Title: ENZYMATIC REMOVAL OF STERYL GLYCOSIDES

Abstract: The present invention provides compositions and methods related to the production and use of enzymes suitable for reducing the amount of steryl glycosides or saturated monoacyl glycerols in a lipid mixture.
before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

with sequence listing part of description (Rule 5.2(a))

Published:

— with international search report (Art. 21(3))
ENZYMATIC REMOVAL OF STERYL GLYCOSIDES

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Application No. 61/61 1,949, filed March 16, 2012, and U.S. Application No. 61/696,588, filed September 4, 2012, which are incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

[0002] There is an urgent demand for sustainable and affordable alternatives to petroleum-based fuels. Biofuels are a promising replacement for petroleum-based fuels. Biofuels can be produced from animal or plant matter, such as from corn, sugar cane, sawgrass, soybeans, or algae. As such, they are a renewable and potentially limitless source of fuel. In particular, biodiesels are useful as fuel for vehicles in replacement or as a supplement to petroleum-based diesel fuels. They can be utilized by traditional fuel-burning engines, produce fewer particulates when burnt, have a higher flash point, and are less toxic than petroleum-based fuels. In 2006, biodiesel production in the United States alone was estimated to be more than 1 billion gallons.

[0003] Chemically, biodiesels primarily comprise a mixture of monoalkyl esters of long chain fatty acids. Biodiesels are typically produced from lipid transesterification of vegetable oils, including those from soybean, jatropha, palm, rapeseed, sunflower, and others; and/or animal fats with a short-chain monohydric alcohol. The longer the carbon chain of the alcohol used, the better the cold-flow properties. For example, biodiesel comprising fatty acid ethyl esters (FAEEs), derived from ethanol, has better cold-flow properties than biodiesel comprising fatty acid methyl esters (FAMEs), derived from methanol.

[0004] However, transesterification produces various unwanted side products, including saturated monoacyl glycerols (SMGs) and steryl glycosides such as steryl glucosides. Acylated steryl glycosides are soluble in oil, but during esterification, they are converted to nonacylated SGs, which are relatively insoluble. If not removed from the biodiesel, steryl glycosides can clog oil filters or cause engine failures. Particles of clumped steryl glycoside molecules can also promote crystallization, aggregation, or precipitation of other compounds in the biodiesel. This further reduces biodiesel flowability and increases the likelihood of clogging. Steryl glycosides typically have a high melting point of around 240°C and thus cannot simply be heated to allow them to pass through an oil filter. Similarly, SMGs can form crystals in the biodiesel, especially at low temperatures, which creates cold-flow problems and can cause blockages in fuel lines under cold conditions. Additionally, the
formation of these precipitates may cause several problems during the biodiesel production process resulting in an increase in production costs.

[0005] Insoluble contaminants containing steryl glycosides may appear as haze, precipitates or sediments in biodiesel, which prevents the product from complying with the requirements on contamination and filterability according to biodiesel quality standards.

[0006] One method capable of completely removing steryl glycosides and SMGs from biodiesel is distillation. Distillation is energy-intensive, which reduces the cost efficiency and net energy gain of biodiesel production. Filtering, such as through diatomaceous earth, is expensive and not easily scalable to large quantities. Adding adsorbents requires an additional removal step, and is similarly expensive and time-consuming. Other methods include the centrifugation methods disclosed in WO 2010 004423.

[0007] Steryl glycosidases can be used to digest steryl glycosides, producing a glycoside and a sterol. Similarly, lipases can be used to eliminate SMGs. However, steryl glycosidases and lipases currently used in the field are inefficient and do not effectively reduce the amount of steryl glycosides and SMGs in biodiesel.

**SUMMARY OF THE INVENTION**

[0008] The present invention provides isolated thermostable enzymes that are capable of hydrolyzing the glycosidic bond of steryl glycosides or acylated steryl glycosides and methods of making and using such enzymes. This solves the challenges of producing biodiesel fuel which is higher quality, more cost-effective, and competitive in the global market.

[0009] The platform disclosed herein uses genetic engineering, synthetic biology and directed evolution to rapidly generate new and improved enzymes that can significantly reduce current production costs and provide premium high-quality biodiesel by eliminating major impurities in an environmental-friendly and commercially competitive way. The invention also provides methods and compositions for generating designer enzymes that eliminate key impurities in plant-based biodiesel, such as steryl glycosides and saturated monoacylglycerols (SMGs) which result in the formation of insoluble materials that compromise quality and performance of the end product.

[0010] In one aspect, the present invention provides a method for reducing steryl glycoside in a sample. The method comprises: mixing a thermostable enzyme with a sample comprising steryl glycoside under a condition suitable for the thermostable enzyme for a
suitable period of time to degrade the steryl glycoside, thereby reducing steryl glycoside in
the sample to obtain a processed sample.

[0011] In some embodiments, the sample comprises oil, fat, or biofuel (e.g. biodiesel).

[0012] In some embodiments, the steryl glycoside comprises steryl glucoside. In some
embodiments, the steryl glycoside has a solubility that is more than 50 ppm, more than 80
ppm, or more than 100 ppm.

[0013] In some embodiments, the thermostable enzyme is capable of hydrolyzing the
glycosidic bond of steryl glucosides or acylated steryl glucosides.

[0014] In some embodiments, the thermostable enzyme comprises a glycosidase enzyme.

[0015] In some embodiments, the thermostable enzyme comprises a glucosidase enzyme.

[0016] In some embodiments, the thermostable enzyme comprises a variant of an enzyme
selected from Table 1.

[0017] In some embodiments, the thermostable enzyme comprises a variant having an amino
acid sequence that has at least 95% identity to a sequence selected from the sequences of
Table 1.

[0018] In some embodiments, the thermostable enzyme comprises an amino acid sequence
selected from the sequences of Table 1.

[0019] In some embodiments, the sample comprises 0.1 % to 30% of water.

[0020] In some embodiments, the mixing step is carried out at a temperature that is between
about 50 °C and about 110 °C, such as above about 65 °C, above about 70 °C, or above about
75 °C.

[0021] In some embodiments, the mixing step is carried for about 30 minutes to 24 hours.

[0022] In some embodiments, the steryl glycoside is reduced by at least 20% to 80%,
inclusive.

[0023] In some embodiments, the processed sample comprises less than 20 ppm of the steryl
glycoside.

[0024] In some embodiments, the mixing step comprises mixing the sample with an enzyme
selected from beta-glucosidases, sterol-esterases, amyloglucosidases, and pectinases.

[0025] In some embodiments, the method further comprises collecting the processed sample.
[0026] In another aspect, the present invention provides an oil produced by the methods provided herein.

[0027] In another aspect, the present invention provides an isolated thermostable enzyme that is capable of hydrolyzing the glycosidic bond of steryl glycosides or acylated steryl glycosides. In some embodiments, the enzyme has an activity of at least 5 g of steryl glycoside per gram of enzyme per hour at a temperature that is between about 50 °C and about 99 °C, such as above about 65 °C, 70 °C, 75, 80, 85, or 90 °C. In some embodiments, the thermostable enzyme comprises a variant of one of the enzymes listed in Table 1. In some embodiments, the thermostable enzyme comprises a variant having an amino acid sequence that is at least 95% identical to the sequence of one of the enzymes listed in Table 1. In some embodiments, the thermostable enzyme comprises an amino acid sequence selected from the sequences of Table 1.

[0028] In another aspect, the present invention provides a method for generating a gene encoding a variant steryl glucosidase, comprising: (a) growing in a cultural medium a plurality of host cells transformed with a library of variant steryl glucosidase genes, wherein expression of each variant steryl glucosidase gene is under the control of a promoter that linearly responds to concentrations of an inducer added to the culture medium, wherein the host cells require ergosterol to grow and are unable to synthesize ergosterol, and wherein the cultural medium comprises steryl glucosides and a first concentration of the inducer so as to permit only a host cell expressing a variant steryl glucosidase with sufficient activity to form a colony; and (b) recovering the variant steryl glucosidase gene from the colony. In some embodiments, the method further comprises repeating the steps (a) and (b) wherein the library of variant steryl glucosidase genes is generated from the variant steryl glucosidase gene recovered from the previous iteration, and wherein in the new iteration cycle a lower concentration of the inducer is added to the cultural medium.

[0029] In some embodiments, the library is generated using error prone PCR or oligonucleotide directed mutagenesis.

[0030] In some embodiments, an expression vector used to transform host cells with variant steryl glucosidase genes comprises a yeast expression vector.

[0031] In some embodiments, an expression vector used to transform host cells with variant steryl glucosidase genes is inducible by an inducer selected from: Cu^{2+} and beta-estradiol.

[0032] In some embodiments, the host cell comprises a yeast mutant cell.
In some embodiments, the method further comprises designing and synthesizing codon optimized sequences encoding the variant steryl glucosidase.

In another aspect, the present invention provides a method for producing a recombinant steryl glucosidase, comprising: (a) expressing codon optimized sequences provided herein or obtained using a method provided herein in a suitable heterologous host cell to generate recombinant steryl glucosidase; and (b) isolating the recombinant steryl glucosidase. In some embodiments, the growing step occurs at or above 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, or 85°C.

In another aspect, the present invention provides a host cell expressing a gene produced by the methods provided herein.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

FIG. 1 depicts enzymatic hydrolysis of an example steryl glycoside (SG), β-sitosteryl-glucoside.

FIG. 2 is a schematic depicting an example process of enzymatic removal of steryl glycosides from biodiesel.

FIG. 3 is a graph depicting the temperature-dependent solubility of steryl glucoside in biodiesel.

FIG. 4A is a collection of photographs depicting the expression of various example steryl glycosidase (SGase) genes in an E. coli host and the purification of expressed proteins via Ni-NTA (nickel-nitrilotriacetic acid) affinity chromatography. FIG. 4B is a collection of photographs depicting the activity of an example SGase evaluated in aqueous media (5 hs incubation at 80°C, pH5.5 with 100 ppm SG).
FIG. 5 is a graph depicting SG hydrolysis in Biodiesel/water emulsions (5% ADMUL) using an example SGase, LacS.

FIG. 6 is a graph and set of photographs depicting the hydrolysis of an SG using example SGases Sulfolobus solfataricus LacS and Thermococcus litoralis TL in 40 ml flasks without emulsifier (3 ug Ez/ml biodiesel, 120 ppm SG).

FIG. 7 is a plot depicting gas chromatography-flame ionization detection (GC-FID) analysis of biodiesel samples before and after enzymatic treatment with an example SGase (TL).

FIG. 8 is a plot and collection of photographs depicting SG hydrolysis using an example SGase (TL).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to compositions and methods for reducing the amount of steryl glycosides in a biodiesel sample. The invention further provides methods to increase efficiency of enzymatic degradation of steryl glycosides. In some embodiments, elevated temperatures may be used to increase efficiency of enzymatic degradation. In some embodiments, a thermostable enzyme may be used, such as a thermostable steryl glycosidase or a thermostable lipase. In some embodiments, pressure, additives, or other techniques may be used to increase efficiency of enzymatic activity.

Biodiesels, such as those generated by lipid transesterification, can contain various contaminating compounds, including but not limited to steryl glycosides and saturated monacetyl glycerols (SMGs). Acylated steryl glycosides are soluble in oil, but during esterification, they are converted to nonacylated steryl glycosides, which are relatively insoluble. Nonacylated steryl glycosides can also be naturally present. Precipitation of steryl glycosides and SMGs can occur at any temperature. Even low levels, such as 10-90 ppm of steryl glycoside in biodiesel, can form aggregates. These aggregates, if present in biodiesels, can clog oil filters and also promote crystallization, aggregation, or precipitation of other compounds.

WO2007/076163 describes filtration methods to remove steryl glucosides, including the use of additives to increase precipitation or aggregation. However, this procedure introduces an extra filtration step which can be costly and/or time consuming. Additionally, removal of steryl glucosides by filtration or centrifugation requires waiting for the steryl glucosides to aggregate and precipitate before they can be removed from the biodiesel or starting oil. These methods also reduce the overall yield of biodiesel.
[0049] WO 2010/102952 and WO 2010/004423, herein incorporated by reference, describe methods using enzymatic catalysis to remove steryl glucosides from biodiesel and oil. However, WO 2010/102952 and WO 2010/004423 describe processes where the optimal reaction occurs at 50°C. At 50°C, the solubility of steryl glucosides in biodiesel is around 50 ppm. However, crude biodiesel typically contains 10-300 ppm of steryl glucosides. Thus, a significant fraction of steryl glucoside is insoluble at 50°C and, therefore, not accessible to the enzymes.

