl-CoA is enzymatically condensed with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). An enzyme known to catalyze this step is, for example, HMG-CoA synthase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include (NC.sub.--001145. complement 19061 . . . 20536; Saccharomyces cerevisiae), (X96617; Saccharomyces cerevisiae), (X83882; Arabidopsis thaliana), (AB037907; Kitasatospora griseola), (BT007302; Homo sapiens), and (NC.sub.--002758, Locus tag SAV2546, GeneID 1122571; Staphylococcus aureus).

In the third step, HMG-CoA is enzymatically converted to mevalonate. An enzyme known to catalyze this step is, for example, HMG-CoA reductase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include (NM.sub.--206548; Drosophila melanogaster), (NC.sub.--002758, Locus tag SAV2545, GeneID 1122570; Staphylococcus aureus), (NM.sub.--204485; Gallus gallus), (AB015627; Streptomyces sp. KO 3988), (AF542543; Nicotiana attenuata), (AB037907; Kitasatospora griseola), (AX128213, providing the sequence encoding a truncated HMGR; Saccharomyces cerevisiae), and (NC.sub.--001145: complement (115734 . . . 118898; Saccharomyces cerevisiae).

In the fourth step, mevalonate is enzymatically phosphorylated to form mevalonate 5-phosphate. An enzyme known to catalyze this step is, for example, mevalonate kinase. Some non-limiting examples of nucleotide sequences encoding such an enzyme include (L77688; Arabidopsis thaliana) and (X55875;

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 Temeculal), 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).

In the fifth step, 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate is converted to 2C-methyl-D-erythritol 2,4-cyclodiphosphate. An enzyme known to catalyze this step is, for example, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AF230738; Escherichia coli), (NC.sub.--007493, locus_tag RSP.sub.--6071; Rhodobacter sphaeroides 2.4.1), and (NC.sub.--002947, locus_tag PP1618; Pseudomonas putida KT2440).

In the sixth step, 2C-methyl-D-erythritol 2,4-cyclodiphosphate is converted to 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate. An enzyme known to catalyze this step is, for example, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AY033515; Escherichia coli), (NC.sub.--002947, locus_tag PP0853; Pseudomonas putida KT2440), and (NC.sub.--007493, locus_tag RSP.sub.--2982; Rhodobacter sphaeroides 2.4.1).

In the seventh step, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate is converted to either IPP or its isomer, DMAPP. An enzyme known to catalyze this step is, for example, isopentyl/dimethylallyl diphosphate synthase. Some non-limiting examples of nucleotide sequences that encode such an enzyme include (AY062212; Escherichia coli) and (NC.sub.--002947, locus_tag PP0606; Pseudomonas putida KT2440).

In some embodiments, "cross talk" (or interference) between the host cell's own metabolic processes and those processes involved with the production of IPP as provided by the present invention are minimized or eliminated entirely. For example, cross talk is minimized or eliminated entirely when the host microorganism relies exclusively on the DXP pathway for synthesizing IPP, and a MEV pathway is introduced to provide additional IPP. Such a host organisms would not be equipped to alter the expression of the MEV pathway enzymes or process the intermediates associated with the MEV pathway. Organisms that rely exclusively or predominately on the DXP pathway include, for example, Escherichia coli.

In some embodiments, the host cell produces IPP via the MEV pathway, either exclusively or in combination with the DXP pathway. In other embodiments, a host's DXP pathway is functionally disabled so that the host cell produces IPP exclusively through a heterologously introduced MEV pathway. The DXP pathway can be functionally disabled by disabling gene expression or inactivating the function of one or more of the DXP pathway enzymes.

C.sub.15 Isoprenoid Starting Material

Like IPP, farnesyl pyrophosphate ("FPP") also can be made biologically. In general, two molecules of IPP and one molecule of DMAPP are condensed to form FPP. In some embodiments, the reaction can be catalyzed by an enzyme known to catalyze this step, for example, farnesyl pyrophosphate synthase.

Some non-limiting examples of nucleotide sequences that encode a farnesyl pyrophosphate synthase include (ATU80605; Arabidopsis thaliana), (ATHFPS2R; Arabidopsis thaliana), (AAU36376; Artemisia annua), (AF461050; Bos taurus), (D00694; Escherichia coli K-12), (AE009951, Locus AAL95523; Fusobacterium nucleatum subsp. nucleatum ATCC 25586), (GFFPPSGEN; Gibberella fujikuroi), (CP000009, Locus AAW60034; Gluconobacter oxydans 621H), (AF019892; Helianthus annuus), (HUMFAPS; Homo sapiens), (KLPFPSQCR; Kluyveromyces lactis), (LAU15777; Lupinus albus), (LAU20771; Lupinus albus), (AF309508; Mus musculus), (NCFPPSGEN; Neurospora crassa), (PAFPSI; Parthenium argentatum), (PAPFS2; Parthenium argentatum), (RATFAPS; Rattus norvegicus), (YSCFPP; Saccharomyces cerevisiae), (D89104; Schizosaccharomyces pombe), (CP000003, Locus

AAT87386; Streptococcus pyogenes), (CP000017, Locus AAZ51849; Streptococcus pyogenes), (NC.sub.--008022, Locus YP.sub.--598856; Streptococcus pyogenes MGAS10270), (NC.sub.--008023, Locus YP.sub.--600845; Streptococcus pyogenes MGAS2096), (NC.sub.--008024, Locus YP.sub.--602832; Streptococcus pyogenes MGAS10750), and (MZEFPS; Zea mays).

Methods for the biological production of both IPP and FPP have been previously described by references including WO 2006/014837 and U.S. Publication Nos. 2003/0148479, 2004/0005678, and, 2006/0079476. Examples 1 and 2 also provide embodiments for making these compounds.

