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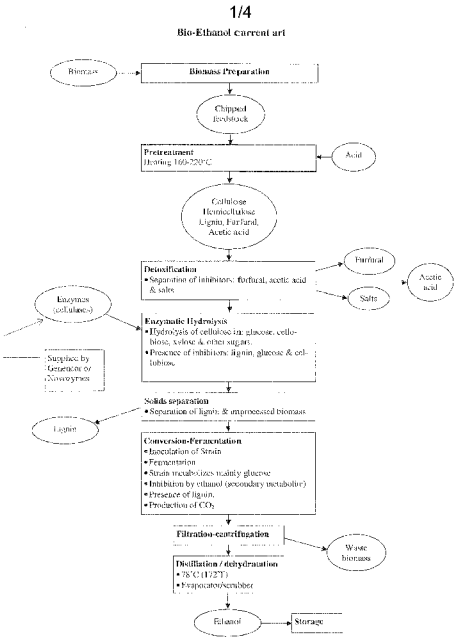

FIG. 1
PRIOR ART

(57) Abstract: The present invention relates to a method of producing fatty acids, by (i) inoculating a mixture of at least one of cellulose, hemicellulose, and lignin with at least one microorganism strain that produces one or more cellulases, hemicellulases and laccase, that hydrolyze at least one of cellulose, hemicellulose and lignin, under conditions to produce at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars; (ii) inhibiting growth of said at least one microorganism strain; (iii) inoculating the mixture of step (ii) with at least one algae strain that metabolizes said at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars, under conditions so that said at least one algae strain produces one or more fatty acids; and optionally (iv) recovering said one or more fatty acids from said at least one algae strain.

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A METHOD OF PRODUCING FATTY ACIDS FOR BIOFUEL, BIODIESEL, AND OTHER VALUABLE CHEMICALS

BACKGROUND OF THE INVENTION

Petroleum is a non-renewable resource. As a result, many people are worried about the eventual depletion of petroleum reserves in the future. World petroleum resources have even been predicted by some to run out by the 21st century (Kerr RA, Science 1998, 281, 1128).

This has fostered the expansion of alternative hydrocarbon products such as ethanol or other microbial fermentation products from plant derived feed stock and waste. In fact, current studies estimate that the United States could easily produce 1 billion dry tons of biomass (biomass feedstock) material (over half of which is waste) per year. This is primarily in the form of cellulosic biomass.

Cellulose is contained in nearly every natural, free-growing plant, tree, and bush, in meadows, forests, and fields all over the world without agricultural effort or cost needed to make it grow.

It is estimated that these cellulosic materials could be used to produce enough ethanol to replace 30% or more of the US energy needs in 2030. The great advantage of this strategy is that cellulose is the most abundant and renewable carbon source

on earth and its efficient transformation into a useable fuel could solve the world's energy problem.

Cellulosic ethanol has been researched extensively. Cellulosic ethanol is chemically identical to ethanol from other sources, such as corn starch or sugar, but has the advantage that the cellulosic materials are highly abundant and diverse. However, it differs in that it requires a greater amount of processing to make the sugar monomers available to the microorganisms that are typically used to produce ethanol by fermentation.

Although cellulose is an abundant plant material resource, its rigid structure makes cellulose a difficult starting material to process. As a result, an effective pretreatment is needed to liberate the cellulose from the lignin seal and its crystalline structure so as to render it accessible for a subsequent hydrolysis step. By far, most pretreatments are done through physical or chemical means. In order to achieve higher efficiency, some researchers seek to incorporate both effects.

To date, the available pretreatment techniques include acid hydrolysis, steam explosion, ammonia fiber expansion, alkaline wet oxidation and ozone pretreatment. Besides effective cellulose liberation, an ideal pretreatment has to minimize the formation of degradation products because of their inhibitory effects on subsequent hydrolysis and fermentation processes.

The presence of inhibitors makes it more difficult to produce ethanol. Even though pretreatment by acid hydrolysis is probably the oldest and most studied pretreatment technique, it produces several potent inhibitors including furfural and hydroxymethyl furfural (HMF) which are by far regarded as the most toxic inhibitors present in lignocellulosic hydrolysate.

The cellulose molecules are composed of long chains of sugar molecules of various kinds. In the hydrolysis process, these chains are broken down to free the sugar, before it is fermented for alcohol production.

There are two major cellulose hydrolysis processes: i) a chemical reaction using acids, or an ii) an enzymatic reaction. However, current hydrolysis processes are expensive and inefficient. For example, enzymatic hydrolysis processes require obtaining costly cellulase enzymes from outside suppliers.

A further problem in transforming cellulosic products into ethanol is that up to 50% of the available carbon to carbon dioxide is inherently lost through the fermentation process. In addition, ethanol is more corrosive than gas and diesel. As a result, it requires a distinct distribution infrastructure as well as specifically designed engines. Finally, ethanol is 20-30% less efficient than fossil gas and as ethanol evaporates

more easily, a higher percentage is lost along the whole production and distribution process.

A process that could produce biodiesel from cellulose would alleviate the problems associated with ethanol and other biodiesel productions.

Biodiesel obtained from microorganisms (e.g., algae and bacteria) is also non-toxic, biodegradable and free of sulfur. As most of the carbon dioxide released from burning biodiesel is recycled from what was absorbed during the growth of the microorganisms (e.g., algae and bacteria), it is believed that the burning of biodiesel releases less carbon dioxide than from the burning of petroleum, which releases carbon dioxide from a source that has been previously stored within the earth for centuries. Thus, utilizing microorganisms for the production of biodiesel may result in lower greenhouse gases such as carbon dioxide.

Some species of microorganisms are ideally suited for biodiesel production due to their high oil content. Certain microorganisms contain lipids and/or other desirable hydrocarbon compounds as membrane components, storage products, metabolites and sources of energy. The percentages in which the lipids, hydrocarbon compounds and fatty acids are expressed in the microorganism will vary depending on the type of microorganism that is grown. However, some strains have been discovered where

up to 90% of their overall mass contain lipids, fatty acids and other desirable hydrocarbon compounds (e.g., Botryococcus).

Algae such as *Chlorela sp.* and *Dunaliella* are a source of fatty acids for biodiesel that has been recognized for a long time. Indeed, these eukaryotic microbes produce a high yield of fatty acids (20-80% of dry weight), and can utilize CO_2 as carbon with a solar energy source.

However, the photosynthetic process is not efficient enough to allow this process to become a cost effective biodiesel source. An alternative was to use the organoheterotrophic properties of Algae and have them grow on carbon sources such as glucose. In these conditions, the fatty acid yield is extremely high and the fatty acids are of a high quality. The rest of the dry weight is mainly constituted of proteins. However, the carbon sources used are too rare and expensive to achieve any commercial viability.

Lipid and other desirable hydrocarbon compound accumulation in microorganisms can occur during periods of environmental stress, including growth under nutrient-deficient conditions. Accordingly, the lipid and fatty acid contents of microorganisms may vary in accordance with culture conditions.

The naturally occurring lipids and other hydrocarbon compounds in these microorganisms can be isolated and transesterified to obtain a biodiesel. The transesterification

of a lipid with a monohydric alcohol, in most cases methanol, yields alkyl esters, which are the primary component of biodiesel.

The transesterification reaction of a lipid leads to a biodiesel fuel having a similar fatty acid profile as that of the initial lipid that was used (e.g., the lipid may be obtained from animal or plant sources). As the fatty acid profile of the resulting biodiesel will vary depending on the source of the lipid, the type of alkyl esters that are produced from a transesterification reaction will also vary. As a result, the properties of the biodiesel may also vary depending on the source of the lipid. (e.g., see Schuchardt, et al, TRANSESTERIFICATION OF VEGETABLE OILS: A REVIEW, J. Braz. Chem. Soc., vol. 9, 1, 199-210, 1998 and G. Knothe, FUEL PROCESSING TECHNOLOGY, 86, 1059-1070 (2005), each incorporated herein by reference).