[0050] Steryl glycosides and SMG aggregates in biodiesels are resistant to enzymatic degradation because their aggregation prevents the enzymes from efficiently accessing or digesting the compounds. Enzymatic digestion works the most efficiently when the substrates are free in solution. Steryl glycosides solubility in biodiesel increases with temperature, which reduces aggregation and increases accessibility. However, high temperatures can reduce enzymatic activity, such as by heat denaturation of the enzymes.

[0051] The present invention overcomes problems related to the presence of steryl glycosides in biodiesel by using newly discovered or artificially generated enzymes capable of hydrolyzing steryl glycosides at high temperature, providing methods for the inexpensive production of such enzymes and using such enzymes to remove steryl glycosides from biodiesel, biodiesel precursors, or biodiesel derivatives.

1. Samples

[0052] Samples as described herein can refer to any oil, fat or biofuel. Biofuels can include any energy source derived from organic material, including but not limited to cellulosic ethanol and biodiesels. In some embodiments, a sample is a starting material, precursor or intermediate product used for biofuel or biodiesel production, processing, or refinement. For example, a biofuel precursor can refer to any oil or other sample suitable for generating biofuel. A biodiesel precursor can refer to any oil or other sample suitable for generating biodiesel. In some embodiments, the oils, fats, biofuels, or precursors thereof are derived from an organic source, including but not limited to animal fats, such as tallow, lard, chicken fat, yellow grease, fish oil, and byproducts of animal fat processing; plant oils, including but not limited to oils from rapeseed, soybeans, flax, sunflower, safflower, nasturtium, palm, coconut, hemp, olive, sesame, peanut, babassu nut, castor, corn, canola, jatropha, mustard, jojoba, rice bran, cottonseed, pennycress, lupin, algae, halophytes such as dwarf saltwort; waste vegetable or other oils, such as oils left over from food production, or products generated therefrom. In some embodiments, the sample is an intermediate product, a waste product, or a by-product of oil or fat refining, including but not limited to soap stock, acid oil,
fatty acid distillates, gums, by-products of Omega-3 fatty acid derivates from fish oil, fat trap
grease, free fatty acids, fractions of oil obtained by physical separations, or any combinations
thereof. In some embodiments, samples for biodiesel generation are derived from algae.

[0053] In some embodiments, the sample comprises steryl glycosides. "Steryl glycosides" as
used herein refers to molecules comprising one or more carbohydrate units linked to a
hydroxyl group of a sterol molecule. Examples of sterol molecule include but are not limited
to phytosterols such as campesterol, stigmasterol, sitosterol, avenasterol, desmosterol,
fucosterol, sargasterol, brassicasterol and dihydrositosterol; zoosterols such as cholesterol; or
saturated "stanol" versions of such sterols. A carbohydrate may be a sugar moiety with
examples that include but are not limited to glucose, sucrose, xylose, arabinose, fructose,
galactose, mannose, glucuronides, sulfated steryl glycosides or diglycosides. A sugar moiety
may be linked to a sterol moiety via a glycosidic bond. In some embodiments, a sugar moiety
is acylated at the carbon 6 position. Examples of steryl glycosides include but are not limited
to acylated steryl glycosides, nonacylated steryl glycosides, steryl glucosides, and β-
sitosteryl-glucoside. When a sugar moiety is glucose, the steryl glycoside may be referred to
as a steryl glucoside. In the present invention the term steryl glycoside is meant to encompass
steryl glucoside.

[0054] As used herein, solubility refers to the amount of a solute that can be dissolved within
a solvent. A solute's solubility generally varies based on temperature, pressure and on the
composition of the solvent. Solubility of steryl glycosides in biodiesels and oils generally
increases with temperature. FIG.3 depicts the solubility of a steryl glucoside mixture in
biodiesel generated from soybean oil at different temperatures. Steryl glucoside solubility
was evaluated in distilled soybean biodiesel. 100 parts-per-million ("ppm") of steryl
glucoside was added to biodiesel and incubated at 100 °C for 24 hours ("h") to the indicated
temperatures and incubated for 4h prior to steryl glycoside solubility determination. In some
embodiments, solubility of steryl glucoside in the sample is at least 30 ppm, at least 40 ppm,
at least 50 ppm, at least 60 ppm, at least 70 ppm, at least 80 ppm, at least 90 ppm, at least 100
ppm, or at least 110 ppm. Solubility can be measured by determining the amount of steryl
glycoside in the biodiesel or biodiesel precursor, excluding any precipitated steryl glycoside.
The amount of steryl glycoside in the oil or fat (e.g. biofuel substrate) and/or the biofuel may
be determined by any conventional process.

[0055] The amount of steryl glycoside in an oil or fat may vary depending on the sample
source. The amount of steryl glycosides in crude soybean oil is higher than in some other oils
that are commonly used to make biodiesel such as, for example, rape seed, corn, cotton or
sunflower oil. In some embodiments, the concentration of steryl glycoside in a sample is at least 30 ppm, at least 40 ppm, at least 50 ppm, at least 60 ppm, at least 70 ppm, at least 80 ppm, at least 90 ppm, at least 100 ppm, at least 110 ppm, at least 120 ppm, at least 130 ppm, at least 140 ppm, at least 150 ppm, at least 200 ppm, at least 250 ppm, or at least 300 ppm by weight. Concentration as used herein generally refers to the total amount of a substance (e.g., steryl glycoside) in a sample, including both precipitates and dissolved species. Concentrations may be determined, for example, by solid-phase extraction and gas chromatography as described in Phillips et al. (2005), Journal of Food Lipids, 12(2), 124-140, which is incorporated by reference in its entirety.

[0056] The quality of the biodiesel strongly depends on the amount of insoluble material that it contains. This may be measured using a standard filter blocking test such as that according to ASTM method D 2068 "Standard Test Method for Filter Blocking Tendency of Distillate Fuel Oils", Total Contamination Test according to EN12662: 1998 or ASTM D7321-11, and Cold Soak Filtration Test according to ASTM D7501-12. In general, when steryl glycosides are removed in accordance with the present invention, the biodiesel is of better quality when compared with a comparable control biodiesel in which steryl glycosides have not been removed.

[0057] In some embodiments, the sample comprises other insoluble compounds, such as sterol esters, sterol alkyl esters, sulfated sterol glucosides, and waxes.

[0058] In some embodiments, the methods as described herein are used to remove steryl glycosides from a sample prior to processing to produce biodiesel. In other embodiments, the methods described are used after biodiesel production. In some embodiments, the methods are used in combination with other methods for removing unwanted components, such as distillation or filtration.

II. Enzymes

[0059] The invention as described herein encompasses a variety of enzymes for reducing the amounts of steryl glycosides. As used herein, an enzyme refers to a polypeptide or ribozyme that can catalyze a chemical reaction.

[0060] Steryl glycosidases are enzymes capable of hydrolyzing the glycosidic bond in a steryl glycoside and/or an acylated steryl glycoside to produce a free sugar residue and a free sterol, an example of which is shown in FIG.1. Steryl glycosidases include but are not limited to glucosidase enzymes, such as β-glucosidase or amylglucosidase enzymes.

[0061] To efficiently perform enzymatic reduction of SGs at high temperatures, thermostable steryl glycosidases are needed. Thermostable enzymes are enzymes that retain at least a
portion of their activity at high temperatures. For example, thermostable enzymes can retain a percentage of its peak activity above the temperature required for peak activity. Such temperatures may be at or above about 50°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 98°C, 100°C, 105°C, 110°C, or 115°C. In some embodiments, the percentage of activity retained at any of the above temperatures is least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, or 99%, or more than 99% of peak activity. In some embodiments, thermostable enzymes are thermophilic enzymes, wherein the peak activity of the enzyme occurs at relatively higher temperature such as at or above 50°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 98°C, 100°C, 105°C, 110°C, or 115°C. In some embodiments, thermostable enzymes are thermostable enzymes, wherein the peak activity of the enzyme occurs at between 80 and 90°C.

[0062] Enzymes may be of naturally occurring wild-type sequences, or natural or artificially generated variants. Variants may have an amino acid sequence that is at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a wild-type enzyme. In some embodiments, the enzyme has at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 92%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identity, or 100% identity to a sequence listed in Table 1. Variants can comprise any type of variation, including but not limited to genetic mutations such as point mutations, insertions, deletions, or transversions.

[0063] In some embodiments, the enzymes are variants of naturally occurring, wild-type enzymes. In some embodiments, the variant enzymes have increased activity compared to wild-type enzymes. In some embodiments, the variant enzymes have better activity than wild-type enzymes under the conditions used to purify biodiesel, such as ionic content, pressure, or temperature. In some embodiments, the variant enzymes are more stable or have better activity in certain temperature ranges compared to wild-type enzymes, such as at temperatures above about 25°C, above about 37°C, above about 45°C, above about 50°C, above about 65°C, above about 70°C, between about 25°C and about 50°C, between about 25°C and about 65°C, between about 37°C and about 50°C, between about 37°C and about 65°C, between about 45°C and about 65°C, between about 25°C and about 110°C, between about 37°C and about 110°C, between about 45°C and about 110°C, between about 50°C and about 110°C, between about 60°C and about 110°C, between about 70°C and about 110°C, between about 80°C and about 110°C, between about 37°C and about 95°C, between about 45°C and about 95°C, between about 50°C and about 95°C, between about 60°C and about
95°C, and between about 70°C and about 95°C. In some embodiments, the variant enzymes are thermostable or thermophilic versions of wild-type enzymes. In some embodiments, thermostable or thermophilic enzymes can be wild-type enzymes, such as those isolated from thermophiles.

[0064] Sterol glycosidase activity can be determined by measuring the glucose resulting from the hydrolysis of sterol glycoside, by means of colorimetric methods such as the Glucose Oxidase assay. In some embodiments, sterol glycosidase activity is measured by mixing a sterol glycosidase in appropriate buffer with water and the enzyme to be tested. The reaction mixture is incubated at a selected temperature in a shaking incubator. Sterol products are extracted with chloroform, and the chloroform phase removed and evaporated, such as under nitrogen. The resulting sample is then analyzed, for example by using HPTLC or mass spectroscopy, to determine the presence and, optionally, the amount of sterol produced. Further details on an example method of how to assay sterol glycosidase activity can be found in WO2010/004423. In some embodiments, enzyme activity is measured over a range of temperatures. In some embodiments, peak activity is calculated as enzyme activity at an optimal temperature at which the enzyme has highest activity.

A. Identifying proteins with sterol glycosidase activity

[0065] Preparations containing mixtures of unidentified enzymes with sterol glucosidase activity have been described but no polypeptides carrying such specific activity have been identified. There also various enzymes that are known to have beta-glusidase activity. However, there is no known report that these enzymes possess sterol glycosidase. Inventors of the present invention surprisingly discovered that certain beta-glusidase also have sterol glycosidase, some of which are provided herein in Table I. Presented in Table I are polypeptide sequences of non-limiting examples of enzymes capable of hydrolyzing the glycosidic bond of sterol glycosides or acylated sterol glycosides to form a sugar and a corresponding sterol or acylated sterol. Other sterol glycosidase enzymes can be isolated and/or identified from cells or cellular extracts using methods known in the art.

[0066] Enzymes suitable for use with this invention can be found and isolated from a variety of species, including animals, plants, protists, microbes, and fungi. In some embodiments, suitable enzymes can be isolated from thermophilic species. Examples of species that may contain lipases or sterol glycosidase suitable for use with the invention include species of the genus Sulfolobus, including S. acidocaldarius, S. islandicus and S. solfataricus; Pyrococcus, including P. horikoshii and P. furiosus, Caldivirga such as C. maquilingensis; Vulcanisaeta,
including *V. distributa* and *V. moutnovskia*; *Acidilobus* such as *A. saccharovorans*; *Thermoproteus* such as *T. uzoniensis*; *Thermoplasma* such as *T. volcanium*; *Ignisphaera* such as *I. aggregans*; *Thermosphaera* such as *T. aggregans*; *Thermococcus*, including *T. litoralis, T. kodakarensis, T. barophilus, T. alcaliphilus* and *T. sibiricus*; *Aciduliprofundum* such as *A. boonei*; *Aspergillus*, including *A. niger, A. aculeatus, A. flavus, A. kawachii, A. oryzae, A. terreus*; *Thermomyces* such as *T. lanuginosa*; *Candida*, including *C. Antarctica* and *C. albicans*; *Saccharomyces*, such as *S. cerevisiae*.