FPP can be subsequently converted to a variety of C.sub.15 isoprenoids. In general, acyclic (branched or linear) and cyclic (with or without side chain) C.sub.15 isoprenoids can be used as starting materials. However, acyclic C.sub.15 isoprenoids require fewer chemical steps to produce the desired compounds for the practice of the invention. Some non-limiting examples of suitable C.sub.15 isoprenoid starting materials include but are not limited to:

##STR00027## .alpha.-Farnesene

.alpha.-Farnesene, whose structure is

##STR00028## is found in various biological sources including, but not limited to, the Dufour's gland in ants and in the coating of apple and pear peels. Biochemically, .alpha.-farnesene is made from FPP by .alpha.-farnesene synthase. Some non-limiting examples of suitable nucleotide sequences that encode such an enzyme include (DQ309034; Pyrus communis cultivar d'Anjou) and (AY182241; Malus domestica). See Pechouus et al., Planta 219(1):84-94 (2004). .beta.-Farnesene

.beta.-Farnesene, whose structure is

##STR00029## is found in various biological sources including, but not limited to, aphids and essential oils such as peppermint oil. In some plants such as wild potato, .beta.-farnesene is synthesized as a natural insect repellent. Biochemically, .beta.-farnesene is made from FPP by .beta.-farnesene synthase. Some non-limiting examples of suitable nucleotide sequences that encode such an enzyme include (AF024615; Mentha.times.piperita) and (AY835398; Artemisia annua). See Picaud et al., Phytochemistry 66(9): 961-967 (2005). Farnesol

Farnesol, whose structure is

##STR00030## is found in various biological sources including insects and essential oils from cintronella, neroli, cyclamen, lemon grass, tuberose, and rose. Biochemically, farnesol is made from FPP by a hydroxylase such as farnesol synthase. Some non-limiting examples of suitable nucleotide sequences that encode such an enzyme include (AF529266; Zea mays) and (YDR481c; Saccharomyces cerevisiae). See Song, L., Applied Biochemistry and Biotechnology 128:149-158 (2006). Nerolidol

Nerolidol, whose structure is

##STR00031## is also known as peruviol which is found in various biological sources including essential oils from neroli, ginger, jasmine, lavender, tea tree, and lemon grass. Biochemically, nerolidol is made from FPP by a hydroxylase such as nerolidol synthase. A non-limiting example of a suitable nucleotide sequence that encodes such an enzyme includes AF529266 from Zea mays (maize; gene tps1).

In some embodiments, the isoprenoid starting materials can be obtained or prepared from naturally

occurring terpenes that can be produced by a wide variety of plants, such as Copaifera langsdorfii, conifers, and spurges; 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 spurges. 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. Spurges, also known as Euphorbia, are a very diverse worldwide genus of plants, belonging to the spurge family (Euphorbiaceae). Consisting of about 2160 species, spurges 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 cintronella, enroli, 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-methyllumiflavinium 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

corresponding C--C bonds by hydrogenation in the presence of a catalyst and hydrogen at room temperature. In a further embodiment, the catalyst is a 10% Pd/C as shown in Scheme 1 below.

##STR00033##

The fully saturated C.sub.15 alcohols obtained according to Scheme 1 above can be further modified to produce the corresponding saturated C.sub.15 esters by any known esterification agent such as carboxylic acids, carboxylic acid halides (e.g., fluoride, chloride, bromide, and iodide), and carboxylic acid anhydrides. The esterification reactions can be carried out in any reaction conditions recognized by skilled artisans. In some embodiments, the C.sub.15 alcohol starting materials are esterified by reacting with the desired carboxylic acid in the presence of an acid or a base catalyst, or using either Fischer or Steglich esterification conditions. In other embodiments, the C.sub.15 alcohol starting materials are esterified by reacting with the desired carboxylic acid halides in the presence or absence of a base catalyst such as an amine or pyridine compound. In other embodiments, the C.sub.15 alcohol starting materials are esterified by reacting with the desired carboxylic acid anhydrides in the presence of a base catalyst such as an amine compound (e.g., triethylamine), as depicted in Scheme 2 below. The completed reaction mixture can be concentrated, washed, and dried to produce the corresponding ester.

##STR00034##

Alternatively, the saturated C.sub.15 esters can be obtained from the saturated C.sub.15 alcohols and a desired ester via a trans-esterification reaction as shown in Scheme 3 below. The trans-esterification reaction can be carried out in any reaction conditions recognized by skilled artisans. In some embodiments, the trans-esterification reaction is catalyzed by a base catalyst such as alkali (e.g., Li, Na, K, Rb, and Cs) or alkaline (e.g., Mg, Ca, Sr, and Ba) hydroxide, carbonate or acetate, or a combination thereof.

##STR00035##

In some embodiments, the fully saturated C.sub.15 alcohols can be further modified to produce the corresponding ether by any known alkylating agent such as R--X wherein R is alkyl and X is a good leaving group such as halo, sulfonyl, sulfate group and the like. Some non-limiting examples of the alkylating agent include alkyl halides, alkyl sulfonates, and alkyl sulfates. In general, the C.sub.15 alcohols may be converted to C.sub.15 alkoxides first by a base and then the C.sub.15 alkoxides subsequently may be reacted with R--X where X is Cl, Br, or I to form the corresponding ethers as shown in Scheme 4 below. In some embodiments, the base can be an active metal such as metallic sodium or a metal hydride such as sodium hydride, lithium aluminum hydride, and sodium borohydride.

##STR00036##

Alternatively, C.sub.15 olefinic alcohols can be first alkylated or esterified as described above and then subsequently hydrogenated, as depicted in Scheme 5 below where R' is R or C(.dbd.O)R and R is H or alkyl.

##STR00037##

Referring to Scheme 6 below, the esterification can be carried out in the same manner as described above. The subsequent hydrogenation can be carried out in the same manner as described above. Alternatively, the subsequent hydrogenation of the double bonds can be done selectively by using any hydrogenation catalyst that will not affect the --O--C(.dbd.O)R group. In some embodiments, the hydrogenation catalyst is Pd/C using diphenylsulfide as a catalyst poison which selectively reduces

olefin functionalities without hydrogenolysis of the O--C(.dbd.O)R group, as disclosed in Mori et al., Org. Lett, 8, 3279-3281 (2006), which is incorporated herein by reference. In other embodiments, poly (ethylene glycol) and Adams' catalyst, i.e., PtO.sub.2, can be used as a solvent to selectively hydrogenate the double bonds with hydrogen at 1 atmospheric pressure. The use of the Adams' catalyst is disclosed in Chandrasekhar et al., J. Org. Chem., 71, 2196-2199 (2006), which is incorporated herein by reference.