SUMMARY

The present invention relates to a method for producing fatty acids from biomass, and in particular, a method of producing fatty acids from biomass and for producing a biofuel from said fatty acids. In particular, the present invention relates to a method of producing fatty acids, by:

(i) inoculating a mixture of at least one of cellulose, hemicellulose, and lignin with at least one microorganism strain that produces one or more cellulases, hemicellulases and laccase, that hydrolyze at least one of cellulose, hemicellulose and lignin, under conditions to produce at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars;

(ii) inhibiting growth of said at least one microorganism strain;

(iii) inoculating the mixture of step (ii) with at least one algae strain that metabolizes said at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars, under conditions so that said at least one algae strain produces one or more fatty acids; and

optionally, (iv) recovering said one or more fatty acids from said at least one algae strain.

These and other features of the invention will be further described and exemplified with reference to the drawings and detailed description below.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig 1. is a flowchart illustrating a conventional process for bio-ethanol production.

Fig 2. is a flowchart illustrating the general process for fatty acid production and biofuel production of the invention.

Fig 3. is a flowchart illustrating a specific process for fatty acid production and biofuel production of the invention.

Fig 4. is a flowchart illustrating a preferred embodiment of a specific process for fatty acid production and biofuel production of the invention.

DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to embodiments of the invention. Examples of embodiments are illustrated in the accompanying drawings. While the invention will be described in conjunction with these embodiments, it will be understood that it is not intended to limit the invention to such embodiments. On the contrary, it is intended to cover alternatives, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

In the following description, numerous specific details are set forth in order to provide a thorough understanding of the present invention. The present invention may be practiced without some or all of these specific details. In other instances, well known process operations have not been described

in detail in order not to unnecessarily obscure the present invention.

The present invention relates to a method for producing fatty acids from biomass material. The fatty acids can be used, for example, in biofuel production.

One embodiment of the invention is directed to a method of producing fatty acids, by:

(i) inoculating a mixture of at least one of cellulose, hemicellulose, and lignin with at least one microorganism strain that produces one or more cellulases, hemicellulases and laccase, that hydrolyze at least one of cellulose, hemicellulose and lignin, under conditions to produce at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars;

(ii) inhibiting growth of said at least one microorganism strain and recovering extracellular and/or intracellular cellulase enzymes in the supernatant (recovery of intracellular cellulase enzyme can be performed by disrupting/breaking cells for release of intracellular enzyme utilizing common techniques, including ultrasonication, French press, temperature, chemical process, enzymatic process, homogenizer, microwaves);

(iii) inoculating the mixture of step (ii) with at least one algae strain that metabolizes said at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or

other hemicellulose sugars, under conditions so that said at least one algae strain produces one or more fatty acids; and

optionally, (iv) recovering said one or more fatty acids from said at least one algae strain.

The mixture in step (i) can be obtained from biomass. Biomass is any organic material made from plants or animals, including living or recently dead biological material, which can be used as fuel or for industrial production. Most commonly, biomass refers to plant matter grown for use as biofuel, but it also includes plant or animal matter used for production of fibers, chemicals or heat. Biomass is a renewable energy source.

There are a wide variety of sources of biomass, including tree and grass crops and forestry, agricultural, and urban wastes, all of which can be utilized in the present invention. Examples of domestic biomass resources include agricultural and forestry residues, municipal solid wastes, industrial wastes, and terrestrial and aquatic crops.

There are many types of plants in the world, and many ways they can be used for energy production. In general there are two approaches: growing plants specifically for energy use, and using the residues from plants that are used for other things. The type of plant utilized in the present invention varies from region to region according to climate, soils, geography, population, and so on.

Energy crops (also called "power crops") can be grown on farms in potentially very large quantities. Trees and grasses, including those native to a region, are preferred energy crops, but other, less agriculturally sustainable crops, including corn can also be used.

Trees are a good renewable source of biomass for processing in the present invention. In addition to growing very fast, certain trees will grow back after being cut off close to the ground (called "coppicing"). This allows trees to be harvested every three to eight years for 20 or 30 years before replanting. Such trees (also called "short-rotation woody crops") grow as much as 40 feet high in the years between harvests. In cooler, wetter regions of the northern United States, varieties of poplar, maple, black locust, and willow are preferred. In the warmer Southeast, sycamore and sweetgum are preferred. While in the warmest parts of Florida and California, eucalyptus and pine are likely to grow well.

Grasses are a good renewable source of biomass for use in the present invention. Thin-stemmed perennial grasses are common throughout the United States. Examples include switchgrass, big bluestem, and other native varieties, which grow quickly in many parts of the country, and can be harvested for up to 10 years before replanting. Thick-stemmed perennials including sugar cane and elephant grass can be grown in hot and

wet climates like those of Florida and Hawaii. Annuals, such as corn and sorghum, are another type of grass commonly grown for food.

Oil plants are also a good source of biomass for use in the present invention. Such plants include, for example, soybeans and sunflowers that produce oil, which can be used to make biofuels. Some other oil plants that carry a good yield in oil are poorly used as energy feedstock as their residual bean cake is toxic for mammal nutrition, like jatropha tree or castor bean plant, and are actually good biomass crop. Another different type of oil crop is microalgae. These tiny aquatic plants have the potential to grow extremely fast in the hot, shallow, saline water found in some lakes in the U.S. desert Southwest.

In this regard, biomass is typically obtained from waste products of the forestry, agricultural and manufacturing industries, which generate plant and animal waste in large quantities.

Forestry wastes are currently a large source of heat and electricity, as lumber, pulp, and paper mills use them to power their factories. Another large source of wood waste is tree tops and branches normally left behind in the forest after timber-harvesting operations.

Other sources of wood waste include sawdust and bark from sawmills, shavings produced during the manufacture of furniture, and organic sludge (or "liquor") from pulp and paper mills.

As with the forestry industry, a large volume of crop residue remains in the field after harvest. Such waste could be collected for biofuel production. Animal farms produce many "wet wastes" in the form of manure. Such waste can be collected and used by the present invention to produce fatty acids for biofuel production.

People generate biomass wastes in many forms, including "urban wood waste" (such as shipping pallets and leftover construction wood), the biodegradable portion of garbage (paper, food, leather, yard waste, etc.) and the gas given off by landfills when waste decomposes. Even our sewage can be used as energy; some sewage treatment plants capture the methane given off by sewage and burn it for heat and power, reducing air pollution and emissions of global warming gases.

In one embodiment, the present invention utilizes biomass obtained from plants or animals. Such biomass material can be in any form, including for example, chipped feedstock, plant waste, animal waste, etc.

Such plant biomass typically comprises: about 10-35% lignin; about 15-35% hemicellulose; and about 30-60% cellulose.

The plant biomass that can be utilized in the present invention include at least one member selected from the group consisting of wood, paper, straw, leaves, husks, shells, prunings, grass, including switchgrass, miscanthus, hemp, vegetable pulp, corn, bean cake, corn stover, sugarcane, sugar beets, sorghum, cassava, poplar, willow, potato waste, bagasse, sawdust, and mixed waste of plant, oil palm (palm oil) and forest mill waste.

In one embodiment of the invention, the plant biomass is obtained from at least one plant selected from the group consisting of: switchgrass, corn stover, and mixed waste of plant. In another embodiment, the plant biomass is obtained from switchgrass, due to its high levels of cellulose.

It should be noted that any such biomass material can by utilized in the method of the present invention.

The plant biomass can initially undergo a pretreatment to prepare the mixture utilized in step (i). Pretreatment helps altering the biomass macroscopic and microscopic size and structure, as well as submicroscopic chemical composition and structure, so hydrolysis of the carbohydrate fraction to monomeric sugars can be achieved more rapidly and with greater yields. Common pretreatment procedures are disclosed in Nathan Mosier, Charles Wyman, Bruce Dale, Richard Elander, Y.Y. Lee, Mark Holtzapple, Michael Ladisch, "Features of promising

technologies for pretreatment of lignocellulosic biomass," Bioresource Technology: 96, pp. 673-686 (2005), herein incorporated by reference, and discussed below.