[0067] Steryl glycosidases can be identified by methods known in the art, such as by biochemical purification from fractional extracts with glycosidase activity. Such fractional extracts may be taken from cellular samples, such as lysates, or from mixed compositions comprising glycosidase activity. Some such mixed compositions are commercially available, such as Grindamyl™ Ca 150 (available from Danisco A/S). Other suitable enzymes include but are not limited to amyloglucosidases such as AMG8000 (available from Danisco A/S). Glycosidase activity can be measured by any method described herein or known in the art.

<table>
<thead>
<tr>
<th>Sequence ID No.: 1</th>
<th>Species</th>
<th>Amino Acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEQ ID</td>
<td><em>Sulfolobus acidocaldarius</em></td>
<td>MLSFPKGFKFGSQGSEQMTPGSEDPSND WHVWVDREHINSVQVVGDLPGPYPGWN YKRFHDEAEKIQLNAVRINVEWSRPFLPKP EMQGTDKENPVISVLNKLREMNDNYAN HEALSHYRQILEDLRNRGFHIVLMNYWTLPLI WLHDPIRVRRGDFGSTGWLNSRTYEFARFS AYVAYAWKLDDASEYATMNENPVVWGAGYAF PRAGFPNPYLNSFLSEIAKWNIIIQAHARAYDAI KSVSNKSVIGYANTYRLQPDNEAVEIAER LNRWSFFDSIIKGEITSEGQVNRDELNRRLWDI GVNYYTRTVTAKESGYLTLPYGDRCERNLSLANTPSDFGWFFEPFEGYDLKLYWNYR GLPLYVMENIADDADYQRPPYYLVSHYIQVH RALNEGVDRGILHWSLADNYEWSGSFMRSFGLKVDYLTKRLYWRPSALYREIITRSNGIPEE LEHLNRVPIIKPLRH</td>
</tr>
<tr>
<td>SEQ ID No.: 2</td>
<td><em>Sulfolobus solfatarius</em></td>
<td>MYSFPSNFSGFSQAGFSEQMTPGSEDPSNTD WYKVWHPENMAAGLVSGLPENPGYPGWN NYKTFHDNIAQKMGLKIJARNVWEWSRFIPNLP RPQNFDESKQDVETEVEINEELKRLDEYANKDALNHYREIFDLKSRGLYFLINMYHWPLPWL HDPIRVRRGDFTGPSGLWSTRTYEFARFSAY1 AWKFDDLVDEYSTMNENPVVGGGLGYVGKGFPPYGLSFEFSRAMYNIIQAHARAYDGKSV SKKPVGIIYANNSSFQPLTDKMEAVEMAENDN</td>
</tr>
<tr>
<td>SEQ ID No.: 3</td>
<td><em>Sulfolobus islandicus</em></td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MYSFPKNFRFGWSQAGFQSEMGTGPGSEDNT DWYWVWHDPENIAAGLVSGLDPENGPGYWYNGYKTFFHDNAQKMGKLKMARLNVEWSRIFPNNL PKQNFDESQDVTEVEINQELRLDEHANK DLALNYREIFDLKSRGITYFLNYMHWPSW LHDPIVRRGDLSGPTGWLSRTYEFARFSA YIAWKFDDLVDEYSTMNEPNVVGGLGYGVC RSGFPPGYLSFELSRTKAMYNNIQAHVRAYDGIK SVSKKPPIIYANSSFQPLTEKDAEVAEYID NREWAFFDAIIRGEIMKGEKVVRRDDLRLGD WIGVNYYRTVVKKTEKYVSLGGYGHCER NSVSLAGLPTSDFGEFPEGLYDVLKTVWNRY YLHMYVTENGIADDADYQRPYYLYVSHVYYQ VRHAINSSADVGYLHWSDLNAYEWASGFSM RFGLKVDYTFRKLYWRSALVYREIAATNGGI TDEIEHLLNSVIPPRPLRHL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SEQ ID No.: 4</th>
<th><em>Caldivirga maquilingensis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDISFPKSKFRFGWSQAGFQSEMGTGPGSEDNT DWYWVWHDPENIASGLVSGLDPENGPGYWGL YRMFHDNAVKMGLDIARINVEWSRIFPKPMPD PPQNGNVEKGNDLVAHVDENDLRLDEAA NQEAVRHREIFSMDLAKRIGHFLFNYHWPLPL WVHDPIRVRRKGLDLSGPTGWLDVKTVPINFARF AAAYTAWKFDDLADYEYSTMNEPNVHSVNGYM WVKSGFPSSYLNFELESRTVMVNLIAHARAY DAVKAISKKPPIYANSSFPTLTDKAVAL AEYDSRWIFFDAIIGELMGVTDDDLKGRDL DWIGVNYYSRTVVKLIEKSYVSIPGYGCERNS ISPDBGPCSDFGWEFYEPGLYDVMKYWSRYH LPIYVTENGIADDADYQRPYYLYVSHVYYQ VREIANSSADVGYLHWSDLNAYEWASGFSMRFGL LWQVYSTTKKQYWRPSAYVYYREIAKSAIPEEL MHLNTPPRSLRRL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SEQ ID No.: 5</th>
<th><em>Vulcanisaeta distributa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTLSFPKGFRFGWSQAGFQHEMGIPGDETTNS DWWWVWHDRDNIVSLGVSGLDPENGPGYWS LRYVFFHFNARMLDIARYNVEWSRIFPKPM PEPPNGVVFVKIDVLERDDLRLDETA NAKAAEHYRAIFNDLRNRIFIYNLYHWPLPL WVHDPIRVRRKGLDLSGPTGWLDIKTVINFARFA AYAVAWKLDLVDMYSTMNENPVWAWNGYN VKSFGPPSSYNPLARKALVNLIQAARAYDA IKTVSRKPGIYANNAYTPTKEKSAVELAE QDARWSFFDAVHGNLYGEVREDLRNRLDWI GANYYSRLVVKLIDNSYAIVPGYGHACERNS</td>
</tr>
</tbody>
</table>
Vulcanisaeta moutnovskia

MTLSFPQDFRFGWSQAGFQHEMGIPGDEDPSNS DWWWVVVHVRDNISAGLVSGLDLPENGPGYWS LRYRFHDNAVRMGLDIARINVEWSRFVKPM PEPPSGNVENVGDNVIKVDVERDRLRLDEAA NKAAVEHYRFMNFNLKNRNIFFFILNLHWP LWHIDSPIVRVGRGLSGPTGWLDVKTVINARF AAYVAWFDDLDVDMYSTNEPNVAYAGY ANVKSFGPPGYNPLGLRALLNILQAHARY DAIAISKRPVIIYANNAYPLTEKDAAGVEL AEQDARWSFFDAIIIHGNLYGEVRDDLRLGRLLD WIGVNYYSRLVVRILLGENSYVVPYGHACE RNSVSPDNKPCSDGFWEFYEGLYDVLMKYW RYRLPMYYTGIAAAADYLRPYLVSHVY QVHRALGDAGVRGLHWSLTDNYEWASGF SMRFGLLYVDYSSKKQYWRSPAYIYREIAMN KAIPDELMLNAVPPRPLRR

Vulcanisaeta distributa

MTLSFPKGFGRFGWSQAGFQHEMGIPGDEDNTNS DWWWVVVHVRDNISAGLVSGLDLPENGPGYWS LRYRFHDNAVRMGLDIARINVEWSRFPKPM PEPPNGNVENVGDVVIKVDVERDRLRLDEFA NKAIEHYRAIFNLKNRNIFFFILNLHWP LWHIDSPIVRKGLLSGPTGWLDIKTVINARFA AAYVAKLDLDDVDMYSTNEPNVAVNGYNV VGKSFAPPGLPLARKALVLNIQAHAHAYDA IKTVSRKPVGIYANNAYPTLEKDSKAVEAE QDARWSFFDAIHGNLYGEVRREDLRNLDWI GANYYSRLVVKLIDNSYAIVPGYHACE RNSVSPNRCSDGFWEFYEGLYDVLTKYWRRY HLPYVTENGIADSDAYLRPPYLVSHIYQYVR ALSGDVGVRGYHLHWSLTDNYEWASGFMSRF GLLYVDYTTKQYWRSPAYIYREIALNKAI PD ELMHLNTIPVRSLRK