##STR00038##

The hydrogenation of the C.sub.15 isoprenoid starting materials can be carried out in the presence of an asymmetric hydrogenation catalyst such as rhodium-chiral diphosphine complex to form stereospecific hydrogenated products substantially free of other stereoisomers. A non-limiting example of the asymmetric hydrogenation catalyst includes the rhodium-DIPAMP catalyst. The rhodium-DIPAMP catalyst and other asymmetric hydrogenation catalysts are disclosed in Vineyard et al., J. Am. Chem. Soc. 1977, 99, (18), 5946; Ryoji Noyori, "Asymmetric Catalysis In Organic Synthesis," John Wiley & Sons Inc., New York, Chapter 2, pp. 16-94 (1994); and Blaser et al., "Asymmetric Catalysis on Industrial Scale: Challenges, Approaches and Solutions," Wiley-VCH, Weinheim, pp. 23-52 (2004), all of which are incorporated herein by reference in their entirety.

In some embodiments, .alpha.-farnesene and .beta.-farnesene can be hydrogenated in the presence of an asymmetric hydrogenation catalyst to form one of the four possible stereoisomers of farnesane, i.e., compounds (III-a), (III-b), (III-c), and (III-d), as shown below.

##STR00039##

Similarly, farnesol can be hydrogenated in the presence of an asymmetric hydrogenation catalyst to form one of the four possible stereoisomers of 3,7,11-trimethyldodecan-1-ol as shown below.

##STR00040##

Similarly, nerolidol can be hydrogenated in the presence of an asymmetric hydrogenation catalyst to form one of the four possible stereoisomers of 3,7,11-trimethyldodecan-3-ol as shown below.

##STR00041##

Similarly, C.sub.15 olefinic alcohols or their alkylated, esterified, sulfated, phosphated, sulfonated, or phosphonated products can also be hydrogenated in the presence of an asymmetric hydrogenation catalyst to form the corresponding stereospecific hydrogenated products.

In yet another alternative method, the hydrogenation and the alkylation, esterification, sulfation, sulfonation, phosphation, or phosphonation of the C.sub.15 olefinic alcohol can occur simultaneously.

Fuel Compositions

The fuel composition disclosed herein can be produced in a cost-effective and environmentally friendly manner. Advantageously, the isoprenoid compounds provided herein can be produced by one or more microorganisms. These isoprenoid compounds can thus provide a renewable source of energy for diesel or jet fuels, in particularly the fuel compositions provided herein. Further, these isoprenoid compounds can decrease dependence on non-renewable sources of fuel, fuel components, and/or fuel additives. In certain embodiments, the present invention encompasses a fuel composition comprising a bioengineered farnesane.

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-FsIl-PmeI restriction sites into the pACYC184 vector (GenBank accession number XO6403), and by removing the tetramycin 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

operon (see description for pAM36-MevT66 above) was digested to completion using EcoRI and Hind III restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 4.2 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the EcoRI HindIII restriction site of pAM29, yielding expression plasmid pAM25.

Expression plasmid pMevB-Cm was generated by inserting the MevB operon into the pBBR1MCS-1 vector. The MevB operon encodes the set of enzymes that together convert (R)-mevalonate to IPP, namely mevalonate kinase, phosphomevalonate kinase, and mevalonate pyrophosphate carboxylase. The MevB operon was generated by PCR amplifying from Saccharomyces cerevisiae genomic DNA the coding sequences of the ERG12 gene (GenBank accession number X55875, REGION: 580 . . . 1911) (encodes a mevalonate kinase), the ERG8 gene (GenBank accession number Z49939, REGION: 3363 . . . 4718) (encodes a phosphomevalonate kinase), and the MVD1 gene (GenBank accession number X97557, REGION: 544 . . . 1734) (encodes a mevalonate pyrophosphate carboxylase), and by splicing the PCR fragments together using overlap extensions (SOEing). By choosing appropriate primer sequences, the stop codons of ERG12 and ERG8 were changed from TAA to TAG during amplification to introduce ribosome binding sites. After the addition of 3' A overhangs, the MevB operon was ligated into the TA cloning vector pCR4 (Invitrogen, Carlsbad, Calif.). The MevB operon was excised by digesting the cloning construct to completion using PstI restriction enzyme, resolving the reaction mixture by gel electrophoresis, gel extracting the approximately 4.2 kb DNA fragment, and ligating the isolated DNA fragment into the PstI restriction site of vector pBBRIMCS-1 (Kovach et al., Gene 166(1): 175-176 (1995)), yielding expression plasmid pMevB-Cm.

Expression plasmid pMBI was generated by inserting the MBI operon into the pBBR1MCS-3 vector. In addition to the enzymes of the MevB operon, the MBI operon also encodes an isopentenyl pyrophosphatase isomerase, which catalyzes the conversion of IPP to DMAPP. The MBI operon was generated by PCR amplifying from Escherichia coli genomic DNA the coding sequence of the idi gene (GenBank accession number AF119715) using primers that contained an XmaI restriction site at their 5' ends, digesting the amplified DNA fragment to completion using XmaI restriction enzyme, resolving the reaction mixture by gel electrophoresis, gel extracting the approximately 0.5 kb fragment, and ligating the isolated DNA fragment into the XmaI restriction site of expression plasmid pMevB-Cm, thereby placing idi at the 3' end of the MevB operon. The MBI operon was subcloned into the SailI SacI restriction site of vector pBBR1MCS-3 (Kovach et al., Gene 166(1): 175-176 (1995)), yielding expression plasmid pMBI (see U.S. Pat. No. 7,192,751).

Expression plasmid pMBIS was generated by inserting the ispA gene into pMBI. The ispA gene encodes a farnesyl pyrophosphate synthase, which catalyzes the condensation of two molecules of IPP with one molecule of DMAPP to make FPP. The coding sequence of the ispA gene (GenBank accession number D00694, REGION: 484 . . . 1383) was PCR amplified from Escherichia coli genomic DNA using a forward primer with a SacII restriction site and a reverse primer with a SacI restriction site. The amplified PCR product was digested to completion using SacII and SacI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 0.9 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the SacII SacI restriction site of pMBI, thereby placing the ispA gene 3' of idi and the MevB operon, and yielding expression plasmid pMBIS (see U.S. Pat. No. 7,192,751).