Pretreatment methods are either physical or chemical. Some methods incorporate both effects (McMillan,1994; Hsu, 1996). For the purposes of classification, steam and water are excluded from being considered chemical agents for pretreatment since extraneous chemicals are not added to the biomass. Physical pretreatment methods include comminution (mechanical reduction in biomass particulate size), steam explosion, and hydrothermolysis. Comminution, including dry, wet, and vibratory ball milling (Millett et al., 1979; Rivers and Emert, 1987; Sidiras and Koukios, 1989), and compression milling (Tassinari et al., 1980, 1982) is sometimes needed to make material handling easier through subsequent processing steps. Acids or bases could promote hydrolysis and improve the yield of glucose recovery from cellulose by removing hemicelluloses or lignin during pretreatment. Commonly used acid and base include, for example, H_2SO_4 and NaOH, respectively. Cellulose solvents are another type of chemical additive. Solvents that dissolve cellulose in bagasse, cornstalks, tall fescue, and orchard grass resulted in 90% conversion of cellulose to glucose (Ladisch et al., 1978; Hamilton et al., 1984) and showed enzyme hydrolysis could be greatly enhanced when the biomass structure is

disrupted before hydrolysis. Alkaline H_2O_2, ozone, organosolv (uses Lewis acids, $FeCl_3$, $(Al)_2SO_4$ in aqueous alcohols), glycerol, dioxane, phenol, or ethylene glycol are among solvents known to disrupt cellulose structure and promote hydrolysis (Wood and Saddler, 1988). Concentrated mineral acids (H_2SO_4, HCl), ammonia-based solvents (NH_3, hydrazine), aprotic solvents (DMSO), metal complexes (ferric sodium tartrate, cadoxen, and cuoxan), and wet oxidation also reduce cellulose crystallinity and disrupt the association of lignin with cellulose, as well as dissolve hemicellulose. These methods, while effective, are too expensive for now to be practical when measured against the value of the glucose (approximately 5¢/lb). The following pretreatment methods of steam explosion, liquid hot water, dilute acid, lime, and ammonia pretreatments (AFEX), could have potential as cost-effective pretreatments.

It should be noted that any such pretreatment procedure can be utilized to alter the biomass to make the mixture utilized in the invention. In this regard, the microorganism in step (i) can be adapted to apply all pretreatment procedures and their associated residual compound that can include, for example, furfural, hydroxymethyl furfural(HMF), phenolics like 3,4-dihydroxybenzal-dehyde, 3-methoxy-4-hydroxy-benzoic acid, cinnamic acid, anillin, vanillin alcohol, as well as sodium

combinates like sodium hydroxide, nitrate combinates or ammonia, depending on the elected pretreatment method.

Acid pretreatment is a common pretreatment procedure. Acid pretreatment by acid hydrolysis and heat treatment can be utilized to produce the mixture inoculated in step (i) of the present invention. Any suitable acid can be used in this step, preferably an acid that hydrolyzes hemicelluloses away from cellulose. Some common acids that can be used include a mineral acid selected from hydrochloric acid, phosphoric acid, sulfuric acid, or sulfurous acid. Sulfuric acid, for example at concentration of about 0.5% to 2.0%, is preferred. Suitable organic acids may be carbonic acid, tartaric acid, citric acid, glucuronic acid, acetic acid, formic acid, or similar mono- or polycarboxylic acids. The acid pretreatment also typically involves heating the mixture, for example, in a range of about 70°C to 500°C, or in a range of about 120°C to 200°C.

Such acid pretreatment procedure can be used to generate the mixture utilized in step (i).

It should be noted that, when the biomass is obtained from plants, the mixture comprises at least one of cellulose, hemicellulose, lignin, furfural, phenolics and acetic acid.

In step (i), after the pretreatment procedure, the mixture is inoculated with at least one microorganism strain that is an extracellular cellulase producer. This microorganism can

produce one or more cellulases that hydrolyze (enzymatic hydrolysis) at least one of cellulose and hemicelluloses present in the mixture under conditions to produce at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars.

Cellulase refers to a group of enzymes which hydrolyze cellulose, hemicellulose, and/or lignin. It is typically referred to as a class of enzymes produced by microorganisms (i.e., an extracellular cellulase producer), such as archaea, fungi, bacteria, protozoans, that catalyze the cellulolysis (or hydrolysis) of cellulose. However, it should be noted that there are cellulases produced by other kinds of microorganisms.

It is important to note that the present invention can utilize any extracellular and/or intracellular cellulase producer that produces one or more cellulases selected from the group consisting of: endoglucanase, exoglucanase, and β-glucosidase, hemicellulases, and laccase. Examples of cellulase producing microorganisms that can be utilized in the present invention include those in the attached Table 1.

Accordingly, the cellulase enzymes produced by the microorganism can perform enzymatic hydrolysis on the mixture in step (i). At the end of the enzymatic hydrolysis, the resultant medium can contains glucose, cellobiose, acetic acid, furfural,

lignin, xylose, arabinose, mannose, galactose, and other hemicelluloses sugars.

Again, the present invention can utilize any microorganism that is an extracellular and/or intracellular cellulase enzyme producer to produce the requisite cellulase enzymes for enzymatic hydrolysis in step (i). As such, any prokaryote, including bacteria, archaea, and eukaryote, including fungi, which produces extracellular and/or intracellular cellulase enzymes may be utilized as the microorganism in step (i).

In one embodiment, the extracellular and/or intracellular cellulase producer is a fungus, archaea or bacteria of a genus selected from the group consisting of *Humicola*, *Trichoderma*, *Penicillium*, *Ruminococcus*, *Bacillus*, *Cytophaga* and *Sporocytophaga*. According to still a further embodiment the extracellular and/or intracellular cellulase producer can be at least microorganism selected from the group consisting of *Humicola grisea*, *Trichoderma harzianum*, *Trichoderma lignorum*, *Trichoderma reesei*, *Penicillium verruculosum*, *Ruminococcus albus*, *Bacillus subtilis*, *Bacillus thermoglucosidasius*, *Cytophaga spp.*, and *Sporocytophaga spp*.

In addition, a microorganism that is an extracellular and/or intracellular laccase enzyme producer may also be utilized in the present invention. Accordingly, any prokaryote, including bacteria, archaea, and eukaryote, including fungi,

which produces extracellular and/or intracellular laccase may be utilized as the microorganism in step (i). In one embodiment, the extracellular and/or intracellular laccase producer is a fungus, bacteria or archaea of a genus selected from the group consisting of *Humicola*, *Trichoderma*, *Penicillium*, *Ruminococcus*, *Bacillus*, *Cytophaga* and *Sporocytophaga*. According to still a further embodiment the extracellular and/or intracellular laccase producer can be at least microorganism selected from the group consisting of *Humicola grisea*, *Trichoderma harzianum*, *Trichoderma lignorum*, *Trichoderma reesei*, *Penicillium verruculosum*, *Ruminococcus albus*, *Bacillus subtilis*, *Bacillus thermoglucosidasius*, *Cytophaga spp.*, and *Sporocytophaga spp.* Examples of laccase producing microorganisms that can be utilized in the present invention include those in the attached Table 1.

In one embodiment, the microorganism strain is a fungus, and more preferably, an aerobic fungus, such as *Trichoderma reesei*.

Again, any microorganism that is an extracellular and/or intracellular cellulase enzyme producer or extracellular and/or intracellular laccase enzyme producer can be utilized in the present invention to produce the requisite enzymes for enzymatic hydrolysis in step (i). Examples include those listed in attached Tables 1 and 2.

In the present invention, the type of microorganism can be selected and/or evolved to be specific to the type of plant biomass used.

The microorganism strain is tolerant to one or more compounds produced by the biomass pretreatment procedure, such as acid or alkaline pretreatment. Such compounds produced in the biomass pretreatment step include, for example, furfural, 3,4-dihydroxybenzaldehyde, 3-methoxy-4-hydroxy-benzoic acid, cinnamic acid, vanillin, vanillin alcohol, acetic acid, lignin and other residual salts or impurities.

In a preferred embodiment, the method of present invention utilizes at least one microorganism that has been evolutionarily modified and specialized for the specific type of biomass used. The evolutionarily modified microorganism can metabolize (enzymatic hydrolysis) the pretreated targeted biomass more efficiently and such microorganisms can be better able to tolerate residual compounds, for example, furfural and acetic acid. In this respect, the evolutionarily modified microorganism can have greater tolerance to furfural and acetic acid as compared to the unmodified wild-type version of the microorganism.