Vulcanisaeta moutnovskia

MTLSFPQDFRFGWSQAGFQHEMGIPGDEDPSNS DWWWVVVHVRDNISAGLVSGLDLPENGPGYWS LRYRFHDNAVRMGLDIARINVEWSRFVKPM PEPPSGNVENVGDNVIKVDVERDRLRLDEAA NKAAVEHYRFMNFNLKNRNIFFFILNLHWP LWHIDSPIVRVGRGLSGPTGWLDVKTVINARF AAYVAWFDDLDVDMYSTNEPNVAYAGY ANVKSFGPPGYNPLGLRALLNILQAHARY DAIAISKRPVIIYANNAYPLTEKDAAGVEL AEQDARWSFFDAIIIHGNLYGEVRDDLRLGRLLD WIGVNYYSRLVVRILLGENSYVVPYGHACE RNSVSPDNKPCSDGFWEFYEGLYDVLMKYW RYRLPMYYTGIAAAADYLRPYLVSHVY
<table>
<thead>
<tr>
<th>SEQ ID</th>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.:  9</td>
<td>Acidilobus saccharovorans</td>
<td>MAVTFPKDFLGWSQAGFQOSEMGTPGSEDPSNDWYAWVHDREIAAGLVSGDFPENGPGYWGNYRKFDAAAQAMGTLAARIGVEWSRIFPRPFTDVKVDAEKGGDVLSEYVSVSEALEQDLKMANRDAINHYREMFSDLRSRGITFILNYHWPLPLWLHDPAIARRGANLSPSGWLDRVTVIEFAKSAYVAWKLDLAVYMYSTMNEPVVVWGLGYYAACKSGFPPYGFLCLECAGRAMKNIQLVQAHARAAYDAVKAITKKPVGVIYANSDFTPLTDADREAAERAKFDNRWAFFDAVVRGQLGSTRDDLKGRLDWIGVNYYTRQVRARSGYEIPGYPGHCEPNGVSPAGRPCSDFGEFPEGLYNVLKEYWDRYHLPLLVTENIADEGDYQRPPYLVSHVYQVHRALQDGVNVIGLYHWLSLADNYEWASGFSKRFGLMVYDSTKRLHWPSAFIYREIASKRATIDIEIELNSVPPLRGLSPGHRSEQ ID No.:  10</td>
</tr>
<tr>
<td>SEQ ID No.: 12</td>
<td>Ignisphaera aggregans</td>
<td>MGLKYPKEFIFGSESGFQFEMGLPGSEDPNTD WWWVHDPENIATLVSQDFPENGPGYWHL YRQDHDIAERLGMDGARIGIEWSRIFSKPTFVKVDVARDERGNLYVIDVAEKALEELDRIANKD AVNHYREILSDWKNGKLIINLYHWTLPLWL HDPIKVRKLGDRAPAGWVDERTVIEFLKYA VIAWKLGLDPLDLWCTMNENPVVYYSISYNKIG YGPPGYLSFEAASKAMKHLEVAHAVARYEVLK RFTNKPVGIIYVTTHTHEPLKEDRDVAAAMAQAVFDFLDSITGRSMSIGERKDLEKHDLWVIG NYYSLRLLVVERYGNAWRELPGYPGFACPGGTS LAGRPCNDAWGITYPEGLYIMLKRCWERYRL PIIVTENGTADAIRLPRLYLATHLYWVKAL SEGVIDIRGLHLWALVDNYEWSGFRMRGFLV HVDFTETKRYLRPSALLFREIASSKEIPDFEHHMTQPQILLI</td>
</tr>
<tr>
<td>SEQ ID No.: 13</td>
<td>Thermosphaera aggregans</td>
<td>MKFPKDFMGYSSSPFQFEAGIPGSEDPNSDW WWWVHDPENATAAGLVSQDLPGPwynly KNHDHDKLGVNTIRVGEWSRIFSKPTFNV KPVPERDENGSIHVHDVDKAVLDELANK EAVNHYVEMYKDWVERGKLLINLYHWPILPL WLFIPMVRMRGPDRASGWLNLESSEFVAFAK YAYAIAWKMGLPVMWSTMNENPVVYEQGYYMFVKGGFPYLSFEAADMKARRNMQAHARA YDNIKRFSSKVPLGLYAFQWFLEEGPAEVFD KFKSSSKLYYFTDVSQSGSSIAENYRRDLANRL DWLGVMNYSLYVKIVDDKPIILHGYFLCCTP GGISPAENPSCDSEGWEVYPEGLYLLKELKLYNGERGYNMCAWALVDNDRPRVLYLVSHPVSYV WKA VWNEIGPVKGHLWSLTDNYEQAGQFRQ KFGLVMVDKFKKRYLRPSALVFREIAATHNGIPDELQHNLIIQ</td>
</tr>
<tr>
<td>SEQ ID No.: 14</td>
<td>Caldivirga maquilingensis</td>
<td>MIKFPSDFFRFSTVGTVQHEMGTPGSEFVSDW YVWLHDPENIASGLVSQDLPEHGPYWDLYK QDHSIARDLGLDAAWITIEWARVFKPFPTFDVK VKVDEDDGQVVDVEVNESEALELRLGLDN AVNHYRGLSDLKRGGLVNLHYHWAMPT IWLHDPIAVRKNPGDRASPGLWLDSRVSIEFTKF AAFAIAHELGDMLMYTMNPEGVVICELY YVSFGPGPYLDNLAATAGKHLIEAHARAAYADA IKAYSSRKPVGLYFVDYPLQDGDEAVKEA KGLDDSFDAPIKGELMGVTDRLKGRKDNYVG VNYTRAVLRRQDGARVAVVGFSGYSE PGGVSNDRPCSDSEGWEYPEGVYNVLMLDWR RYRPMYITENGIADEHDKWRSFIVSHLY QIHRAAMEGVVDVRGFLHWLIDNLEWAAGY RMRFGLVYVDYATKRKYFRPSALVMREVAKQ KAIPDYLEHYIKPPRIE</td>
</tr>
<tr>
<td>SEQ ID</td>
<td>Pyrococcus</td>
<td>PLKFPEELFGTATAAHQIEGDNKWNDDWWYY</td>
</tr>
<tr>
<td>No.</td>
<td>Species</td>
<td>Sequence Description</td>
</tr>
<tr>
<td>------</td>
<td>-----------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>15</td>
<td>furiosus</td>
<td>EQIGKLPYKSGKACNHWEFYKEDIQLMASLG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YNAYRFSEWSRLFPEENKFANEAFNYQEIID</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLLANNITPLVTLLHFTSLWFMMKGGFLREE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NLKFWEKYVEKVAELLEKVKLATFNEMPMVY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VMMGTLAYWPPFISKPFKAVSNLLKAH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALAYEILHGFKFQGVIKNVPIMLPATDKERDK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KAAERADNLNFNYFLDAIWGKYRGAFKAYR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VPQSDADFIGINYYTASEVRHSWNPLKFFFDATA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KLADEVRSERKQMGWSVPRGIIALKKASKY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GKPILYIENIATLDDEWRFLIIQHLQYVHKAI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EDGLDVRGYFYWSFDNYEWEHGFRPFGFLVEV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EVDYETEFERRPRKSAIYGGIAKSEIKDEILEK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YGLSSL</td>
</tr>
<tr>
<td>16</td>
<td>horikoshii</td>
<td>PLKFPFMFLFGTATSHQIEGNNRNWNDWYYY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EQIGKLPYRSGBKACNHWEYRDIQMLTSLG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAYRFSEWSRLFPEENKFNAFMDKAEYREIDLE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLLTRGITPLVTLLHFTSLWFMMKGGFLREELN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KHEWYIEKVAELLEKVKLATFNEMPMVYVM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGYLTAYWPPFIRSPFKAFLVAANLLKAI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YELHLHGFKFQGVKNIPILDASDRDKAKAE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KADNLNFWHFLDAIWGKYRGKFYTRIPQPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DADFIGVNYYTASEVRHTWNPLKFFFEVKLAD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ISERKQMGWSVYPIGIYALKKASRYGRPL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YITENIATLDDEWRVFIQHLQYVHKAIEDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LDVRGYFYWSFDNYEWEHGFRPFGFLVEV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EVDYETEFERRPRKSAIYGGIAKSEIKDEILEK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YGLPQL</td>
</tr>
<tr>
<td>17</td>
<td>litoralis</td>
<td>FPEKFLFGTSTAAHVEGDNRWNDWYYEEI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GKLFPYKSGKACNWGELYREDIEMAQLGYNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YRFSEWSRLFPIEVEGKFNEDAFNRYREIELLE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KGTIPNVTLLHFTSLWFMRKGGFLKEENLK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WEKYVDKAELLKGVKLAVTFSNEMPMYVM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MGYLTAYWPPFVKSPFKAFVANNLLKAI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YDLHGNFDVGKIVKNIPLMPASREKDI AQK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KADNLNFWHFLDAIWGKYRGKFYTRIPQPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DVDFIGVNYYTASEVRHSWNPLKFFFDALKAD</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LSRKTDGWSVYPIGIYALKVSYRKPM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YITENIATLEDWEWFIQHLQYVHKALNDG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FDRLGKYFYWSFDNYEWEHGFRPFGFLVEV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YTTFERRPRKSAIYGGIAKSEIKDEILEK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YGLPQL</td>
</tr>
<tr>
<td>18</td>
<td>sibiricus</td>
<td>NAVIVFPKSFLFGTATSHQIEGNNKWNDWW</td>
</tr>
<tr>
<td></td>
<td></td>
<td>YYEIQGKLPYKSGKACNHWEYKEDISLMHSL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GYDGYRFSEWSRFPKENEIDALNRYEIEIE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LLVKSgitPNTLLHFTSLPIFMRQGGFAKEE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NLKAYEQYVETFVAGILKDVKLVTFSNEMPM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VMMGTLAYWPPFVKSPFKAFVANNLLKAI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HAYEILSSRLKGVKIVKNIPLAASYMERDK</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KAAEKADNLNFWHFLDAIWGKYRGKFYTSTY</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VPESEVDFIGVNYYTASEVKYSWNPKFFFEA</td>
</tr>
<tr>
<td>SEQ ID No.:</td>
<td>Termococcus kodakarensis</td>
<td>Aciduliprofundum m boonei</td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>19</td>
<td>KLADLSERKTQMGWSVYPEGIYKAITAVSYE KPMYITENGIATLDDEWRKEFVVQHLQVYQK AIDEGYDVGDFYFWFMDNYYEWEKGFEPFG LIEIDYTHERKPVSSAYVYGEIAQPACKKEISEEL KKYGLKGL</td>
<td>MLSMSFPEKFLFGTSTAHHVQVGEFDNKNWDDWW YYYEMGKLPYKSGKACNHWELYREDIELMAE LGNYAINTSIESWRLFPEEGKFNEDAFNYREI IELLELGITPNVTLHHFTSLWFMKGGFLKLIE ENLKYMEEIVDKAELLKGVKLVATFNEPLY YVTGMYLYTAWYPPFKSFPSLRVANLKAHI AIAYELLHGFQVSIVKIRVLMPLERKGYDEKA AQQADNLNFWYFLDAISWGKYRGAFKTYSP ESADAFIVNYTASTVRRSLNPLKMFEEAKD AEIGERRTQMGWSVYPEGVLALRRASEYGR PLYVTENGIATLDDEWRKEFIIQHLQVLRAR IEGLDVRYFWSMNDNYESWREGFEPRFGLIE VDFETFPRPGSAYLYGEIARTKLPGEEDPD MDLFPDDHFIFGATSSYQIEGDNIWSDWYV AEGKRLPKAGKACNHWELYREDIELMLASNL PAYLSVEWARIFPEEGKLNESALERYQDIINDL LNKKGJTOMPILVHHTQPLMWFALKGGFEKDE NLKYYEYYSVIAELKGVELATFNEPMVY VAGYLMGWMPPFKNPAGKVAANLNAH AIAYELHGFRKVGEKNQHPIATSNKRDER AEARDVRYLFNWAFIDGIGFGHSVEFSKKKYKV NLESDFIGNYNNIQVKKSWNPLVFIVEDA SVSRKTDMGWSVYPEGKIFQHLYRRAIKGYGVN KGYFWSMNDNFEWKGAFPRFGLVEIANY VNFQKRPSAYVYGEISKTKIKDEVLEKYGES</td>
</tr>
<tr>
<td>20</td>
<td>MLKFPNNFIFGATStAHQIEGDINVNSDWYH ENMKGPYKSGKTCNHWNLYRDIELMQSFL YNAYRFSIEWARIFPEEGKIDKKALOYREIIN LLLNKKGIIPMTLHHFTLWFLLEKGGFAKEE NLKYYEYYSVIAELKGVELATFNEPMVYY VAGYLMGWMPPFKNPAGKVAANLNAH AIAYELHGFRKVGEKNQHPIATSNKRDER AEARDVRYLFNWAFIDGIGFGHSVEFSKKKYKV NLESDFIGNYNNIQVKKSWNPLVFIVEDA SVSRKTDMGWSVYPEGKIFQHLYRRAIKGYGVN KGYFWSMNDNFEWKGAFPRFGLVEIANY VNFQKRPSAYVYGEISKTKIKDEVLEKYGES</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>MLKFPDDHFIFGATSSYQIEGDNIWSDWYV AEGKRLPKAGKACNHWELYREDIELMLASNL PAYLSVEWARIFPEEGKLNESALERYQDIINDL LNKKGJTOMPILVHHTQPLMWFALKGGFEKDE NLKYYEYYSVIAELKGVELATFNEPMVY VAGYLMGWMPPFKNPAGKVAANLNAH AIAYELHGFRKVGEKNQHPIATSNKRDER AEARDVRYLFNWAFIDGIGFGHSVEFSKKKYKV NLESDFIGNYNNIQVKKSWNPLVFIVEDA SVSRKTDMGWSVYPEGKIFQHLYRRAIKGYGVN KGYFWSMNDNFEWKGAFPRFGLVEIANY VNFQKRPSAYVYGEISKTKIKDEVLEKYGES</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>MIVFPEFFLGATSSYQIEGDNIWSDWYV AEGKRLPKAGKACNHWELYREDIELMLASNL PAYLSVEWARIFPEEGKLNESALERYQDIINDL LNKKGJTOMPILVHHTQPLMWFALKGGFEKDE NLKYYEYYSVIAELKGVELATFNEPMVY VAGYLMGWMPPFKNPAGKVAANLNAH AIAYELHGFRKVGEKNQHPIATSNKRDER AEARDVRYLFNWAFIDGIGFGHSVEFSKKKYKV NLESDFIGNYNNIQVKKSWNPLVFIVEDA SVSRKTDMGWSVYPEGKIFQHLYRRAIKGYGVN KGYFWSMNDNFEWKGAFPRFGLVEIANY VNFQKRPSAYVYGEISKTKIKDEVLEKYGES</td>
<td></td>
</tr>
</tbody>
</table>

No.: 19

Termococcus kodakarensis

<table>
<thead>
<tr>
<th>SEQ ID No.:</th>
<th>Termococcus barophilus</th>
<th>Thermococcus alcaliphilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>MLKFPDDHFIFGATSSYQIEGDNIWSDWYV AEGKRLPKAGKACNHWELYREDIELMLASNL PAYLSVEWARIFPEEGKLNESALERYQDIINDL LNKKGJTOMPILVHHTQPLMWFALKGGFEKDE NLKYYEYYSVIAELKGVELATFNEPMVY VAGYLMGWMPPFKNPAGKVAANLNAH AIAYELHGFRKVGEKNQHPIATSNKRDER AEARDVRYLFNWAFIDGIGFGHSVEFSKKKYKV NLESDFIGNYNNIQVKKSWNPLVFIVEDA SVSRKTDMGWSVYPEGKIFQHLYRRAIKGYGVN KGYFWSMNDNFEWKGAFPRFGLVEIANY VNFQKRPSAYVYGEISKTKIKDEVLEKYGES</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>MIVFPEFFLGATSSYQIEGDNIWSDWYV AEGKRLPKAGKACNHWELYREDIELMLASNL PAYLSVEWARIFPEEGKLNESALERYQDIINDL LNKKGJTOMPILVHHTQPLMWFALKGGFEKDE NLKYYEYYSVIAELKGVELATFNEPMVY VAGYLMGWMPPFKNPAGKVAANLNAH AIAYELHGFRKVGEKNQHPIATSNKRDER AEARDVRYLFNWAFIDGIGFGHSVEFSKKKYKV NLESDFIGNYNNIQVKKSWNPLVFIVEDA SVSRKTDMGWSVYPEGKIFQHLYRRAIKGYGVN KGYFWSMNDNFEWKGAFPRFGLVEIANY VNFQKRPSAYVYGEISKTKIKDEVLEKYGES</td>
<td></td>
</tr>
</tbody>
</table>
Additional thermophilic D-glucosidases as disclosed in US 6,960,454, which is incorporated by reference in its entirety, can be used directly in the present invention or can used as starting point for further optimization using methods provided herein.