Expression plasmid pAM45 was generated by inserting the MBIS operon into pAM36-MevT66 and adding lacUV5 promoters in front of the MBIS and MevT66 operons. The MBIS operon was PCR amplified from pMBIS using primers comprising a 5' XhoI restriction site and a 3' PacI restriction site, the amplified PCR product was digested to completion using XhoI and PacI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 5.4 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the XhoI PacI restriction site of pAM36-

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 Staphyloccoccus aureus subsp. aureus (ATCC 70069) genomic DNA using primers 4-49 mvaA SpeI (SEQ ID NO: 13) and 449 mvaAR 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 Staphyloccoccus 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 pAM408 was generated by inserting genes encoding enzymes of the "top" DXP pathway into the pAM29 vector. Enzymes of the "top" DXP pathway include 1-deoxy-D-xylulose-5-phosphate synthase (encoded by the dxs gene of Escherichia coli), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (encoded by the dxr gene of Escherichia coli), 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (encoded by the ispD gene of Escherichia coli), and 4-diphosphocytidyl-2C-methyl-D-erythritol synthase (encoded by the ispE gene of Escherichia coli), which together transform pyruvate and D-glyceraldehyde-3-phosphate into 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate. DNA fragments comprising nucleotide sequences that encode enzymes of the "top" DXP pathway were generated by PCR amplifying the coding sequences of the dxs (GenBank accession number U00096 REGION: 437539 . . . 439401), dxr (GenBank accession number U00096 REGION: 193521 . . . 194717), ispD (GenBank accession number U00096 REGION: 2869803 . . . 2870512), and ispE (GenBank accession number U00096 REGION 1261249 . . . 1262100) genes from Escherichia coli strain DH1 (ATCC #33849) with added optimal Shine Dalgarno sequences and 5' and 3' restriction sites using the PCR primers shown in SEQ ID NOS: 19-26. The PCR products were resolved by gel electrophoresis, gel extracted, digested to completion using appropriate restriction enzymes (XhoI and KpnI for the PCR product comprising the dxs gene; KpnI and ApaI for the PCR product comprising the dxr gene; ApaI and NdeI for the PCR product comprising the ispD gene; NdeI and MluI for the PCR product comprising the ispE gene), and purified using a PCR purification kit (Qiagen, Valencia, Calif.). Roughly equimolar amounts of each PCR product were then added to a ligation reaction to assemble the individual genes into an operon. From this ligation reaction, 1 ul of reaction mixture was used to PCR amplify two separate gene cassettes, namely the dxs-dxr and the ispD-ispE gene cassettes. The dxs-dxr gene cassette was PCR amplified using primers 67-1A-C (SEQ ID NO: 19) and 67-1D-C (SEQ ID NO: 22), and the ispD-ispE gene cassette was PCR amplified using primers 67-1E-C (SEQ ID NO: 23) and 67-1H-C (SEQ ID NO: 26). The two PCR products were resolved by gel electrophoresis, and gel extracted. The PCR product comprising the dxs-dxr gene cassette was digested to completion using XhoI and ApaI restriction enzymes, and the PCR product comprising the ispD-ispE gene cassette was digested to completion using ApaI and MluI restriction enzymes. The two PCR products were purified, and the purified DNA fragments were ligated into the SalI MluI restriction site of the pAM29 vector, yielding expression plasmid pAM408 (see FIG. 4 for a plasmid map).

Expression plasmid pAM409 was generated by inserting genes encoding enzymes of the "bottom" DXP pathway into the pAM369 vector. Enzymes of the "bottom" DXP pathway include 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (encoded by the ispF gene of Escherichia coli), 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (encoded by the ispG gene of Escherichia coli), and isopentenyl/dimethylallyl diphosphate synthase (encoded by the ispH gene of Escherichia coli), which together transform 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate to IPP and DMAPP. IPP is also converted to DMAPP through the activity of isopentyl diphosphate isomerase (encoded by the idi gene of Escherichia coli). DMAPP can be further converted to FPP through the activity of a farnesyl diphosphate synthase (such as encoded by the ispA gene of Escherichia coli). An operon encoding enzymes of the "bottom" DXP pathway as well as an isopentyl diphosphate isomerase and a farnesyl diphosphate synthase was generated by PCR amplifying the ispF (GenBank accession number U00096 REGION: 2869323 . . . 2869802), ispG (GenBank accession number U00096 REGION: 2638708 . . . 2639826), ispH (GenBank accession number U00096 REGION: 26277 . . . 27227), idi (GenBank accession number AF119715), and ispA (GenBank accession number D00694 REGION: 484 . . . 1383) genes from Escherichia coli strain DH1 (ATCC #33849) with added optimal Shine Dalgarno sequences and 5' and 3' restriction sites using the PCR primers shown in SEQ ID NOS: 27-36. The PCR products were resolved by gel electrophoresis, gel extracted, digested with the appropriate restriction enzymes (BamHI and ApaI for the PCR product comprising the ispF gene; KpnI and ApaI for the PCR product comprising the ispG gene; SalI and KpnI for the PCR product comprising the ispH gene; SalI and HindIII for the PCR product comprising the idi gene; HindIII and NcoI for the PCR product comprising the ispA gene), and purified. Roughly equimolar amounts of each PCR product were then added to a

ligation reaction to assemble the individual genes into an operon. From this ligation reaction, 1 ul of reaction mixture was used to PCR amplify two separate gene cassettes, namely the ispF-ispG and the ispH-idi-ispA gene cassettes. The ispF-ispG gene cassette was PCR amplified using primers 67-2A-C (SEQ ID NO: 27) and 67-2D-C (SEQ ID NO: 30), and the ispH-idi-ispA gene cassette was PCR amplified using primers 67-2E-C (SEQ ID NO: 31) and 67-2J-C (SEQ ID NO: 36). The two PCR products were resolved by gel electrophoresis, and gel extracted. The PCR product comprising the ispF-ispG gene cassette was digested to completion using BamHI and KpnI restriction enzymes, and the PCR product comprising the ispH-idi-ispA gene cassette was digested to completion using KpnI and NcoI restriction enzymes. The two PCR products were purified. Vector pAM369 was created by assembling the p15A origin of replication from pAM29 and beta-lactamase gene for ampicillin resistance from pZE12-luc (Lutz and Bujard (1997) Nucl Acids Res. 25:1203-1210) with an oligonucleotide-generated lacUV5 promoter. The two isolated PCR products containing the "bottom" DXP pathway operon were ligated into the BamHI NcoI restriction site of the pAM369 vector, yielding expression plasmid pAM409.