The evolutionarily modified microorganism can also produces one or more cellulase and/or laccase enzymes that are less inhibited by lignin and/or have improved capacity to metabolize

lignin. As such, the evolutionarily modified microorganism can have improved capacity to produce enzymes (such as laccase) that metabolize lignin. Thus, the cellulase, hemicellulase and/or laccase enzymes produced by the evolutionarily modified microorganism can have greater capacity to metabolize cellulose and hemicelluloses with lignin as compared to the unmodified wild-type version of the microorganism.

Due to the use of the evolutionarily modified microorganism, the present invention allows for production of cellulases *in situ* in the mixture/medium of step (i). Consequently, there is no need to buy expensive cellulase enzymes from outside suppliers. This reduces operational costs as compared to conventional methods for biofuel production. Further, also due to the use of the evolutionarily modified microorganism, there is no need to wash and detoxify the acid pretreated mixture in the present invention to remove furfural, acetic acid, and salts that would normally inhibit biofuel production (as in conventional methods). By removing the wash and detoxification steps, the present invention can further reduces operational costs as compared to conventional methods for biofuel production.

It is noted that an evolutionarily modified microorganism is defined as a microorganism that has been modified by natural selection techniques. These techniques include, for example,

serial transfer, serial dilution, Genetic Engine, continuous culture, and chemostat. One method and chemostatic device (the Genetic Engine; which can avoid dilution resistance in continuous culture) has been described in U.S. Patent No. 6,686,194-B1, incorporated herein by reference.

In one embodiment, the microorganism is evolutionarily modified by use of the continuous culture procedure as disclosed in PCT Application No. PCT/US05/05616, or United States Patent Application No. 11/508,286, each incorporated herein by reference.

By cultivating a microorganism in this manner, beneficial mutations will occur to produce brand new alleles (*i.e.*, variants of genes) that improve an organism's chances of survival and/or growth rate in that particular environment.

As such, the microorganism (e.g., fungi, archaea, algae, or bacteria) of the present invention can constitute a different strain, which can be identified by the mutations acquired during the course of culture, and these mutations, may allow the new cells to be distinguished from their ancestors' genotype characteristics. Thus, one can select new strains of microorganisms by segregating individuals with improved rates of reproduction through the process of natural selection.

Selection parameters for evolutionarily modifying the microorganism. By way of example, the microorganism in step (i) can be evolutionarily modified, through a natural selection

technique, so that through evolution, it evolves to be adapted to use the particular carbon source selected. This involves identifying and selecting the fastest growing variant microorganisms, through adaptation in the natural selection technique utilized (such as continuous culture), that grow faster than wild-type on a particular carbon source. This also includes selecting those mutant microorganisms that have improved tolerance to furfural and acetic acid when using dilute acid pre-treatment; or selecting variant microorganisms that produce one or more cellulase and/or laccase enzymes that are less inhibited by lignin and/or have improved capacity to metabolize lignin. This would also involve selecting those microorganisms producing the above-discussed requisite cellulose enzymes.

It should be noted that, by using such parameters, any one of the natural selection techniques could be used in the present invention to evolutionarily modify the microorganism in the present invention.

Accordingly, the microorganisms can be evolutionarily modified in a number of ways so that their growth rate, viability, and utility as a biofuel, or other hydrocarbon product can be improved. Thus, the microorganisms can be evolutionarily modified to enhance their ability to grow on a particular substrate, constituted of the biomass and residual chemical related to chemical pre-treatment if any. In this regard, the microorganisms

can be evolutionarily modified for a specific biomass plant and eventually associated residual chemicals.

The microorganisms (e.g., fungi, algae or bacteria) are preferably naturally occurring and have not been modified by recombinant DNA techniques. In other words, it is not necessary to genetically modify the microorganism to obtain a desired trait. Rather, the desired trait can be obtained by evolutionarily modifying the microorganism using the techniques discussed above. Nonetheless, even genetically modified microorganisms can be evolutionarily modified to increase their growth rate and/or viability of a modified by recombinant DNA techniques.

In one embodiment of the invention, the microorganism is a fungus, and in particular, *Trichoderma reesei* **(also known as *Hypocrea jecorina)***, which has been evolutionarily modified by continuous culture.

The cellulase activity in step (i) can also be measured using common techniques to assess the level of cellulose activity to determine when to inhibit and/or stop the growth of the microorganism by proceeding to step (ii).

In step (ii) of the invention, growth and enzyme production of the microorganism is inhibited by one or more common techniques, such as those selected from the group consisting of: heat shock, UV exposure, radiation exposure, gas injection, and

genetic modification of said at least one microorganism, (prior to step (i)) so that growth of said at least one genetically modified microorganism can be inhibited, for example, when temperature is increased to 45°C. Also, cells could be broken, using common techniques, for the release of intracellular cellulase enzymes in the supernatant.

Step (iii) of the invention involves inoculating the mixture of step (ii) with at least one algae strain that metabolizes said at least one of glucose, cellobiose, xylose or other hemicellulose sugars, under conditions so that said at least one algae strain produces one or more fatty acids.

Preferably, the growth of said at least one algae strain is not substantially inhibited by the presence of one or more of lignin, furfural, salts and cellulases enzymes present in the mixture.

The algae strain can also grow in one or more of the conditions selected from the group consisting of aerobic, anaerobic, phototrophic, and heterotrophic conditions.

Similar to the microorganism, the algae in step (iii) may be evolutionarily modified (using the natural selection techniques discussed above) to serve as an improved source of fatty acids, biofuel, biodiesel, and other hydrocarbon products. In this regard, the algae can be cultivated for use as a biofuel, biodiesel, or hydrocarbon based product.

Most algae need some amount of sunlight, carbon dioxide, and water. As a result, algae are often cultivated in open ponds and lakes. However, when algae are grown in such an "open" system, the systems are vulnerable to contamination by other algae and bacteria.

In one embodiment, the present invention can utilize heterotrophic algae (Stanier et al, Microbial World, Fifth Edition, Prentice-Hall, Englewood Cliffs, New Jersey, 1986, incorporated herein by reference), which can be grown in a closed reactor.

While a variety of algal species can be used, algae that naturally contain a high amount of lipids, for example, about 15-90%, about 30-80%, about 40-60%, or about 25-60% of lipids by dry weight of the algae is preferred. Prior to the work of the present invention, algae that naturally contained a high amount of lipids and high amount of bio-hydrocarbon were associated as having a slow growth rate. Evolutionarily modified algae strains can be produced in accordance with the present invention that exhibit an improved growth rate.

The conditions for growing the algae can be used to modify the algae. For example, there is considerable evidence that lipid accumulation takes place in algae as a response to the exhaustion of the nitrogen supply in the medium. Studies have analyzed samples where nitrogen has been removed from the

culture medium and observed that while protein contents decrease under such conditions, the carbohydrate content increases, which are then followed by an increase in the lipid content of the algae. (Richardson et al, EFFECTS OF NITROGEN LIMITATION ON THE GROWTH OF ALGAE ON THE GROWTH AND COMPOSITION OF A UNICELLULAR ALGAE IN CONTINUOUS CULTURE CONDITIONS, Applied Microbiology, 1969, volume 18, page 2245-2250, 1969, incorporated herein by reference).

The algae can be evolutionarily modified by a number of techniques, including, for example, serial transfer, serial dilution, genetic engine, continuous culture, and chemostat. Any one of these techniques can be used to modify the algae. In one embodiment, the algae can be evolutionarily modified by continuous culture, as disclosed in PCT Application No. PCT/US05/05616, or United States Patent Application No. 11/508,286, each incorporated herein by reference.

In doing so, the algae can be evolutionarily modified in a number of ways so that their growth rate, viability, and utility as a biofuel, or other hydrocarbon product can be improved. Accordingly, the algae can be evolutionarily modified to enhance their ability to grow on a particular substrate.