B. Developing a variant steryl glycosidase

In some embodiments, an identified enzyme with steryl glycosidase activity is used as a basis for generating a mutant enzyme with increased activity, such with greater catalytic efficiency, or stability, including but not limited to thermostability. In some embodiments, an identified enzyme or variant enzyme is modified to increase activity or stability, such as by post-translational modification. In some embodiments, the variant has an epigenetic difference from the original strain. In some embodiments, the variant is a mutant, e.g. the variant contains a mutation in the gene encoding the steryl glycosidase. A gene refers to a deoxyribonucleotide (DNA) sequence that encodes a polypeptide, such as a template steryl glycosidase. DNA can be natural, artificial, or a combination of both.

In some embodiments, the mutant enzyme may be generated by targeted mutation. In some embodiments, the mutations may be determined based on structural information about the enzyme or its homologs. For example, the enzyme can be rendered more stable under higher heat conditions by using cysteine mutations to create stabilizing disulfide bridges. In some embodiments, stabilizing mutations can be based on increasing charged or hydrophobic interactions between residues. In some embodiments, catalytic activity can be increased by mutations that affect the active site of the enzyme. Such mutations can, for example, be based on increasing homology to another, more active enzyme. In some embodiments, the mutant enzyme can be truncated, for example to remove an inhibitory domain. In some embodiments, the mutant enzyme can be a fusion protein, such as by fusion to a polypeptide, including but not limited to whole or partial proteins or domains or short peptide sequences.

In some embodiments, the variant enzyme is generated by random mutation, followed by selection for the desired activity. In some embodiments, the variant enzyme is generated
by directed evolution. Directed evolution generally consists of producing a population of variants around a template or starting sequence, then choosing variants with a desired property, such as steryl glycosidase activity, lipase activity, and/or thermostability.

[0072] In some embodiments, a gene encoding a previously identified enzyme is used as a template for directed evolution. The template gene may be used to generate mutated copies. Methods suitable for generating mutated copies include but are not limited to error-prone replication, targeted mutagenesis, or oligonucleotide directed mutagenesis.

[0073] In some embodiments, the genes are inserted into vectors under control of a promoter. In some embodiments, the promoter is used to control expression of a variant gene encoding the steryl glycosidase or lipase. Promoters for use with the invention may be, for example, inducible or constitutively active. By "inducible" is meant that promoter activity can be controlled by an inducing agent, such as a compound, peptide, ion, or other additive. Inducing agents include but are not limited to organic agents; inorganic agents; alcohols; neurotransmitters; antibiotics; peptides; carbohydrates; nucleic acids; hormones; drugs; light; toxins; and temperature. In some embodiments, inducible promoters are activated by an activator. In some embodiments, inducible promoters are repressed by a repressor.

[0074] In some embodiments, the inducible promoter is a switch, e.g. either active or silenced depending on whether an inducing agent is present. In some embodiments, the inducible promoter is tunable, e.g. the level of promoter activity varies based on the amount of inducing agent that is present. In some embodiments, the promoter is linearly tunable. A tunable promoter can control enzyme expression based on the concentration of inducing agent added to the screening medium, which is useful for controlling the degree of selection pressure as described herein.

[0075] Examples of promoters suitable for use in the invention include but are not limited to copper-inducible promoters and beta-estradiol dependent expression system (UASGALIO/GEV).

Transformation

[0076] In some embodiments, mutated copies of the template gene may be transformed into host cells. Host cells of the present invention can be of different types and from different organisms, which include, but are not limited to, bacteria, fungi (e.g. yeast), algae, plants, and animals. In some embodiments, the cell is a microorganism, such as yeast or microalgae. In some embodiments, the cells are yeast cells, including but not limited to *Saccharomyces cerevisiae*, *Saccharomyces boulardi*, *Pichia pastoris*, *Hansenula polymorpha* and *Schizosaccharomyces pombe*. Host cells comprising any of the variant genes of the invention
can form separate strains. Strains are, for example, clonal strains, e.g. isolated from an individual colony, or non-clonal, e.g. derived from a liquid culture sample.

[0077] Transformation can be performed by any method known in the art, including but not limited to electroporation, chemical transformation, transfection, use of a Ti plasmid, particle bombardment, transduction, or use of infectious agents. Methods of modifying gene expression or introducing one or more exogenous genes into a cell are known in the art. For example, methods of stably transforming cells and compositions comprising isolated nucleic acids of use are well known in the art and any such methods and compositions may be used in the practice of the present invention. Exemplary transformation methods of use may include microprojectile bombardment, electroporation, protoplast fusion, PEG-mediated transformation, DNA-coated silicon carbide whiskers or use of viral mediated transformation (see, e.g., Sanford et al., 1993, Meth. Enzymol. 217:483-509; Dunahay et al., 1997, Meth. Molec. Biol. 62:503-9; U.S. Pat. Nos. 5,270,175; 5,661,017). The method used can vary with the type or species of host cell. For example, particle bombardment may be more suitable for crossing through cell walls of plant cells.

[0078] In some embodiments, one or more exogenous genes are introduced into the host cells using a vector. In general, the vector comprises the nucleotide sequences encoding the exogenous gene and the regulatory elements necessary for the transformation and/or expression of gene in the host cell, such as the promoter sequences provided herein. In some embodiments, vectors are selected to optimize expression in the host cells used. For example, yeast expression vector YES2 can be used to express the genes in yeast host cells. In some embodiments, the vectors of the present invention comprise a backbone sequence. In some embodiments, the vectors of the present invention comprise a multiple cloning site, one or more regulatory elements to control the expression of the insert gene, as well as one or more markers for selection. Markers included are paromomycin resistance (Sizova et al, Gene 181:13-8 (1996)) and hygromycin B resistance (Berthold et al, Protist 153:401-12 (2002)).

[0079] In some embodiments, one or more exogenous genes are integrated into the genome of the host cells. In some embodiments, a vector containing an exogenous gene is introduced into the host cell, and the exogenous gene subsequently integrated into the host cell's genome. In some embodiments, homologous recombination is used for integration. In other embodiments, site-specific recombination is used, including but not limited to methods such as Cre-Lox recombination. In some embodiments, a retroviral or transposon-based system is used.
In some embodiments, mutated genes are generated in a host cell. Methods for generating such a variant population include but are not limited to inducing mutations in host cells containing the template gene, such as growing the host cells in a mutation-inducing environment. Mutation-inducing environments include but are not limited to UV radiation or treatment with mutagens, such as methylmethane sulphonate (MMS). Many other methods for generating a population of mutant genes are known in the art and are usable with the invention.

Generating mutations in the host cell may be advantageous because it does not require a transformation step before initial screening. However, generating random mutations in the host cell genome may also cause mutations in other genes, which may cause phenotypic effects that affect the screening process. In some embodiments, a gene encoding the target steryl glycosidase or lipase is isolated from a mutated host cell. In some embodiments, the gene is isolated from the mutated host cell before screening. In some embodiments, the gene is then transformed into one or more other host cells for screening in a consistent genetic background. In some embodiments, the gene is isolated from the mutated host cell after screening. In some embodiments, the isolated gene is tested for mutations, such as by single nucleotide polymorphism (SNP) detection, restriction analysis, or sequencing. In some embodiments, the gene is not isolated from a mutated host cell.

Screening

In some embodiments, after generating a population of variants, variants with the desired property are chosen by screening or selection methods, such as by positive selection. The number of investigated mutants is typically very large, up to $10^{11}$.

In some embodiments, the host cell used for screening is a yeast cell. In some embodiments, the host cell is an auxotrophic yeast cell that is unable to synthesize ergosterol, such as DY1457 Aheml (Crisp et al, J. Biol. Chem 278: 45499-45506). Ergosterol is a natural sterol that is a component of the yeast envelope. In the absence of an external source of sterols, such auxotrophic yeast cells are unable to form colonies.

In some embodiments, auxotrophic yeast cells are plated on a medium containing steryl glycoside. For example, to test for a steryl glycosidase, ergosterol glucoside can be included in the medium. Some amount of inducer is also included to stimulate expression of the variant steryl glycosidase gene. The lower the amount of inducer, the more selective the method will be. Only host cells expressing a steryl glycosidase with sufficient activity to digest steryl glycoside into a viable amount of sterols will form colonies. In some embodiments, cells from successful colonies are isolated, used for subsequent rounds of
directed evolution, and/or used to produce enzyme for treating biodiesel. In some embodiments, steryl glycosidase genes from successful colonies are also isolated for analysis, sequencing, transformation into another type of host cell, or used for subsequent rounds of directed evolution.

[0085] In some embodiments, the auxotrophic host cells are grown at a specific temperature to select for enzyme thermostability. In some embodiments, the host cells are grown at or above about 45°C, at or above about 50°C, at or above 55°C, at or above 60°C, at or above 65°C, at or above 70°C, at or above 75°C, at or above 80°C, at or above 85°C, at or above 90°C, or at or above 95°C.

[0086] Corresponding lipid auxotroph mutants, supplemental nutrients, and inducer levels as would be known to one of skill in the art may be used to select for variant lipases.

[0087] In some embodiments, the mutation and selection steps are repeated multiple times. In some embodiments, the subsequent selection step is performed with decreasing amounts of promoter activator, which allows each round of directed evolution to gradually increase enzyme activity. In some embodiments, the selection step is performed with increasing amounts of a promoter inhibitor. In some embodiments, subsequent selection steps are performed at increasing temperatures to gradually increase selective pressure for high thermostability. In other embodiments, the initial screening is performed with a low level of ergosterol or other corresponding essential nutrient, and subsequent screening rounds are performed with decreasing levels of the essential nutrient. This screening method is more suitable for use when the starting enzyme is relatively inefficient.

[0088] In some embodiments, between each round of selection, the variant gene is placed under the control of a weaker promoter. In these embodiments, the promoters can be inducible or constitutive. In one example, the initial selection step is performed while the variant gene is under the control of a strong promoter. In a subsequent selection step, the variant gene or a variant thereof is under the control of a medium promoter. In a still later selection step, the variant gene or a variant thereof is under the control of a weak promoter.

[0089] Exemplary strong promoters include promoters from the following genes: Photosystem II stability/assembly factor, Peptidyl-Prolyl cis-trans isomerase, histidinol dehydrogenase, malate dehydrogenase (NAD+) (Mdh2), and LHC (LhcII-1.3).

[0090] Exemplary medium promoters are the promoters from the following genes: Formate Nitrite transporter, ATP-dependent CLP protease proteolytic subunit, serine carboxypeptidase I, and 40S ribosomal protein S19.
[0091] An exemplary weak promoter is the promoter from the following gene: sterol-C-methyltransferase Erg6 like protein.

[0092] Similar methods as would be obvious to one of skill in the art can be used to select for lipases or phospholipases with greater activity or thermostability.

C. Enzyme production

[0093] Enzymes can be produced by small-scale methods, large-scale methods, industrial methods, or any other methods known in the art. In some embodiments, the sequence of the gene encoding the enzyme is a sequence known in the public domain, specifically the sequence that codes for the protein sequences provided herein (e.g. in Table 1). In some embodiments, the sequence is a sequence that is obtained by codon optimization. In some embodiments, the gene sequence is isolated from a screened colony as described herein, or is designed to encode such a protein.

[0094] In some embodiments, the enzyme is produced by in vitro translation. In some embodiments, the enzyme is chemically synthesized. In some embodiments, the enzyme is expressed in a host cell. Synthetic or isolated genes expressing an steryl glycosidase of the invention can be transformed and expressed in suitable host cells, including, but are not limiting to bacteria (e.g. E. coli), yeast, algae, filamentous fungi, plant, and mammalian cells.

[0095] In some embodiments, genes encoding the enzymes are optimized for expression in the host cell, for example by designing and synthesizing codon optimized sequences encoding the polypeptides. A summary of codon usage of C. reinhardtii is provided in Mayfield and Kindle, PNAS (1990) 87:2987-2991. Additional codon usage for different organisms are available at the Codon Usage Database (web address: www.kazusa.or.jp/codon/).

[0096] In some embodiments, the cells produce both the raw materials for generating biofuels and a variant enzyme of the invention. Host cells can be heterologous or homologous to the source of the original gene. In some embodiments, the host cells have normal or near-normal growth rates. In some embodiments, the variant gene is controlled by an inducible promoter, and the genetically engineered cells have a normal or near-normal growth rate while the promoter is not active.