Expression plasmid pAM424, a derivative of expression plasmid pAM409 containing the broad-host range RK2 origin of replication, was generated by transferring the lacUV5 promoter and the ispFGH-idi-ispA operon of pAM409 to the pAM257 vector. Vector pAM257 was generated as follows: the RK2 par locus was PCR-amplified from RK2 plasmid DNA (Meyer et al. (1975) Science 190:1226-1228) using primers 9-156A (SEQ ID NO: 37) and 9-156B (SEQ ID NO: 38), the 2.6 kb PCR product was digested to completion using AatII and XhoI restriction enzymes, and the DNA fragment was ligated into a plasmid containing the p15 origin of replication and the chloramphenicol resistance conferring gene from vector pZA31-luc (Lutz and Bujard (1997) Nucl Acids Res. 25:1203-1210), yielding plasmid pAM37-par; pAM37-par was digested to completion using restriction enzymes SacI and HindIII, the reaction mixture was resolved by gel electrophoresis, the DNA fragment comprising the RK2 par locus and the chloramphenicol resistance gene was gel extracted, and the isolated DNA fragment was ligated into the SacI HindIII site of the mini-RK2 replicon pRR10 (Roberts et al. (1990) J Bacteriol. 172:6204-6216), yielding vector pAM133; pAM133 was digested to completion using BglII and HindIII restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 6.4 kb DNA fragment lacking the ampicillin resistance gene and oriT conjugative origin was gel extracted, and the isolated DNA fragment was ligated with a synthetically generated DNA fragment comprising a multiple cloning site that contained PciI and XhoI restriction sites, yielding vector pAM257. Expression plasmid pAM409 was digested to completion using XhoI and PciI restriction enzymes, the reaction mixture was resolved by gel electrophoresis, the approximately 4.4 kb DNA fragment was gel extracted, and the isolated DNA fragment was ligated into the XhoI PciI restriction site of the pAM257 vector, yielding expression plasmid pAM424 (see FIG. 5 for a plasmid map).

Example 4

This example describes methods for making vectors for the targeted integration of nucleic acids encoding enzymes including enzymes of the MEV pathway into specific chromosomal locations of Saccharomyces cerevisiae.

Genomic DNA was isolated from Saccharomyces cerevisiae strains Y002 (CEN.PK2 background; MATA; ura3-52; trp1-289; leu2-3,112; his3.DELTA.1; MAL2-8C; SUC2), Y007 (S288C background MATA trp1.DELTA.63), Y051 (S288C background; MAT.alpha. his3.DELTA.1 leu2.DELTA.0 lys2.DELTA.0 ura3.DELTA.0 P.sub.GAL1-HMG1.sup.1586-3323 P.sub.GAL1-upc2-1 erg9::P.sub.MET3-ERG9::HIS3 P.sub.GAL1-ERG20 P.sub.GAL1-HMG1.sup.1586-3323) and EG123 (MATA ura3; trp1; leu2; his4 can1). The strains were grown overnight in liquid medium containing 1% Yeast extract, 2% Bacto-peptone, and 2% Dextrose (YPD medium). Cells were isolated from 10 mL liquid cultures by centrifugation at 3,100 rpm, washing of cell pellets in 10 mL ultra-pure water, and re-

centrifugation. Genomic DNA was extracted using the Y-DER yeast DNA extraction kit (Pierce Biotechnologies, Rockford, Ill.) as per manufacturer's suggested protocol. Extracted genomic DNA was re-suspended in 100 uL 10 mM Tris-Cl, pH 8.5, and OD.sub.260/280 readings were taken on a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Del.) to determine genomic DNA concentration and purity.

DNA amplification by Polymerase Chain Reaction (PCR) was done in an Applied Biosystems 2720 Thermocycler (Applied Biosystems Inc, Foster City, Calif.) using the Phusion High Fidelity DNA Polymerase system (Finnzymes OY, Espoo, Finland) as per manufacturer's suggested protocol. Upon the completion of a PCR amplification of a DNA fragment that was to be inserted into the TOPO TA pCR2.1 cloning vector (Invitrogen, Carlsbad, Calif.), A nucleotide overhangs were created by adding 1 uL of Qiagen Taq Polymerase (Qiagen, Valencia, Calif.) to the reaction mixture and performing an additional 10 minute, 72.degree. C. PCR extension step, followed by cooling to 4.degree. C. Upon completion of a PCR amplification, 8 uL of a 50% glycerol solution was added to the reaction mix, and the entire mixture was loaded onto a 1% TBE (0.89 M Tris, 0.89 M Boric acid, 0.02 M EDTA sodium salt) agarose gel containing 0.5 ug/mL ethidium bromide.

Agarose gel electrophoresis was performed at 120 V, 400 mA for 30 minutes, and DNA bands were visualized using ultraviolet light. DNA bands were excised from the gel with a sterile razor blade, and the excised DNA was gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Orange, Calif.) according to manufacturer's suggested protocol. The purified DNA was eluted into 10 uL ultra-pure water, and OD.sub.260/280 readings were taken on a ND-1000 spectrophotometer to determine DNA concentration and purity.

Ligations were performed using 100-500 ug of purified PCR product and High Concentration T4 DNA Ligase (New England Biolabs, Ipswich, Mass.) as per manufacturer's suggested protocol. For plasmid propagation, ligated constructs were transformed into Escherichia coli DH5.alpha. chemically competent cells (Invitrogen, Carlsbad, Calif.) as per manufacturer's suggested protocol. Positive transformants were selected on solid media containing 1.5% Bacto Agar, 1% Tryptone, 0.5% Yeast Extract, 1% NaCl, and 50 ug/mL of an appropriate antibiotic. Isolated transformants were grown for 16 hours in liquid LB medium containing 50 ug/mL carbenicillin or kanamycin antibiotic at 37.degree. C., and plasmid was isolated and purified using a QIAprep Spin Miniprep kit (Qiagen, Valencia, Calif.) as per manufacturer's suggested protocol. Constructs were verified by performing diagnostic restriction enzyme digestions, and resolving and visualizing DNA fragments on an agarose gel. Select constructs were also verified by DNA sequencing, which was done by Elim Biopharmaceuticals Inc. (Hayward, Calif.).