Selection parameters for evolutionarily modifying the algae. By way of example, the algae in step (iii) can be evolutionarily modified, through a natural selection technique, such as

continuous culture, so that through evolution, the algae evolves to be adapted to use the particular carbon source selected. This involves identifying and selecting the fastest growing variant algae, through adaptation in the natural selection technique utilized, that grow faster than wild-type on a particular carbon source. This also includes, for example, selecting those algae that use acetic acid as a carbon source with improved tolerance to lignin, furfural and salts. It should be noted that, by using such parameters, any one of the natural selection techniques could be used in the present invention to evolutionarily modify the algae in the present invention.

In the present invention, such evolutionarily modified algae metabolize one or more compounds selected from the group consisting of: glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars and/or waste glycerol, and the algae use acetic acid a carbon source, under conditions so that said at least one algae strain produces one or more fatty acids. Such evolutionarily modified algae can also grow in one or more of the conditions selected from the group consisting of aerobic, anaerobic, phototrophic, and heterotrophic conditions.

In one embodiment, when step (iii) of the invention is performed under aerobic and heterotrophic conditions, the algae uses respiration.

In step (iii), the algae using the same amount of carbon source as an organism producing fermentation by-product producer, will produce only up to about 10% carbon dioxide. In this regard, more sugar is used by the algae for growth than is transformed to carbon dioxide. Alternatively, the microorganism or algae can be one that does not use fermentation, and as such much less carbon dioxide is made as a by-product in respiration.

Also, at least one algae strain in step (iii) preferably produces little or no inhibitory by-product, for growth inhibition of said algae.

Types of algae that can be utilized in the invention is one or more selected from the group consisting of green algae, red algae, blue-green algae, cyanobacteria and diatoms.

It should be noted that the present invention can utilize any algae strain that metabolizes at least one of glucose, cellobiose, xylose or other hemicellulose sugars, under conditions so that algae strain produces one or more fatty acids.

By way of example, the algae utilized in step (iii) can be from the following taxonomic divisions of algae:

(1) Division *Chlorophyta* (green algae);

(2) Division *Cyanophyta* (blue-green algae);

(3) Division *Bacillariophyta* (diatoms);

(4) Division *Chrysophyta;*

(5) Division *Xanthophyta;*

(6) Division *Cryptophyta;*

(7) Division *Euglenophyta;*

(8) Division *Ochrophyta* ;

(9) Division *Haptophyta*; and

(10) Division *Dinophyta*.

More specifically, the algae can be from the following species of algae, included within the above divisions (wherein number in parenthesis corresponds to the division):

Biddulphia (8);

Pinguiococcus (8);

Skeletonema (8);

Emiliania (9);

Prymnesium (9);

Crypthecodinium (10);

Anabaenopsis circularis (2);

Ankistrodesmus braunii (1);

A. falcatus (1);

Botrydiopsis intercedens (5);

Bracteacoccus cinnabarinus (1);

B. engadiensis (1);

B. minor (Chodat) Petrova (1);

B. terrestris (1);

Bracteacoccus sp. (1);

Bracteacoccus sp. (1);

Bumilleriopsis brevis (5);

Chilomonas paramecium (6);

Chlamydobotrys sp. (1);

Chlamydomonas agloeformis (1);

C. dysosmos (1);

C. mundana Mojave strain Boron strain (1);

C. reinhardi (-) strain (1);

Chlorella ellipsoidea (1);

C. protothecoides (1);

C. pyrenoidosa (1);

C. pyrenoidosa ATCC 7516 (1);

C. pyrenoidosa C-37-2 (1);

C. pyrenoidosa Emerson (1);

C. pyrenoidosa 7-11-05 (1);

C. vulgaris (1);

C. vulgaris ATCC 9765 (1);

C. vulgaris Emerson (1);

C. vulgaris Pratt-Trealease (1);

C. vulgaris var. viridis (1);

Chlorellidium tetrabotrys (5);

Chlorocloster engadinensis (5);

Chlorococcum macrostigmatum (1);

Chlorococcum sp. (1);

Chlorogloea fritschii (2);

Chlorogonium elongatum (1);

Coccomyxa elongata (1);

Cyclotella sp. (3);

Dictyochloris fragrans (1);

Euglena gracilis (7);

E. gracilis Vischer (7);

E. gracilis var. bacillaris (7);

E. gracilis var. saccharophila (7);

Haematococcus pluvialis (1);

Navicula incerta Grun. (3);

N. pelliculosa (3);

Neochloris alveolaris (1);

N. aquatica Starr (1);

N. gelatinosa Herndon (1);

N. pseudoalveolaris Deason (1);

Neochloris sp. (1);

Nitzschia angularis var. affinis (3) (Grun.) perag.;

N. chlosterium (Ehr.) (3);

N. curvilineata Hust. (3);

N. filiformis (3);

N. frustulum (Kürtz.) (3);

N. laevis Hust. (3);

Nostoc muscorum (2);

Ochromonas malhamensis (4);

Pediastrum boryanum (1);

P. duplex (1);

Polytoma obtusum (1);

P. ocellatum (1);

P. uvella (1);

Polytomella caeca (or coeca) (1);

Prototheca zopfii (1);

Scenedesmus acuminatus (1);

S. acutiformis (1);

S. costulatus Chod, var. chlorelloides (1);

S. dimorphus (1);

S. obliquus (1);

S. quadricauda (1);

Spongiochloris excentrica (1);

S. lamellata Deason (1);

S. spongiosus (1);

Spongiochloris sp. (1);

Spongiococcum alabamense (1);

S. excentricum (1);

S. excentricum Deason et Bold (1)

S. multinucleatum (1);

Stichococcus bacillaris (1);

S. subtilis (1);

Tolypothrix tenuis (2);

Tribonema aequale (5); and

T. minus (5).

In one embodiment, the algae can be from *Chlorophyta (Chlorella and Prototheca), Prasinophyta (Dunaliella), Bacillariophyta (Navicula and Nitzschia), Ochrophyta (Ochromonas), Dinophyta (Gyrodinium) and Euglenozoa (Euglena).* More preferably, the algae is one selected from the group consisting of: *Monalanthus Salina; Botryococcus Braunii; Chlorella prototecoides; Outirococcus sp.; Scenedesmus obliquus; Nannochloris sp.; Dunaliella bardawil (D. Salina); Navicula pelliculosa; Radiosphaera negevensis; Biddulphia aurita; Chlorella vulgaris; Nitzschia palea; Ochromonas dannica; Chrorella pyrenoidosa; Peridinium cinctum; Neochloris oleabundans; Oocystis polymorpha; Chrysochromulina spp.; Scenedesmus acutus; Scenedesmus spp.; Chlorella minutissima; Prymnesium parvum; Navicula pelliculosa; Scenedesmus dimorphus; Scotiella sp.; Chorella spp.; Euglena gracilis;* and *Porphyridium cruentum.*

In another embodiment, the algae strain is *Chlorella protothecoides* and has been evolutionarily modified by continuous culture using the techniques and procedures described above.

Cyanobacteria may also be used with the present invention. Cyanobacteria are prokaryotes (single-celled organisms) often referred to as "blue-green algae." While most algae are eukaryotic, cyanobacteria are the most common exception. Cyanobacteria are generally unicellular, but can be found in colonial and filamentous forms, some of which differentiate into varying roles. For purposes of the claimed invention, cyanobacteria are considered algae.

Chlorella protothecoides and *Dunaliella Salina* are species that have been evolutionarily modified, cultivated, and harvested for production of a biodiesel.

The following publications relate to growing different types of algae and then harvesting algae for the purpose of producing biodiesel are incorporated herein by reference:

- Xu et al, HIGH QUALITY BIODESEL PRODUCTION FROM A MICROALGA CHLORELLA PROTHECOIDES BY HETEROTROPHIC GROWTH IN FERMENTERS, Journal of Biotechnology, vol. 126, 499-507, 2006,

- Kessler, Erich, PHYSIOLOGICAL AND BIOCHEMICAL CONTRIBUTIONS TO THE TAXONOMY OF THE GENUS PROTOTHECA, III. UTILIZATION OF ORGANIC CARBON AND NITROGEN COMPOUNDS, Arch Microbiol, volume 132, 103-106, 1982,

- Johnson D, 1987, OVERVIEW OF THE DOE/SERI AQUATIC SPECIES PROGRAM FY 1986 SOLAR ENERGY INSTITUTE,

- Pratt et al, PRODUCTION OF PROTEIN AND LIPID BY CHLORELLA VULGARIS AND CHLORELLA PYRENOIDOSA, Journal of Pharmaceutical Sciences, volume 52, Issue 10, 979-984 2006, and

- Sorokin, MAXIMUM GROWTH RATES OF CHLORELLA IN STEADY-STATE AND IN SYNCHRONIZED CULTURES, Proc. N.A.S, volume 45, 1740-1743, 1959.