[0097] In some embodiments, the variant gene is integrated into the host cell's genome in a location under the control of an endogenous promoter. In some embodiments, the variant gene is on a vector or integrated in the host cell's genome along with an exogenous promoter sequence.
In some embodiments, the expression of the transformed gene in the host cell is stable. By "stable expression" herein is meant that the transformed gene is retained in the host cell for at least 5, 10, 20, 50, 100, 200, 300, 400, or 500 generations, and being transcribed into RNA and/or expresses the protein it encodes. In general, a stable transformed gene is retained in the host cell for at least 1, 2, 3, 4, 5, 10, 15, 20 or 25 days, or for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months, and being transcribed into RNA and/or expresses the protein it encodes. In some embodiments, stable expression is in the presence of a promoter activator. Cells that do not express the transformed gene but retain the ability to express the gene such as in the presence of sufficient amounts of activator, are included as stably expressing cells. In some embodiments, the cells are stored, for example as a frozen stock for at least 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 months.

Expression includes constitutive or inducible expression. In some embodiments, the gene encoding a recombinant enzyme is under the control of an inducible promoter, including but not limited to promoters that can be activated by IPTG or Cu^{2+}. The host cell containing the inducible enzyme may be grown in liquid culture, in the absence of activators, until the host cell population is undergoing exponential growth. In bacterial host cells, the exponential growth phase is typically determined by light absorbance of 0.6-0.8 O.D. at 600 nm. The activator is then added to the liquid culture to induce expression of the enzyme. After several hours, the cells are harvested and typically lysed to release the enzyme. In some embodiments, protease inhibitors, reducing agents, or other additives are added to the lysed cells to preserve enzymatic activity.

In some embodiments, the gene encoding the recombinant enzyme is under the control of a constitutive promoter. In some embodiments, the promoter is a strong promoter. In these embodiments, cells can be directly lysed to release enzyme, without any induction step.

In some embodiments, the present invention describes production facilities to be used in large-scale enzyme production. In some embodiments, the facilities use yeast, bacteria, or algae cells that express the variant enzyme. In embodiments where yeast or bacteria are used to produce the enzymes, methods of growing the yeast and bacteria include fermenters, such as industrial-scale fermenters.

In embodiments where algae is used to produce the enzymes, methods of growing the algae include but are not limited to open raceway ponds, also known as high rate ponds (HRPs), or enclosed growth vessels, also known as photobioreactors (PBRs). Some
examples of PBRs include transparent plastic bags or plastic tubes with pumps to promote circulation.

[0103] In some embodiments, expressed enzymes are used without substantial isolation, or without substantial purification from the host cells. In some embodiments, expressed enzymes are isolated from host cells. Isolated enzymes refer to enzymes that are substantially free of at least one component of the host cell. In some embodiments, isolated enzymes are further purified, for example to at least 50% purity, at least 60% purity, at least 75%, purity, at least 80% purity, at least 90% purity, at least 95% purity, at least 98% purity, or about 100% purity. Purity refers to the total amount of enzyme in the composition by mass or molarity.

[0104] In some embodiments, isolated enzymes are further formulated for storage or for use in treating biodiesels or their oil precursors. Formulation steps include but are not limited to adding cofactors, chaperones, or other additives, performing post-translational modification of the enzyme, or adding preservatives such as protease inhibitors or reducing agents.

D. Using enzymes

[0105] The enzymes of the invention are suitable for use in reducing the amount of steryl glycosides in a sample. Methods for using the enzymes are also encompassed by the invention. Methods for using the enzymes generally comprise generating a reaction mixture comprising the enzyme and a sample comprising some amount of steryl glycosides.

[0106] In some embodiments, the method comprises the following steps: (i) adding a steryl glycosidase to biodiesel or oil sample with some amount of water, (ii) agitating the mixture at given temperature and shear rate for a period of time and (iii) separating the biodiesel or oil free of steryl glycosides. A schematic of an example procedure is illustrated in FIG. 2. In some embodiments, the steryl glycosidase is attached to a solid substrate. In some embodiments, the steryl glycosidase is attached to a bead or resin that is mixed with the sample, which allows for easier removal of the enzyme after the reaction is complete. In other embodiments, the resin-bound steryl glycosidase is packed into a column, and the sample flows through the column. In some embodiments, the steryl glycosidase is attached to a surface, such as the sides of a reaction vat or to a filter, and the sample allowed to flow across or through the surface.

[0107] In some embodiments, the steryl glycosidase is not isolated from the cell or cell lysate before use. For example, in some embodiments where the host cell produces both a biofuel precursor and the recombinant enzyme, the steryl glycosidase reaction is performed directly
in the cell or cell lysate. In some embodiments, such host cells secrete or excrete biofuel precursor that has already been treated by the steryl glycosidase. In other embodiments, the host cells are lysed to free the biofuel precursor.

[0108] In some embodiments, host cells expressing the enzyme can secrete or excrete the enzyme. In some embodiments, the enzyme can be present on the external surface of the cell. In some embodiments, the host cell is placed in a reaction container or on a reaction surface, and the sample is introduced to the container or surface. In one example, host cells expressing the enzyme are placed in a cylinder, and the biodiesel or biodiesel precursor is forced to flow through the cylinder.

[0109] In some embodiments, water comprises at least 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.5%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, or 30% by weight of the reaction mixture. In some embodiments, water comprises about 0.1% to about 15%, about 0.5% to about 15%, about 1% to about 15%, about 7% to about 15%, about 10% to about 15%, about 0.1% to about 10%, about 0.5% to about 10%, about 1% to about 10%, about 0.1% to about 5%, about 0.5% to about 5%, about 1% to about 5%, or about 0.1% to about 3% by weight of the reaction mixture. Without being bound by any theory, water is used in enzymatic hydrolysis of molecules.

[0110] In some embodiments, methanol comprises at least 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.5%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% by weight of the reaction mixture. In some embodiments, methanol is present as a carryover of the transesterification process in biodiesel.

[0111] In some embodiments, the method comprises further adding other enzymes. In some embodiments, a lipase or phospholipase is added to reduce the amounts of SMGs. In some embodiments, the lipase or phospholipase reaction occurs simultaneously with the glycosidase reaction. In some embodiments, the method uses a lipase or phospholipase without using a steryl glycosidase. In some embodiments, the method uses more than one steryl glycosidase, lipase, and/or phospholipase. In some embodiments, an acyltransferase is use with or in place of a steryl glycosidase. An acyltransferase acylates a nonacylated steryl glycoside to form an acylated steryl glycoside, which is more soluble than nonacylated steryl glycosides. In some embodiments, another enzyme or additive is added to facilitate the reaction(s) or otherwise treat the biodiesel or biodiesel precursor. In some embodiments, enzymes or catalysts responsible for the transesterification reaction are added. In some
embodiments, additives such as emulsificants or cofactors are added. In some embodiments, such enzymes or additives are thermostable.

[0112] Enzymatic reactions of the invention are conducted under conditions suitable for enzyme activity, optionally with mixing. In some embodiments, the reactions are conducted at a temperature between about 50°C and about 110°C. In some embodiments, the reaction are conducted at temperatures above about 50°C, above about 55°C, above about 60°C, above about 65°C, above about 70°C, above about 75°C, above about 80°C, above about 85°C, above about 90°C, above about 95°C, or above about 100°C. In some embodiments, the reaction is performed at about 80°C or higher. Other appropriate temperatures may be selected based on the specific enzyme used, such as based on the peak activity of a thermostable or thermophilic enzyme. In some embodiments, the reaction occurs over at more than one temperature over the course of the reaction, such as over a range of temperatures.

[0113] Agitation of the reaction can be performed at any acceptable shear rate. In some embodiments, the shear rate is vigorous enough to allow full circulation of the liquid in the mixing vat. In some embodiments, the shear rate is between 10 and 5000 s⁻¹.

[0114] The reaction can be performed for any duration suitable for reducing the amount of steryl glycosides in the sample. The reaction time may depend on a variety of factors, including but not limited to the composition or volume of the sample to be treated, the viscosity of the sample, the rate of mixing, the amount of steryl glycosides to be digested, the temperature, and the amount or activity of the enzyme used. As some non-limiting examples, the reaction can be performed for at least 10, 15, 20, 25, or 30 minutes, at least 1 hour, at least 2 hours, at least 3 hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 7 hours, at least 8 hours, at least 9 hours, at least 10 hours, at least 11 hours, at least 12 hours, at least 16 hours, at least 20 hours, at least 24 hours, or for more than 24 hours. Similarly, the sample volume, the rate of mixing, the amount of enzyme, and the temperature used in the reaction may depend on any of the factors listed herein, including the length of the reaction. In some embodiments, the pH of the reactions is between about 3.0 and 8.0, such as between about 4.0 and about 7.6, between 5.0 and 7.0, or about pH 7.5.

[0115] In some embodiments, treatment with a steryl glycosidase, including but not limited to a thermostable steryl glycosidase, occurs before, during, and/or after the transesterification reaction. In some embodiments, a steryl glycosidase is added to a suitable biodiesel precursor at the same time as a catalyst used for transesterification.
In some embodiments, there is less steryl glycoside after steryl glycosidase treatment compared with untreated biodiesel or biodiesel precursor. Steryl glycosidase treatment can reduce the amount of a steryl glycoside in the biodiesel or biodiesel precursor by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or by about 100%. When referring to "reducing" or a "reduction" of the amount of steryl glycoside in an oil or fat (e.g. a biofuel substrate) or a biofuel - the term "reducing" or "reduction" means in comparison to a comparable oil or fat (e.g. biofuel substrate) or biofuel which is the same as the claimed biofuel substrate or biofuel except that no enzyme(s) in accordance with the present invention have been added.

In some embodiments, after treatment, the concentration of steryl glycosides is less than 100 ppm, less than 90 ppm, less than 80 ppm, less than 70 ppm, less than 60 ppm, less than 50 ppm, less than 40 ppm, less than 30 ppm, less than 29 ppm, less than 28 ppm, less than 27 ppm, less than 26 ppm, less than 25 ppm, less than 24 ppm, less than 23 ppm, less than 22 ppm, less than 21 ppm, less than 20 ppm, less than 15 ppm, less than 10 ppm, or less than 5 ppm by weight.

In some embodiments, after treatment with steryl glycosidase, the resulting biodiesel or biodiesel precursor is collected for sale, storage, transport, or further processing or refining.

### III. Biodiesel Production

Biodiesel production methods of the invention comprise producing biofuels from fatty acids or oils, and for enzymatic reduction of the amount of steryl glycosides in the biofuel.

The majority of biodiesel is produced by interesterification of triglycerides (e.g. oil and/or fats) with an alcohol, often in the presence of a catalyst, to form esters and glycerol. The catalyst is usually sodium or potassium hydroxide. As methanol and ethanol are the most commonly used alcohols in commercial biodiesel production, most commercially produced biodiesel comprises methyl or ethyl esters of fatty acids (called FAME and FAEE, respectively). However, longer chain alcohols may also be used. In some embodiments, the sample to be treated comprises a biofuel or biodiesel, such as FAME or FAEE. In some embodiments, the sample to be treated comprises a biofuel or biodiesel precursor.

In some embodiments, sample containing steryl glycosides, and at least one steryl glycosidase are combined in a reaction or mixing vat and mixed to form a reaction mixture, where enzymatic removal of steryl glycosides takes place. In some embodiments, other additives to enhance enzyme activity, enzyme thermostability, or steryl glycosides solubility
are included in the reaction mixture. In some embodiments, a chaperone protein is added to
enhance the enzyme's thermostability.

[0122] In some embodiments, such as that depicted in FIG.2, water is also added to the
reaction mixture. In some embodiments, water is included with the input material used to
produce the biofuel. In some embodiments, water or other additives are removed from the
biodiesel after the enzymatic reaction is complete. As some non-limiting examples, the
reaction can be performed for at least 30 minutes, at least 1 hour, at least 2 hours, at least 3
hours, at least 4 hours, at least 5 hours, at least 6 hours, at least 7 hours, at least 8 hours, at
least 9 hours, at least 10 hours, at least 11 hours, at least 12 hours, at least 16 hours, at least
20 hours, at least 24 hours, or for more than 24 hours.

[0123] In some embodiments, the reaction is maintained at a set temperature. The set
temperature can be determined based on the temperature-dependent activity of the steryl
glycosidase. Non-limiting examples of suitable temperatures include temperatures at about
or above 32°C, 37°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C, 98°C,
100°C, 105°C, 110°C, or 115°C.

[0124] In some embodiments, the concentration of steryl glycosidase is about 0.1-15g/ton
(gram of enzyme per ton of biodiesel.

[0125] In some embodiments, the reaction is carried out in the presence of a proper additive,
such as polyglycerol polyrincinoleate (ADMUL™), and emulsificants, such as lecithin.