Plasmid pAM489 was generated by inserting the ERG20-P.sub.GAL-tHMGR insert of vector pAM471 into vector pAM466. Vector pAM471 was generated by inserting DNA fragment ERG20-P.sub.GAL-tHMGR, which comprises the open reading frame (ORF) of ERG20 (ERG20 nucleotide positions 1 to 1208; A of ATG start codon is nucleotide 1) (ERG20), the genomic locus containing the divergent GAL1 and GAL10 promoter (GAL1 nucleotide position -1 to -668) (P.sub.GAL), and a truncated ORF of HMG1 (HMG1 nucleotide positions 1586 to 3323) (tHMGR), into the TOPO Zero Blunt II cloning vector (Invitrogen, Carlsbad, Calif.). Vector pAM466 was generated by inserting DNA fragment TRP1.sup.-856 to +548, which comprises a segment of the wild-type TRP1 locus of Saccharomyces cerevisiae that extends from nucleotide position -856 to position 548 and harbors a non-native internal XmaI restriction site between bases -226 and -225, into the TOPO TA pCR2.1 cloning vector (Invitrogen, Carlsbad, Calif.). DNA fragments ERG20-P.sub.GAL-tHMGR and TRP1.sup.-856 to +548 were generated by PCR amplification as outlined in Table 1. For the construction of pAM489, 400 ng of pAM471 and 100 ng of pAM466 were digested to completion using XmaI restriction enzyme (New England Biolabs, Ipswich, Mass.), DNA fragments corresponding to the ERG20-P.sub.GAL-tHMGR insert and the linearized pAM466 vector were gel purified, and 4 molar equivalents of the purified insert

was ligated with 1 molar equivalent of the purified linearized vector, yielding pAM489 (see FIG. 6A for a map and SEQ ID NO: 3 for the nucleotide sequence of the ERG20-P.sub.GAL-tHMGR insert).

TABLE-US-00001 TABLE 1 PCR amplifications performed to generate pAM489 PCR Round Template Primer 1 Primer 2 PCR Product 1 100 ng of Y051 genomic 61-67-CPK001- 61-67-CPK002- TRP1.sup.-856 to -226 DNA G (SEQ ID NO: G (SEQ ID NO: 39) 40) 61-67-CPK003- 61-67-CPK004- TRP1.sup.-225 to +548 G (SEQ ID NO: G (SEQ ID NO: 41) 42) 100 ng of EG123 genomic 61-67-CPK025- 61-67-CPK050- ERG20 DNA G (SEQ ID NO: G (SEQ ID NO: 62) 70) 100 ng of Y002 genomic 61-67-CPK051- 61-67-CPK052- P.sub.GAL DNA G (SEQ ID NO: G (SEQ ID NO: 71) 72) 61-67-CPK053- 61-67-CPK031- tHMGR G (SEQ ID NO: G (SEQ ID NO: 73) 63) 2 100 ng each of TRP1.sup.-856 to -226 61-67-CPK001- 61-67-CPK004- TRP1.sup.-856 to +548 and TRP1.sup.-225-to +548 purified G (SEQ ID NO: G (SEQ ID NO: PCR products 39) 42) 100 ng each of ERG20 and 61-67-CPK025- 61-67-CPK052- ERG20-P.sub.GAL P.sub.GAL purified PCR products G (SEQ ID NO: G (SEQ ID NO: 62) 72) 3 100 ng each of ERG20-P.sub.GAL 61-67-CPK025- 61-67-CPK031- ERG20-P.sub.GAL- and tHMGR purified PCR G (SEQ ID NO: G (SEQ ID NO: tHMGR products 62) 63)

Plasmid pAM491 was generated by inserting the ERG13-P.sub.GAL-tHMGR insert of vector pAM472 into vector pAM467. Vector pAM472 was generated by inserting DNA fragment ERG13-P.sub.GAL-tHMGR, which comprises the ORF of ERG13 (ERG13 nucleotide positions 1 to 1626) (ERG13), the genomic locus containing the divergent GAL1 and GAL10 promoter (GAL1 nucleotide position -1 to -668) (P.sub.GAL), and a truncated ORF of HMG1 (HMG1 nucleotide position 1586 to 3323) (tHMGR), into the XmaI restriction site of TOPO Zero Blunt II cloning vector. Vector pAM467 was generated by inserting DNA fragment URA3.sup.-723 to 701, which comprises a segment of the wild-type URA3 locus of Saccharomyces cerevisiae that extends from nucleotide position -723 to position -224 and harbors a non-native internal XmaI restriction site between bases -224 and -223, into the TOPO TA pCR2.1 cloning vector. DNA fragments ERG13-P.sub.GAL-tHMGR and URA3.sup.-723 to 701 were generated by PCR amplification as outlined in Table 2. For the construction of pAM491, 400 ng of pAM472 and 100 ng of pAM467 were digested to completion using XmaI restriction enzyme, DNA fragments corresponding to the ERG13-P.sub.GAL-tHMGR insert and the linearized pAM467 vector were gel purified, and 4 molar equivalents of the purified insert was ligated with 1 molar equivalent of the purified linearized vector, yielding pAM491 (see FIG. 6B for a map and SEQ ID NO: 4 for the nucleotide sequence of the ERG13-P.sub.GAL-tHMGR insert).

TABLE-US-00002 TABLE 2 PCR amplifications performed to generate pAM491 PCR Round Template Primer 1 Primer 2 PCR Product 1 100 ng of Y007 genomic 61-67-CPK005- 61-67-CPK006- URA3.sup.-723 to -224 DNA G (SEQ ID NO: G (SEQ ID NO: 43) 44) 61-67-CPK007- 61-67-CPK008- URA3.sup.-223 to 701 G (SEQ ID NO: G (SEQ ID NO: 45) 46) 100 ng of Y002 genomic 61-67-CPK032- 61-67-CPK054- ERG13 DNA G (SEQ ID NO: G (SEQ ID NO: 64) 74) 61-67-CPK052- 61-67-CPK055- P.sub.GAL G (SEQ ID NO: G (SEQ ID NO: 72) 75) 61-67-CPK031- 61-67-CPK053- tHMGR G (SEQ ID NO: G (SEQ ID NO: 63) 73) 2 100 ng each of URA3.sup.-723 to -224 61-67-CPK005- 61-67-CPK008- URA3.sup.-723 to 701 and URA3.sup.-223 to 701 purified G (SEQ ID NO: G (SEQ ID NO: PCR products 43) 46) 100 ng each of ERG13 and 61-67-CPK032- 61-67-CPK052- ERG13-P.sub.GAL P.sub.GAL purified PCR products G (SEQ ID NO: G (SEQ ID NO: 64) 72) 3 100 ng each of ERG13-P.sub.GAL 61-67-CPK031- 61-67-CPK032- ERG13-P.sub.GAL- and tHMGR purified PCR G (SEQ ID NO: G (SEQ ID NO: tHMGR products 63) 64)