- J.E. Zajic and Y.S. Chiu, HETEROTROPHIC CULTURE OF ALGAE, Biochemical Engineering, Faculty of Engineering Science, University of Western Ontario, London.

By employing the methods of the instant invention, the inoculation of the mixture with the at least one algae strain in step (iii) results in the algae metabolizing at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars, under conditions so that said at least one algae strain produces one or compounds, including fatty acids. In particular, the present invention in step (iii) involves culturing and growing the evolutionarily modified algae for extracellular and/or intracellular production of one or more compounds, such as fatty acids, hydrocarbons, proteins, pigments, sugars, such as polysaccharides and monosaccharides, and glycerol.

The resultant fatty acids, hydrocarbons, proteins, pigments, sugars, such as polysaccharides and monosaccharides, and glycerol in the algae can be used for biofuel, cosmetic,

alimentary, mechanical grease, pigmentation, and medical use production.

In step (iv), the fatty acids, hydrocarbons, proteins, pigments, sugars, such as polysaccharides and monosaccharides, and glycerol can be recovered from the algae. The recovery step can be done by conventional techniques including one or more of fractionating the algae in the culture to obtain a fraction containing the compound, and other techniques including filtration-centrifugation, flocculation, solvent extraction, acid and base extraction, ultrasonication, microwave, pressing, distillation, thermal evaporation, homogenization, hydrocracking (fluid catalytic cracking), and drying of said at least one algae strain containing fatty acids.

In one embodiment, the resultant supernatant recovered in step (iv) can be reused.

Moreover, the recovered fatty acids can be optionally isolated and chemically treated (e.g., by transesterification), and thereby made into a biofuel (biodiesel) that can be incorporated into an engine fuel.

In this regard, the algae strain of the present invention produces hydrocarbon chains which can be used as feedstock for hydrocracking in an oil refinery to produce one or more compounds selected from the group consisting of octane,

gasoline, petrol, kerosene, diesel and other petroleum product as solvent, plastic, oil, grease and fibers.

Direct transesterification can be performed on cells of the algae strain to produce fatty acids for biodiesel fuel. Methods of direct transesterification are well known and include breaking the algae cells, releasing fatty acids and transesterification through a base or acid method with methanol or ethanol to produce biodiesel fuel.

A further advantage of the method of the present invention is that the algae strain can be adapted to use waste glycerol, as a carbon source, produced by the transesterification reaction without pretreatment or refinement to produce fatty acids for biodiesel production.

Raw glycerol is the by-product of a transesterification reaction comprising glycerol and impurities such as fatty acid components, oily components, acid components, alkali components, soap components, alcohol component (e.g., methanol or ethanol) solvent (N-hexane) salts and/or diols. Due to the number and type of impurities present in raw glycerol, microorganisms exhibit little to no growth on the raw glycerol itself. However, the microorganism (e.g., algae or bacteria) can be evolutionarily modified to utilize raw glycerol as a primary carbon source.

The initial test for determining whether a particular type of microorganism will be able to grow in the presence of raw glycerol is the Refined Glycerol Test. The Refined Glycerol Test comprises culturing the microorganism in a medium comprising refined glycerol. The medium utilized in the Refined Glycerol Test may or may not have another carbon source such as glucose. However, the medium in the Refined Glycerol Test must contain a sufficient amount of glycerol so that it can be determined that the microorganism exhibits a minimum metabolizing capacity of the microorganism. The medium preferably contains 10ml-50 ml per liter of refined glycerol, 0.1ml-100ml per liter of refined glycerol, and 2ml-15ml per liter of refined glycerol.

If a positive result (i.e., the microorganism grows in the medium) is obtained with the Refined Glycerol Test, the microorganism can be evolutionarily modified to grow in a medium comprising raw glycerol. The culture medium preferably comprises, for example, 10-100% raw glycerol as a carbon source, 20-90% raw glycerol as a carbon source, 30-75% raw glycerol as a carbon source, 40-75% raw glycerol as a carbon source, or 50.01-55% raw glycerol as a carbon source. Indeed, some strains of microorganisms have been evolutionary modified to grow on a culture medium containing 100% raw glycerol.

An evolutionarily modified microorganism which produces extracellular and/or intracellular cellulase, hemicellulase, and laccase obtained in accordance with the present invention can have a maximum growth rate using the specific carbon sources in the pretreated biomass mixture of at least 5%, preferably 10%, 15%, 25%, 50%, 75%, 100%, 200%, 25%-100%, 25%-100%, 50%-150%, 25-200%, more than 200%, more than 300%, or more than 400% greater than microorganism of the same species that has not been evolutionarily modified to perform in the present invention.

An evolutionarily modified algae obtained in accordance with the present invention can have a maximum growth rate using, as a carbon source, the released polysaccharide and monosaccharide sugars from step (i) in the pretreated biomass mixture of at least 5%, preferably 10%, 15%, 25%, 50%, 75%, 100%, 200%, 25%-100%, 25%-100%, 50%-150%, 25-200%, more than 200%, more than 300%, or more than 400% greater than algae of the same species that has not been evolutionarily modified to perform in the present invention.

While it is envisioned that the most important commercial use for microorganisms grown from the by-products of biodiesel production will be to use the microorganisms themselves for products such as biofuel, biodiesel, "bio"-hydrocarbon products, renewable hydrocarbon products, and fatty acid based products, the invention is not limited to this embodiment. For example, if

the microorganism is an algae, the algae could be grown from the by-products of biofuel production and harvested for use as a food, medicine, and nutritional supplement.

The biofuel obtained from the present invention may be used directly or as an alternative to petroleum for certain products.

In another embodiment, the biofuel (e.g., biodiesel) of the present invention may be used in a blend with other petroleum products or petroleum alternatives to obtain fuels such as motor gasoline and distillate fuel oil composition; finished nonfuel products such as solvents and lubricating oils; and feedstock for the petrochemical industry such as naphtha and various refinery gases.

For example, the biofuel as described above may be used directly in, or blended with other petroleum based compounds to produce solvents; paints; lacquers; and printing inks; lubricating oils; grease for automobile engines and other machinery; wax used in candy making, packaging, candles, matches, and polishes; petroleum jelly; asphalt; petroleum coke; and petroleum feedstock used as chemical feedstock derived from petroleum principally for the manufacture of chemicals, synthetic rubber, and a variety of plastics.

In a preferred embodiment, biodiesel produced in accordance with the present invention may be used in a diesel engine, or may be blended with petroleum-based distillate fuel oil

composition at a ratio such that the resulting petroleum substitute may be in an amount of about 5-95%, 15-85%, 20-80%, 25-75%, 35-50% 50-75%, and 75-95% by weight of the total composition. The components may be mixed in any suitable manner.

The process of fueling a compression ignition internal combustion engine, comprises drawing air into a cylinder of a compression ignition internal combustion engine; compressing the air by a compression stroke of a piston in the cylinder; injecting into the compressed air, toward the end of the compression stroke, a fuel comprising the biodiesel; and igniting the fuel by heat of compression in the cylinder during operation of the compression ignition internal combustion engine.

In another embodiment, the biodiesel can be used as a lubricant or in a process of fueling a compression ignition internal combustion engine.

Alternatively, the biofuel may be further processed to obtain other hydrocarbons that are found in petroleum such as paraffins (e.g., methane, ethane, propane, butane, isobutane, pentane, and hexane), aromatics (e.g., benzene and naphthalene), cycloalkanes (e.g., cyclohexane and methyl cyclopentane), alkenes (e.g., ethylene, butene, and isobutene), alkynes (e.g., acetylene, and butadienes).