[0126] In some embodiments, waste heat, such as that produced from electrical generators, is
used to maintain or help maintain the temperature of the reaction. In some embodiments,
solar or geothermal heat is used to maintain or help maintain the temperature of the reaction.

[0127] In some embodiments, the chemical products resulting from the steryl glycosidase
reaction are removed from the treated sample. In other embodiments, products resulting from
the enzymatic reactions are not removed. In some embodiments, other methods for removing
steryl glycosides or other precipitates, such as filtration, centrifugation or distillation, are also
used. Some non-limiting examples of supplemental methods for removing steryl glycosides
are described in PCT Publication Nos. WO2007/076163, WO2007/0 175091, and
WO2008/05 1984 herein incorporated by reference in its entirety. Such supplemental
methods can be performed prior to, after, or during enzymatic methods of the invention. In
some embodiments, filtration is performed on the biodiesel, such as using a filter with a
molecular weight cut-off of less than 1,000,000 g/mol. In some embodiments, filter aids are
used, such as adsorbents, boric acid, soap, sugars (including sucrose and glucose), salts such
as sodium chloride, citric acid, magnesium silicate, clay, diatomaceous earth, lecithin,
proteins, carbon, cellulose, silica hydrogel, or combinations thereof, to help remove steryl glycosides from the biodiesel. Filter aids tend to increase precipitation or aggregation of the steryl glycosides, which reduces the time needed to filter the entire mixture. In some embodiments, centrifugation is used to separate precipitates from the biodiesel. In some embodiments, filter aids are used to reduce the centrifugation time.

[0128] Biodiesel production systems of the invention can incorporate systems or system components of other biodiesel or biofuel production systems known in the art. In some embodiments, the biodiesel production system comprises a mixing vat for containing and/or mixing the reaction. In some embodiments, the biodiesel production system comprises a solid substrate to which the enzyme is attached. In some embodiments, the enzyme is bound to a filter and the enzymatic reaction occurs as sample flows through the filter. In some embodiments, the enzyme is attached to a bead or resin, such as in a column, and the enzymatic reaction occurs as sample flows through the resin. In some embodiments, the reaction occurs inside a host cell that produces both the sample and the enzyme. Examples of systems and methods for processing lipids into biofuel, can be found in the following patent publications, the entire contents of each of which are incorporated by reference herein: U.S. Patent Publication Nos. 2007/0010682, 2007/0131579, 2007/0135316, 2007/0135663, 2007/0135666, 2007/0135669, and 2007/0299291.

[0129] Biodiesels produced by the methods described herein can be used as an alternative fuel to petroleum diesel, or can be used as an additive in petroleum diesel. Often, a biodiesel/petroleum diesel blend comprises 20% biodiesel.

[0130] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.
EXAMPLES

Example 1

**[0131] Measuring steryl glycosidase activity**

**[0132]** Steryl glycosidase activity is measured as follows:

a) Prepare a stock solution of steryl glycoside by dissolving 10 milligrams (mg) of steryl glycoside in 1 milliliter (ml) of a 3:1 mixture of tetrahydrofuran:water.

b) Add the steryl glycoside to a final concentration of 100 microgram per milliliter (µg/ml) in 1 ml of a reaction mixture containing 50 millimolar (mM) Phosphate buffer pH 6.5 and 5 µg of steryl glycosidase in an Eppendorf tube.

c) Place the reaction mixture on a shaker incubator and incubate at 80°C for 4 h.

d) Extract the reaction mixture with 1 ml of ethyl acetate and evaporate to dryness under vacuum.

e) Re-dissolve the sample in 10 microliter (µl) of ethyl acetate

f) Analyze by thin layer chromatography (TLC) the presence of free sterols, generated as a result of steryl glycoside hydrolysis after the enzymatic treatment, using Hexane:Methanol 85:15 as running buffer and develop with p-anisaldehyde

d) Alternatively, analyze 50 µl sample of the reaction mixture for the presence of glucose using an assay based on the Hexokinase/Glucose 6-P-dehydrogenase method: the glucose generated by steryl glycoside hydrolysis is converted by Hexokinase (or Glucokinase) into glucose 6-phosphate. Glucose 6-phosphate is further oxidized by Glucose 6-P dehydrogenase, simultaneously reducing NADP+ to NADPH. The NADPH generated is detected by fluorescence (λεχ: 338 nm, λεη: 461 nm) and allows detection of glucose levels below 1 ppm. The assay can be adapted to microplates scale and detected in a Synergy microplate reader.

Example 2

**[0133] Identifying a steryl glycosidase**

**[0134]** The following is an example of how to identify a steryl glycosidase:

a) Clone a putative steryl glycosidase gene into a pET28a *E. coli* expression vector.

b) Transform the resulting plasmids by chemical transformation into a BL21(DE3) *E. coli* strain.
Grow a colony of the recombinant clone on 100 ml of LB at 37°C until cell density reaches an \( \text{OD}_{600} = 0.6 \).

Add 0.5 mM IPTG to the culture, and incubate at 24°C for 10 h.

Disrupt the \textit{E. coli} cells broth with three cycles of compression/decompression at 1000 bar in an APV homogenizers.

Heat the resulting liquid to 80°C, incubate for 10 minutes and centrifuge until clarification to separate solid materials in a sharpless centrifuge at 5000 g.

Collect the supernatant and analyze for steryl glycosidase activity as described in Example 1.

Some of the example proteins listed in Table 1 were determined to be steryl glycosidases, as depicted in FIG. 4.

Example 3

Generating a more active steryl glycosidase

The following is an example of how to generate a more active steryl glycosidase using directed evolution:

a) Create a library of mutated steryl glycosidase genes using error prone PCR.

b) Insert the library of mutated genes into a pCU1 yeast expression vector, which is under the control of a promoter that is linearly inducible by \( \text{Cu}^{2+} \).

c) Transform the resulting plasmids by chemical transformation into an auxotrophic yeast mutant cell unable to synthesize ergosterol, the natural sterol present in yeast envelope.

d) Plate the transformed cells onto a medium supplemented with steryl glycosides to a concentration of 20 mg/L and an initial concentration of the inducer of 100 micromolar (\( \mu \text{M} \)) \( \text{CuSO}_4 \).

e) Recover the steryl glycosidase gene from a resulting colony.

f) Use the steryl glycosidase for a new round of random mutagenesis and repeat the process described above in an iterative fashion. In each new selection, add a 10 \( \mu \text{M} \) lower concentration of the inducer to the medium.

Example 4

Large-scale steryl glycosidase production

The following is an example of how to produce a thermostable steryl glycosidase in a large-scale:
a) A synthetic DNA encoding a codon optimized version of a gene encoding any protein listed in Table 1 is cloned into the Ndel-EcoRI sites of the pET24b plasmid (Novagen, USA).

b) The resulting plasmid is transformed by electroporation into the BL21(DE3) E. coli strain.

c) A colony of the recombinant clone is grown on 100 ml of LB at 37°C until cell density reaches an OD₆₀₀=2.

d) The culture obtained above is transferred to a seed fermentor containing 10 liters (L) of HM medium (described below) and grown for 10 h at 35°C.

e) The culture is transferred to a 1000 L fermentor containing 600 L of HM medium and grown at 35°C until glucose exhaustion. An exponential feeding of a nutrient solution containing 600 g/L glucose and 15 g/L MgSO₄ is then initiated at a rate sufficient to maintain the specific growth rate at a value of 0.35 h⁻¹ ± 0.05. When OD₆₀₀ reaches a value of 80, 1 mM IPTG is added and the nutrient solution is fed at a constant rate of 25 ± 1 L/h for 10 h. Dissolved Oxygen concentration is kept at all time above 30% of saturation by enrichment of the air stream with pure oxygen when necessary. pH is maintained at 7 by the addition of NH₄OH.

f) At the end of the fermentation process, the broth is treated with three cycles of compression/decompression at 100 bar in an APV homogenizers to disrupt the E. coli cells.

g) The resulting liquid is heated to 80°C, incubated for 10 minutes and centrifuged until clarification to separate solid materials in a sharpless centrifuge at 5000 g.

h) (NH₄)₂SO₄ is added to 80% saturation to the clarified liquid, the mixture is incubated at 8°C for 3 h and the centrifuged in a sharpless centrifuge at 5000 g to obtain a brown paste.

i) The obtained paste is air dried and the resulting powder contains a steryl glycosidase with a purity above 70% as determined by polyacrylamide gel electrophoresis (PAGE) analysis.

**[0140]** HM medium: Glucose 10 g/L, Na₂HPO₄·7H₂O 0.6 g/L, KH₂PO₄ 6 g/L, K₂HPO₄ 4 g/L, (NH₄)₂HPO₄ 3 g/L, MgCl₂·6H₂O 2 g/L, and 1 mL/L of trace element solution containing (in g/L): Fe 10, ZnSO₄·7H₂O 2.5, CuSO₄·5H₂O 1, MnSO₄·5H₂O 1, Na₂B₄O₇·10H₂O 0.2, CaCl₂·2H₂O 5, NaMoO₄·2H₂O 1, CoCl₂·6H₂O 1; dissolved in 5 M HCl.
Example 5

[0141] Using steryl glycosidase to remove steryl glycosides from a biodiesel mixture

[0142] The following is an example of how to use a steryl glycosidase to remove steryl glycosides from a biodiesel mixture:

a) A 42.5 ml distilled biodiesel sample containing 100 ppm of steryl glycosides is mixed with 7.5 ml of a water solution containing 50 mM Phosphate buffer pH 6.5 and 300 µg of steryl glycosidase. Alternatively, 5% of the emulsifier polyglycerol polyricinoleate (ADMUL™) is added to the reaction mixture.

b) Transfer the mixture into a 50 ml vessel and incubate in a heating block with a magnetic stirrer for 4 h at 80°C, accompanied by stirring.

c) While the reaction takes place, take 1 ml samples every hour, separate the aqueous phase and analyze for the presence of glucose as described in the Example 1.

d) After the reaction ends, separate the aqueous and organic phases and analyze for the presence of glucose in the aqueous phase as described in the Example 1, and for the SG consumption by GC-FID detection in the organic phase as described elsewhere (J. Food Lipids 12 (2005) 124-140).

[0143] Experiments were performed using two different steryl glycosidase selected among those described in Table 1: LacS from S. solfataricus (SEQ ID NO.: 2) and TL from T. litoralis (SEQ ID NO.: 17). LacS could only exhibit steryl glycoside hydrolysis when reactions were performed in the presence of ADMUL™ (FIG. 5 and FIG. 6). Experiments performed with TL enzyme showed a higher and faster hydrolysis in Bio/water + ADMUL emulsions than LacS (data not shown). Moreover, TL could hydrolize steryl glycosides without the addition of any emulsifier, reaching almost 100% hydrolysis in 3 h (FIG. 6 and FIG. 7).

Example 6

[0144] Evaluation of steryl glycoside hydrolysis in crude biodiesel

[0145] The following is an example of how to use a steryl glycosidase to remove steryl glycosides from crude biodiesel:

a) A 850 ml crude biodiesel sample containing about 70 ppm of steryl glycosides is mixed with 150 ml of a water solution containing 50 mM Phosphate buffer pH 6.5 and 8 mg of steryl glycosidase.

b) Transfer the mixture into a 2 L erlenmeyer and incubate for 4 h at 80°C, accompanied by stirring.
c) While the reaction takes place, take 1ml samples every hour, separate the aqueous phase and analyze for the presence of glucose as described in the Example 1.

d) After the reaction ends, separate the organic phase and analyze for the presence of steryl glycoside by GC-FID and evaluate the quality of the resulting biodiesel by the methods currently used in the biodiesel industry (i.e. Total Contamination Test according to EN1 2662: 1998 and Cold Soak Filtration Test according to ASTM D75G1-12).

[0146] Experiments were performed using TL from \textit{T. litoralis} (SEQ ID No.: 17). TL could completely hydrolize steryl glycosides in 3 h (FIG. 8).
WHAT IS CLAIMED IS:

1. A method for reducing steryl glycoside in a sample, comprising:
   mixing a thermostable enzyme with a sample comprising steryl glycoside under a condition suitable for said thermostable enzyme for a suitable period of time to degrade said steryl glycoside, thereby reducing steryl glycoside in said sample to obtain a processed sample.