Plasmid pAM493 was generated by inserting the IDI1-P.sub.GAL-tHMGR insert of vector pAM473 into vector pAM468. Vector pAM473 was generated by inserting DNA fragment IDI1-P.sub.GAL-tHMGR, which comprises the ORF of IDI1 (IDI1 nucleotide position 1 to 1017) (IDI1), the genomic locus containing the divergent GAL1 and GAL10 promoter (GAL1 nucleotide position -1 to -668) (P.sub.GAL), and a truncated ORF of HMG1 (HMG1 nucleotide positions 1586 to 3323) (tHMGR), into

the TOPO Zero Blunt II cloning vector. Vector pAM468 was generated by inserting DNA fragment ADE1.sup.-825 to 653, which comprises a segment of the wild-type ADE1 locus of Saccharomyces cerevisiae that extends from nucleotide position -225 to position 653 and harbors a non-native internal XmaI restriction site between bases -226 and -225, into the TOPO TA pCR2.1 cloning vector. DNA fragments IDI1-P.sub.GAL-tHMGR and ADE1.sup.-825 to 653 were generated by PCR amplification as outlined in Table 3. For the construction of pAM493, 400 ng of pAM473 and 100 ng of pAM468 were digested to completion using XmaI restriction enzyme, DNA fragments corresponding to the IDI1-P.sub.GAL-tHMGR insert and the linearized pAM468 vector were gel purified, and 4 molar equivalents of the purified insert was ligated with 1 molar equivalent of the purified linearized vector, yielding vector pAM493 (see FIG. 6C for a map and SEQ ID NO: 5 for the nucleotide sequence of the IDI1-P.sub.GAL-tHMGR insert).

TABLE-US-00003 TABLE 3 PCR amplifications performed to generate pAM493 PCR Round Template Primer 1 Primer 2 PCR Product 1 100 ng of Y007 genomic DNA 61-67-CPK009- 61-67-CPK010- ADE1.sup.-825 to -226 G (SEQ ID NO: G (SEQ ID NO: 47) 48) 61-67-CPK011- 61-67-CPK012- ADE1.sup.-225 to 653 G (SEQ ID NO: G (SEQ ID NO: 49) 50) 100 ng of Y002 genomic DNA 61-67-CPK047- 61-67-CPK064- IDI1 G (SEQ ID NO: G (SEQ ID NO: 69) 84) 61-67-CPK052- 61-67-CPK065- P.sub.GAL G (SEQ ID NO: G (SEQ ID NO: 72) 85) 61-67-CPK031- 61-67-CPK053- tHMGR G (SEQ ID NO: G (SEQ ID NO: 63) 73) 2 100 ng each of ADE1.sup.-825 to -226 61-67-CPK009- 61-67-CPK012- ADE1.sup.-825 to 653 and ADE1.sup.-225 to 653 purified PCR G (SEQ ID NO: G (SEQ ID NO: products 47) 50) 100 ng each of IDI1 and P.sub.GAL 61-67-CPK047- 61-67-CPK052- IDI1-P.sub.GAL purified PCR products G (SEQ ID NO: G (SEQ ID NO: 69) 72) 3 100 ng each of IDI1-P.sub.GAL and 61-67-CPK031- 61-67-CPK047- IDI1-P.sub.GAL-tHMGR tHMGR purified PCR products G (SEQ ID NO: G (SEQ ID NO: 63) 69)

Plasmid pAM495 was generated by inserting the ERG10-P.sub.GAL-ERG12 insert of pAM474 into vector pAM469. Vector pAM474 was generated by inserting DNA fragment ERG10-P.sub.GAL-ERG12, which comprises the ORF of ERG10 (ERG10 nucleotide position 1 to 1347) (ERG10), the genomic locus containing the divergent GAL1 and GAL10 promoter (GAL1 nucleotide position -1 to -668) (P.sub.GAL), and the ORF of ERG12 (ERG12 nucleotide position 1 to 1482) (ERG12), into the TOPO Zero Blunt II cloning vector. Vector pAM469 was generated by inserting DNA fragment HIS3.sup.-32 to -1000-HISMX-HIS3.sup.504 to -1103, which comprises two segments of the wild-type HIS locus of Saccharomyces cerevisiae that extend from nucleotide position -32 to position -1000 and from nucleotide position 504 to position 1103, a HISMX marker, and a non-native XmaI restriction site between the HIS3.sup.504 to -1103 sequence and the HISMX marker, into the TOPO TA pCR2.1 cloning vector. DNA fragments ERG10-P.sub.GAL-ERG12 and HIS3.sup.-32 to -1000-HISMX-HIS3.sup.504 to -1103 were generated by PCR amplification as outlined in Table 4. For construction of pAM495, 400 ng of pAM474 and 1100 ng of pAM469 were digested to completion using XmaI restriction enzyme, DNA fragments corresponding to the ERG10-P.sub.GAL-ERG12 insert and the linearized pAM469 vector were gel purified, and 4 molar equivalents of the purified insert was ligated with 1 molar equivalent of the purified linearized vector, yielding vector pAM495 (see FIG. 6D for a map and SEQ ID NO: 6 for the nucleotide sequence of the ERG10-P.sub.GAL-ERG12 insert).