The resulting hydrocarbons can then in turn be used in petroleum based products such as solvents; paints; lacquers; and printing inks; lubricating oils; grease for automobile engines and other machinery; wax used in candy making, packaging, candles, matches, and polishes; petroleum jelly; asphalt; petroleum coke; and petroleum feedstock used as chemical feedstock derived from petroleum principally for the manufacture of chemicals, synthetic rubber, and a variety of plastics.

The following examples illustrate embodiments of the invention. It will be apparent that various changes and modifications can be made without departing from the scope of the invention as defined in the claims.

Examples

One exemplified embodiment of the method of the present invention can be found in the chart in Fig. 4 and is discussed below.

In this example, a plant biomass material of chipped switchgrass was subjected to pretreatment by acid hydrolysis (sulfuric acid 0.5% to 2.0%) and heat treatment (120°C -200°C).

This pretreatment procedure produced a mixture for use in the above-discussed step (i). This mixture contained cellulose, hemicellulose, lignin, furfural, and acetic acid.

In step (i), (Enzymatic Production *in situ*) the mixture was inoculated with an evolutionarily modified microorganism strain of *Trichoderma Reesei* having the following properties and under the following conditions:

- The modified *Trichoderma Reesei* strain was evolved to metabolize pretreated switchgrass more efficently and to tolerate furfural & acetic acid better (as was designated EVG22030).
- The strain produces external cellulase enzymes specific for switchgrass.
- Inoculation & growth of *Trichoderma Reesei* EVG22030 occurred in aerobic environment.
- Hydrolysis of crystalline cellulose into glucose, cellobiose.
- Hydrolysis of the hemicellulose sugars that was not sufficiently processed through pretreatment.

After the growth and enzymes production phase, *Trichoderma Reesei* EVG22030 growth is stopped by heat shock at 50°C (step (ii)).

In step (iii), the mixture from step (ii) was inoculated withan evolutionarily modified algae strain of *Chlorella protothecoides* having the following properties and under the following conditions:

- *Chlorella protothecoides* was evolved in heterotrophic environment to use the carbon sources released from the pretreated switchgrass (by EVG22030 enzymes) and designated EVG15018.
- Inoculation and growth of *Chlorella Protothecoides* EVG15018 in heterotrophic environment.
- EVG15018 metabolizes: glucose, cellobiose, xylose & other hemicellulose sugars, waste glycerol and uses acetic acid as a carbon source.
- Presence of lignin, furfural and salts do not inhibit growth.
- EVG15018 produces 40% and more fatty acid (cell dry weight).
- The algae were then grown under conditions and produced produces fatty acids.

The algae cells and fatty acids were then recovered by filtration and cell drying.

Direct transesterification was then performed on the dry cells (ultrasonication, through a base or acid method with methanol or ethanol) to produce biodiesel fuel. Waste glycerol was also recovered and recycled. The resultant biodiesel fuel can be directly used in any diesel engine for cars, trucks, generators, boats, etc.

While the invention has been described and pointed out in detail with reference to operative embodiments thereof it will be understood by those skilled in the art that various changes, modifications, substitutions and omissions can be made without departing from the spirit of the invention. It is intended, therefore, that the invention embrace those equivalents within the scope of the claims which follow.

TABLE 1 – EXAMPLES OF MICRO-ORGANISMS PRODUCING EXTRA- AND/OR INTRA-CELLULAR CELLULASE ENZYMES

	Division	Organism
Archaea	Crenarchaeota	Caldivirga maquilingensis
Archaea	Crenarchaeota	Sulfolobus acidocaldarius
Archaea	Crenarchaeota	Sulfolobus solfataricus
Archaea	Crenarchaeota	Thermofilum pendens
Archaea	Euryarchaeota	Picrophilus torridus
Archaea	Euryarchaeota	Pyrococcus abyssi
Archaea	Euryarchaeota	Pyrococcus furiosus
Archaea	Euryarchaeota	Pyrococcus horikoshii
Archaea	Euryarchaeota	Thermoplasma volcanium
Bacteria	Acidobacteria	Acidobacterium capsulatum
Bacteria	Actinobacteria	Acidothermus cellulolyticus
Bacteria	Actinobacteria	Actinomadura sp.
Bacteria	Actinobacteria	Actinomyces sp.
Bacteria	Actinobacteria	Amycolatopsis orientalis
Bacteria	Actinobacteria	Arthrobacter aurescens
Bacteria	Actinobacteria	Arthrobacter sp.
Bacteria	Actinobacteria	Bifidobacterium adolescentis
Bacteria	Actinobacteria	Bifidobacterium animalis
Bacteria	Actinobacteria	Bifidobacterium bifidum
Bacteria	Actinobacteria	Bifidobacterium longum
Bacteria	Actinobacteria	Cellulomonas fimi
Bacteria	Actinobacteria	Cellulomonas flavigena
Bacteria	Actinobacteria	Cellulomonas pachnodae
Bacteria	Actinobacteria	Cellulomonas uda
Bacteria	Actinobacteria	Cellulosimicrobium sp.
Bacteria	Actinobacteria	Clavibacter michiganensis subsp. michiganensis
Bacteria	Actinobacteria	Clavibacter michiganensis subsp. sepedonicus
Bacteria	Actinobacteria	Frankia alni
Bacteria	Actinobacteria	Frankia sp.
Bacteria	Actinobacteria	Jonesia sp.
Bacteria	Actinobacteria	Kineococcus radiotolerans
Bacteria	Actinobacteria	Leifsonia xyli subsp. xyli
Bacteria	Actinobacteria	Microbispora bispora
Bacteria	Actinobacteria	Micromonospora cellulolyticum
Bacteria	Actinobacteria	Mycobacterium abscessus
Bacteria	Actinobacteria	Mycobacterium avium
Bacteria	Actinobacteria	Mycobacterium avium subsp. Paratuberculosis

	Division	Organism
Bacteria	Actinobacteria	Mycobacterium bovis
Bacteria	Actinobacteria	Mycobacterium gilvum
Bacteria	Actinobacteria	Mycobacterium marinum
Bacteria	Actinobacteria	Mycobacterium smegmatis
Bacteria	Actinobacteria	Mycobacterium sp.
Bacteria	Actinobacteria	Mycobacterium tuberculosis
Bacteria	Actinobacteria	Mycobacterium ulcerans
Bacteria	Actinobacteria	Mycobacterium vanbaalenii
Bacteria	Actinobacteria	Mycobacterium vanbaalenii
Bacteria	Actinobacteria	Nocardioides sp.
Bacteria	Actinobacteria	Propionibacterium acnes
Bacteria	Actinobacteria	Rhodococcus equi
Bacteria	Actinobacteria	Saccharopolyspora erythraea
Bacteria	Actinobacteria	Saccharothrix australiensis
Bacteria	Actinobacteria	Salinispora arenicola
Bacteria	Actinobacteria	Salinispora tropica
Bacteria	Actinobacteria	Streptomyces ambofaciens
Bacteria	Actinobacteria	Streptomyces avermitilis
Bacteria	Actinobacteria	Streptomyces chartreusis
Bacteria	Actinobacteria	Streptomyces chattanoogensis
Bacteria	Actinobacteria	Streptomyces coelicolor
Bacteria	Actinobacteria	Streptomyces fradiae var.
Bacteria	Actinobacteria	Streptomyces griseus
Bacteria	Actinobacteria	Streptomyces griseus subsp. griseus
Bacteria	Actinobacteria	Streptomyces halstedii
Bacteria	Actinobacteria	Streptomyces lividans
Bacteria	Actinobacteria	Streptomyces nanchangensis
Bacteria	Actinobacteria	Streptomyces olivaceoviridis
Bacteria	Actinobacteria	Streptomyces reticuli
Bacteria	Actinobacteria	Streptomyces roseiscleroticus
Bacteria	Actinobacteria	Streptomyces sp.
Bacteria	Actinobacteria	Streptomyces thermocyaneoviolaceus
Bacteria	Actinobacteria	Streptomyces thermoviolaceus
Bacteria	Actinobacteria	Streptomyces turgidiscabies
Bacteria	Actinobacteria	Streptomyces viridosporus
Bacteria	Actinobacteria	Thermobifida alba
Bacteria	Actinobacteria	Thermobifida fusca
Bacteria	Actinobacteria	Thermopolyspora flexuosa
Bacteria	Bacteroidetes	Bacteroides cellulosolvens
Bacteria	Bacteroidetes	Bacteroides fragilis
Bacteria	Bacteroidetes	Bacteroides ovatus
Bacteria	Bacteroidetes	Bacteroides thetaiotaomicron
Bacteria	Bacteroidetes	Bacteroides vulgatus
Bacteria	Bacteroidetes	Cytophaga hutchinsonii