2. The method of claim 1, wherein said sample comprises oil, fat, or biofuel.

3. The method of claim 2, wherein said biofuel comprises biodiesel.

4. The method of claim 1, wherein said steryl glycoside comprises steryl glucoside.

5. The method of any one of claims 1 to 4, wherein said steryl glycoside has a solubility that is more than 50 ppm.

6. The method of any one of claims 1 to 4, wherein said steryl glycoside has a solubility that is more than 80 ppm.

7. The method of any one of claims 1 to 4, wherein said steryl glycoside has a solubility that is more than 100 ppm.

8. The method of any one of claims 1 to 7, wherein said thermostable enzyme is capable of hydrolyzing the glycosidic bond of a steryl glucoside or acylated steryl glucoside.

9. The method of any one of claims 1 to 7, wherein said thermostable enzyme comprises a glycosidase enzyme.

10. The method of any one of claims 1 to 7, wherein said thermostable enzyme comprises a glucosidase enzyme.

11. The method of any one of claims 1 to 7, wherein said thermostable enzyme comprises a variant of an enzyme selected from Table 1.

12. The method of any one of claims 1 to 7, wherein said thermostable enzyme comprises a variant having an amino acid sequence that has at least 95% identity to a sequence selected from the sequences of Table 1.
13. The method of any one of claims 1 to 7, wherein said thermostable enzyme comprises an amino acid sequence selected from the sequences of Table 1.

14. The method of any one of claims 1 to 13, wherein said sample comprises 0.1% to 30% of water.

15. The method of any one of claims 1 to 13, wherein said contacting step is carried out at a temperature that is between about 50 °C and about 110 °C.

16. The method of claim 15, wherein said contacting step is carried out at a temperature that is above about 65 °C.

17. The method of claim 15, wherein said contacting step is carried out at a temperature that is above about 70 °C.

18. The method of claim 15, wherein said contacting step is carried out at a temperature that is above about 85 °C.

19. The method of any one of claims 1 to 13, wherein said contacting step is carried for about 30 minutes to 24 hours.

20. The method of any one of claims 1 to 19, wherein the amount of said steryl glycoside is reduced by at least 20%.

21. The method of any one of claims 1 to 19, wherein the amount of said steryl glycoside is reduced by at least 80%.

22. The method of any one of claims 1 to 19, wherein said processed sample comprises less than about 20 ppm of said steryl glycoside.

23. The method of any one of claims 1 to 19, wherein said reaction mixture comprises an enzyme selected from the group consisting of beta-glucosidases, steryl-esterases, amyloglucosidases, and pectinases.

24. The method of any one of claims 1 to 23, further comprising collecting said processed sample.

25. An oil produced by the method of any one of claims 1 to 24.

26. An isolated thermostable enzyme that is capable of hydrolyzing the glycosidic bond of steryl glycosides or acylated steryl glycosides.
27. The thermostable enzyme of claim 26, wherein said enzyme has a hydrolytic activity of at least 5 g of steryl glycoside per gram of enzyme per hour at a temperature that is between about 50 °C and about 99 °C.

28. The thermostable enzyme of claim 26, wherein said thermostable enzyme has an activity of at least 5 g of steryl glycoside per gram of enzyme per hour at a temperature that is above about 65 °C.

29. The thermostable enzyme of claim 26, wherein said thermostable enzyme has an activity of at least 5 g of steryl glycoside per gram of enzyme per hour at a temperature that is above about 70 °C.

30. The thermostable enzyme of claim 26, wherein said thermostable enzyme has an activity of at least 5 g of steryl glycoside per gram of enzyme per hour at a temperature that is above about 75 °C.

31. The thermostable enzyme of claim 26, wherein said thermostable enzyme comprises a variant of one of the enzymes listed in Table 1.

32. The thermostable enzyme of claim 26, wherein said thermostable enzyme comprises a variant having an amino acid sequence that is at least 95% identical to the sequence of one of the enzymes listed in Table 1.

33. The thermostable enzyme of claim 26, wherein said thermostable enzyme comprises an amino acid sequence selected from the sequences of Table 1.

34. A method for generating a gene encoding a variant steryl glucosidase, comprising:

(a) growing in a cultural medium a plurality of host cells transformed with a first library of variant steryl glycosidase genes, wherein expression of each variant steryl glycosidase genes is under control of a promoter that linearly responds to concentrations of an inducer added to said culture medium, wherein said host cells require a sterol to grow and are unable to synthesize ergosterol, and wherein said cultural medium comprises steryl glycosides and a first concentration of said inducer so as to permit only a host cell expressing a variant steryl glycosidase with sufficient activity to form a colony; and

(b) recovering the variant steryl glycosidase gene from said colony.

35. The method of claim 34, further comprising iteratively repeating steps (a) and (b), wherein a subsequent library of variant steryl glucosidase genes is generated in each new iteration of said steps (a) and (b) from a previous variant steryl glycosidase gene recovered
from the previous iteration of said steps (a) and (b), and wherein a lower concentration of
said inducer is added to said cultural medium in each successive iteration of steps (a) and (b).

36. The method of claim 34, wherein said first library is generated using error prone PCR
or oligonucleotide directed mutagenesis.

37. The method of claim 34, wherein an expression vector comprising a yeast expression
vector is used to transform said cells.

38. The method of claim 34, wherein an expression vector is used to transform said cells,
and wherein said expression vector is inducible by Cu$^{2+}$ and beta-estradiol.

39. The method of claim 34, where said host cell comprises a yeast mutant cell.

40. The method of claim 34, further comprising designing and synthesizing codon
optimized sequences encoding said variant steryl glucosidase.

41. A method for producing a recombinant steryl glucosidase, comprising:

expressing said codon optimized sequences of claim 40 in a suitable heterologous host
cell to generate recombinant steryl glucosidase; and

isolating said recombinant steryl glucosidase.

42. The method of claim 34, wherein said growing occurs at or above 50°C.

43. The method of claim 34, wherein said growing occurs at or above 65°C.

44. The method of claim 34, wherein said growing occurs at or above 75°C.

45. The method of claim 34, wherein said growing occurs at or above 85°C.

46. A host cell expressing a gene produced by the method of claim 34.
Figure 2
Soybean biodiesel / SG (100 ppm)

Figure 3
Ni-NTA affinity chromatography purified proteins

1. GH Sulfolobus islandicus
2. GH Caldivirga maquiltingensis (YP_001540243)
3. GH Vulcanisaeta distibuta
4. GH Vulcanisaeta moutnovskia
5. BGal Acidilobus saccharovorans
6. BGal Thermoproteus uzoniensis
7. BGal Thermoplasma volcanium
8. GH Ignisphaera aggregans
9. GH Thermoplasma aggregans
10. GH Caldivirga maquiltingensis (YP_001540482)
11. LacS Sulfolobus solfataricus
12. BGal Thermococcus litoralis

Total amount of protein used in each assay:

<table>
<thead>
<tr>
<th>Amount (μg)</th>
<th>Protein Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - 1.1 μg</td>
<td>7 - 5.2 μg</td>
</tr>
<tr>
<td>2 - 4.2 μg</td>
<td>8 - 10.0 μg</td>
</tr>
<tr>
<td>3 - 1 μg</td>
<td>9 - 9.0 μg</td>
</tr>
<tr>
<td>4 - 0.9 μg</td>
<td>10 - 0.1 μg</td>
</tr>
<tr>
<td>5 - x</td>
<td>11 - 13 μg</td>
</tr>
<tr>
<td>6 - 1.4 μg</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4
Figure 5
Figure 6

SG hydrolysis in biodiesel/water emulsions without addition of emulsifier (40 ml vessel with horizontal magnetic stirring) - 80°C, pH 6.5
GC-FID analysis of biodiesel samples before and after enzymatic treatment

Figure 7
Control

TL treatment

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Contamination (ppm)</th>
<th>CSFT (seg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>61</td>
<td>457</td>
</tr>
<tr>
<td>TL</td>
<td>11</td>
<td>108</td>
</tr>
</tbody>
</table>

Figure 8
A. CLASSIFICATION OF SUBJECT MATTER
IPG(8) - C12P 19/16, 19/20; C12N 9/24 (2013.01)
USPC - 435/96, 435/98, 435/200
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12P 19/16, C12P 19/20; C12N 9/24 (2013.01)
USPC - 435/96, 435/98, 435/200

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB, USPT, USOC, EPAB, JPAB); PatBase; Google Scholar and Google.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>US 201 1/0173876 A1 (SOE) 21 July 201 (21.07.201) 1) para [0014], [0025], [0033], [0052], [0067], [0068], [0071], [0077], [01 13], [0121-[0124], [0148], [0167], [0168]</td>
<td>1-7</td>
</tr>
<tr>
<td>A</td>
<td>US 201 1/0059130 A1 (YUSIBOV) 10 March 201 (10.03.201) 1) SEQ ID No: 59; Table 3; para [0084]</td>
<td>1-7</td>
</tr>
</tbody>
</table>

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filling date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "Z" document member of the same patent family

Date of the actual completion of the international search: 26 June 2013 (26.06.2013)
Date of mailing of the international search report: 10 JUL 2013

Name and mailing address of the ISA/US
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-3201

Authorized officer: Lee W. Young
PCT Helpldesk: 571-272-4300
PCT OSP: 571-272-7774
### Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

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

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

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

3. [x] Claims Nos. 8-25
   - because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

- **Group I**: Claims 1-7, drawn to a method for reducing sterol glycosides in a sample, comprising: mixing a thermostable enzyme with a sample comprising sterol glycoside under a condition suitable for said thermostable enzyme for a suitable period of time to degrade said sterol glycoside, thereby reducing sterol glycoside in said sample to obtain a processed sample.

- **Group II**: Claims 26-33, drawn to an isolated thermostable enzyme that is capable of hydrolyzing the glycosidic bond of sterol glycosides or acylated glycosides.

Continued in Supplemental Box

- [ ] All required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

- [ ] All searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

- [ ] Only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

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

**Claims 1-7**

### Remark on Protest

- [ ] The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.

- [ ] The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.

- [ ] No protest accompanied the payment of additional search fees.
Continuation of Box No. III - Observations where unity of invention is lacking:

Group III: Claims 34-46, drawn to a method for generating a gene encoding a variant sterol glucosidase, comprising: (a) growing in a cultural medium a plurality of host cells transformed with a first library of variant sterol glycosidase genes, wherein expression of each variant sterol glycosidase genes is under control of a promoter that linearly responds to concentrations of an inducer added to said culture medium, wherein said host cells require a sterol to grow and are unable to synthesize ergosterol, and wherein said cultural medium comprises sterol glycosides and a first concentration of said inducer so as to permit only a host cell expressing a variant sterol glycosidase with sufficient activity to form a colony; and (b) recovering the variant sterol glycosidase gene from said colony.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features
Group I includes the special technical feature of a method for reducing sterol glycoside in a sample comprising: mixing a thermostable enzyme with a sample comprising sterol glycoside, not required by Group II-III.

Group II includes the special technical feature of an isolated thermostable enzyme, not required by Groups III.

Group II includes the special technical feature that the enzyme being isolated, not required by Group I.

Group III includes the special technical feature of a method for generating a gene encoding a variant sterol glucosidase comprising growing and recovering sterol glucosidase, not required by Groups I-II.

Common Technical Features
Groups I-III share the technical feature of an enzyme that is capable of hydrolyzing the glycosidic bond of sterol glycosides (a sterol glycosidase).

Groups I-II share the technical feature of a thermostable enzyme that is capable of hydrolyzing the glycosidic bond of sterol glycosides.

However, these shared technical features do not represent a contribution over the prior art, as being anticipated by WO 2010/004423 A2 (Soe).

Soe teaches the technical feature of an enzyme, sterol glycosidase (pg 16, in 20-33), that is capable of hydrolyzing the glycosidic bond of sterol glycosides (pg 3, in 31 to pg 4, in 10) and the technical feature of a thermostable enzyme (pg 20, in 26-29; temperature about 55 to 80°C, which is within the same range as in the instant specification para [0123] and instant claims 15-17) that is capable of hydrolyzing the glycosidic bond of sterol glycosides (pg 1, in 5-11, pg 3, in 31 to pg 4, in 10).

In addition, these common features are also anticipated by US 2011/0059130 A1 (Yusibov). Yusibov teaches a thermostable galactosidase enzyme that is capable of hydrolyzing/cleaving the glycosidic bond of glycosides (para [0084], Table 3, seq id no 59). Yusibov does not specifically identify either the glycosides or glycosidases as "steryl." However, Yusibov's glycosidases are inherently "steryl glycosidases," because Yusibov's galactosidase having seq id no 59 has 100% homology with the claimed instant "steryl glycosidase? having seq id no 1 listed in Table 1 in claim 33.

As the common technical features were known at the time of the invention, these cannot be considered special technical feature that would otherwise unify the groups.

Therefore, Groups I-III lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.