TABLE-US-00004 TABLE 4 PCR reactions performed to generate pAM495 PCR Round Template Primer 1 Primer 2 PCR Product 1 100 ng of Y007 genomic 61-67-CPK013-G 61-67-CPK014alt-HIS3.sup.-32 to -1000 DNA (SEQ ID NO: 51) G (SEQ ID NO: 52) 61-67-CPK017-G 61-67-CPK018-G HIS3.sup.504 to -1103 (SEQ ID NO: 54) (SEQ ID NO: 55) 61-67-CPK035-G 61-67-CPK056-G ERG10 (SEQ ID NO: 65) (SEQ ID NO: 76) 61-67-CPK057-G 61-67-CPK058-G P.sub.GAL (SEQ ID NO: 77) (SEQ ID NO: 78) 61-67-CPK040-G 61-67-CPK059-G ERG12 (SEQ ID NO: 66) (SEQ ID NO: 79) 10 ng of plasmid pAM330 61-67-CPK015alt- 61-67-CPK016-G HISMX DNA** G (SEQ ID NO: (SEQ ID NO: 92) 53) 2 100 ng each of HIS3.sup.504 to -1103 61-67-CPK015alt- 61-67-CPK018-G HISMX-

HIS3.sup.504 to -1103 and HISMX PCR G (SEQ ID NO: (SEQ ID NO: 55) purified products 53) 100 ng each of ERG10 and 61-67-CPK035-G 61-67-CPK058-G ERG10-P.sub.GAL P.sub.GAL purified PCR products (SEQ ID NO: 65) (SEQ ID NO: 78) 3 100 ng each of HIS3.sup.-32 to -1000 61-67-CPK013-G 61-67-CPK018-G HIS3.sup.-32 to -1000- and HISMX-HIS3.sup.504 to -1103 (SEQ ID NO: 51) (SEQ ID NO: 55) HISMX-HIS3.sup.504 to -1103 purified PCR products 100 ng each of ERG10-61-67-CPK035-G 61-67-CPK040-G ERG10-P.sub.GAL-ERG12 P.sub.GAL and ERG12 purified (SEQ ID NO: 65) (SEQ ID NO: 66) PCR products **The HISMX marker in pAM330 originated from pFA6a-HISMX6-PGAL1 as described by van Dijken et al. ((2000) Enzyme Microb. Technol. 26(9-10): 706-714).

Plasmid pAM497 was generated by inserting the ERG8-P.sub.GAL-ERG19 insert of pAM475 into vector pAM470. Vector pAM475 was generated by inserting DNA fragment ERG8-P.sub.GAL-ERG19, which comprises the ORF of ERG8 (ERG8 nucleotide position 1 to 1512) (ERG8), the genomic locus containing the divergent GAL1 and GAL10 promoter (GAL1 nucleotide position -1 to -668) (P.sub.GAL), and the ORF of ERG19 (ERG19 nucleotide position 1 to 1341) (ERG19), into the TOPO Zero Blunt II cloning vector. Vector pAM470 was generated by inserting DNA fragment LEU2.sup.-100 to 450-HISMX-LEU2.sup.1096 to 1770, which comprises two segments of the wild-type LEU2 locus of Saccharomyces cerevisiae that extend from nucleotide position -100 to position 450 and from nucleotide position 1096 to position 1770, a HISMX marker, and a non-native XmaI restriction site between the LEU21096 to 1770 sequence and the HISMX marker, into the TOPO TA pCR2.1 cloning vector. DNA fragments ERG8-P.sub.GAL-ERG19 and LEU2.sup.-100 to 450-HISMX-LEU2.sup.1096 to 1770 were generated by PCR amplification as outlined in Table 5. For the construction of pAM497, 400 ng of pAM475 and 100 ng of pAM470 were digested to completion using XmaI restriction enzyme, DNA fragments corresponding to the ERG8-P.sub.GAL-ERG19 insert and the linearized pAM470 vector were purified, and 4 molar equivalents of the purified insert was ligated with 1 molar equivalent of the purified linearized vector, yielding vector pAM497 (see FIG. 6E for a map and SEQ ID NO: 7 for the nucleotide sequence of the ERG8-P.sub.GAL-ERG19 insert).

TABLE-US-00005 TABLE 5 PCR reactions performed to generate pAM497 PCR Round Template Primer 1 Primer 2 PCR Product 1 100 ng of Y007 genomic DNA 61-67-CPK019- 61-67-CPK020- LEU2.sup.-100 to 450 G (SEQ ID NO: G (SEQ ID NO: 56) 57) 61-67-CPK023- 61-67-CPK024- LEU2.sup.1096 to 1770 G (SEQ ID NO: G (SEQ ID NO: 60) 61) 10 ng of plasmid pAM330 DNA** 61-67-CPK021- 61-67-CPK022- HISMX G (SEQ ID NO: G (SEQ ID NO: 58) 59) 100 ng of Y002 genomic DNA 61-67-CPK041- 61-67-CPK060- ERG8 G (SEQ ID NO: G (SEQ ID NO: 67) 80) 61-67-CPK061- 61-67-CPK062- P.sub.GAL G (SEQ ID NO: G (SEQ ID NO: 81) 82) 61-67-CPK046- 61-67-CPK063- ERG19 G (SEQ ID NO: G (SEQ ID NO: 68) 83) 2 100 ng each of LEU2.sup.1096 to 1770 61-67-CPK021- 61-67-CPK024- HISMX-LEU2.sup.1096 to 1770 and HISMX purified PCR G (SEQ ID NO: G (SEQ ID NO: products 58) 61) 100 ng each of ERG8 and P.sub.GAL 61-67-CPK041- 61-67-CPK062- ERG8-P.sub.GAL purified PCR products G (SEQ ID NO: G (SEQ ID NO: 67) 82) 3 100 ng of LEU2.sup.-100 to 450 and 61-67-CPK019- 61-67-CPK024- LEU2.sup.-100 to 450- HISMX-LEU2.sup.1096 to 1770 purified G (SEQ ID NO: G (SEQ ID NO: HISMX-LEU2.sup.1096 to 1770 PCR products 56) 61) 100 ng each of ERG8-P.sub.GAL and 61-67-CPK041-G 61-67-CPK046-G ERG8-P.sub.GAL- ERG19 purified PCR products (SEQ ID NO: (SEQ ID NO: ERG19 67) 68) **The HISMX marker in pAM330 originated from pFA6a-HISMX6-PGAL1 as described by van Dijken et al. ((2000) Enzyme Microb. Technol. 26(9-10): 706-714).

Example 5

This example describes methods for making expression plasmids that encode enzymes that convert FPP.

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.47H.sub.2O 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.244H.sub.2O 3.7 ug H.sub.3BO.sub.4 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