Bacteria	Bacteroidetes	Cytophaga xylanolytica
	Division	Organism
Bacteria	Bacteroidetes	Flavobacterium johnsoniae
Bacteria	Bacteroidetes	Flavobacterium psychrophilum
Bacteria	Bacteroidetes	Flavobacterium sp.
Bacteria	Bacteroidetes	Gramella forsetii
Bacteria	Bacteroidetes	Parabacteroides distasonis
Bacteria	Bacteroidetes	Prevotella bryantii
Bacteria	Bacteroidetes	Prevotella ruminicola
Bacteria	Bacteroidetes	Rhodothermus marinus
Bacteria	Chlorobi	Chlorobium chlorochromatii
Bacteria	Chlorobi	Pelodictyon luteolum
Bacteria	Chloroflexi	Chloroflexus aurantiacus
Bacteria	Chloroflexi	Herpetosiphon aurantiacus
Bacteria	Chloroflexi	Roseiflexus castenholzii
Bacteria	Chloroflexi	Roseiflexus sp.
Bacteria	Cyanobacteria	Anabaena variabilis
Bacteria	Cyanobacteria	Nostoc punctiforme
Bacteria	Cyanobacteria	Nostoc sp.
Bacteria	Cyanobacteria	Synechococcus elongatus
Bacteria	Cyanobacteria	Synechococcus sp.
Bacteria	Cyanobacteria	Synechocystis sp.
Bacteria	Deinococcus-Thermus	Deinococcus geothermalis
Bacteria	Deinococcus-Thermus	Thermus caldophilus
Bacteria	Dictyoglomi	Dictyoglomus thermophilum
Bacteria	Fibrobacteres	Fibrobacter intestinalis
Bacteria	Fibrobacteres	Fibrobacter succinogenes
Bacteria	Fibrobacteres	Fibrobacter succinogenes subsp. succinogenes
Bacteria	Firmicutes	Acetivibrio cellulolyticus
Bacteria	Firmicutes	Alicyclobacillus acidocaldarius
Bacteria	Firmicutes	Alkaliphilus metalliredigens
Bacteria	Firmicutes	Anoxybacillus kestanbolensis
Bacteria	Firmicutes	Bacillus agaradhaerens
Bacteria	Firmicutes	Bacillus alcalophilus
Bacteria	Firmicutes	Bacillus amyloliquefaciens
Bacteria	Firmicutes	Bacillus anthracis
Bacteria	Firmicutes	Bacillus cereus
Bacteria	Firmicutes	Bacillus circulans
Bacteria	Firmicutes	Bacillus clausii
Bacteria	Firmicutes	Bacillus firmus
Bacteria	Firmicutes	Bacillus halodurans
Bacteria	Firmicutes	Bacillus licheniformis
Bacteria	Firmicutes	Bacillus plakortiensis

Bacteria	Firmicutes	Bacillus pumilus
	Division	Organism
Bacteria	Firmicutes	Bacillus sp.
Bacteria	Firmicutes	Bacillus subtilis
Bacteria	Firmicutes	Bacillus subtilis subsp. subtilis
Bacteria	Firmicutes	Bacillus thuringiensis serovar alesti
Bacteria	Firmicutes	Bacillus thuringiensis serovar canadensis
Bacteria	Firmicutes	Bacillus thuringiensis serovar darmstadiensis
Bacteria	Firmicutes	Bacillus thuringiensis serovar israelensis
Bacteria	Firmicutes	Bacillus thuringiensis serovar morrisoni
Bacteria	Firmicutes	Bacillus thuringiensis serovar san diego
Bacteria	Firmicutes	Bacillus thuringiensis serovar sotto
Bacteria	Firmicutes	Bacillus thuringiensis serovar thompsoni
Bacteria	Firmicutes	Bacillus thuringiensis serovar tochigiensis
Bacteria	Firmicutes	Butyrivibrio fibrisolvens
Bacteria	Firmicutes	Caldicellulosiruptor saccharolyticus
Bacteria	Firmicutes	Caldicellulosiruptor sp.
Bacteria	Firmicutes	Clostridium acetobutylicum
Bacteria	Firmicutes	Clostridium beijerinckii
Bacteria	Firmicutes	Clostridium cellulolyticum
Bacteria	Firmicutes	Clostridium cellulovorans
Bacteria	Firmicutes	Clostridium difficile
Bacteria	Firmicutes	Clostridium josui
Bacteria	Firmicutes	Clostridium lentocellum
Bacteria	Firmicutes	Clostridium longisporum
Bacteria	Firmicutes	Clostridium phytofermentans
Bacteria	Firmicutes	Clostridium phytofermentans
Bacteria	Firmicutes	Clostridium saccharobutylicum
Bacteria	Firmicutes	Clostridium sp.
Bacteria	Firmicutes	Clostridium stercorarium
Bacteria	Firmicutes	Clostridium thermocellum
Bacteria	Firmicutes	Eubacterium cellulosolvens
Bacteria	Firmicutes	Eubacterium ruminantium
Bacteria	Firmicutes	Geobacillus caldoxylosilyticus
Bacteria	Firmicutes	Geobacillus stearothermophilus
Bacteria	Firmicutes	Geobacillus thermodenitrificans
Bacteria	Firmicutes	Geobacillus thermoleovorans
Bacteria	Firmicutes	Lactobacillus acidophilus
Bacteria	Firmicutes	Lactobacillus brevis

Bacteria	Firmicutes	Lactobacillus gasseri
Bacteria	Firmicutes	Lactobacillus johnsonii
	Division	Organism
Bacteria	Firmicutes	Lactobacillus reuteri
Bacteria	Firmicutes	Lactococcus lactis subsp. cremoris
Bacteria	Firmicutes	Lactococcus lactis subsp. lactis
Bacteria	Firmicutes	Leuconostoc mesenteroides subsp. Mesenteroides
Bacteria	Firmicutes	Listeria innocua
Bacteria	Firmicutes	Listeria monocytogenes
Bacteria	Firmicutes	Paenibacillus barcinonensis
Bacteria	Firmicutes	Paenibacillus curdlanolyticus
Bacteria	Firmicutes	Paenibacillus fukuinensis
Bacteria	Firmicutes	Paenibacillus lautus
Bacteria	Firmicutes	Paenibacillus pabuli
Bacteria	Firmicutes	Paenibacillus polymyxa
Bacteria	Firmicutes	Paenibacillus sp.
Bacteria	Firmicutes	Ruminococcus albus
Bacteria	Firmicutes	Ruminococcus flavefaciens
Bacteria	Firmicutes	Streptococcus mutans
Bacteria	Firmicutes	Streptococcus sanguinis
Bacteria	Firmicutes	Syntrophomonas wolfei subsp. wolfei
Bacteria	Firmicutes	Thermoanaerobacter pseudethanolicus
Bacteria	Firmicutes	Thermoanaerobacter sp.
Bacteria	Firmicutes	Thermoanaerobacter tengcongensis
Bacteria	Firmicutes	Thermoanaerobacterium polysaccharolyticum
Bacteria	Firmicutes	Thermoanaerobacterium saccharolyticum
Bacteria	Firmicutes	Thermoanaerobacterium sp.
Bacteria	Firmicutes	Thermoanaerobacterium thermosulfurigenes
Bacteria	Firmicutes	Thermobacillus xylanilyticus
Bacteria	Fusobacteria	Fusobacterium mortiferum
Bacteria	Planctomycetes	Rhodopirellula baltica
Bacteria	Proteobacteria	Acidiphilium cryptum
Bacteria	Proteobacteria	Acidovorax avenae subsp. citrulli
Bacteria	Proteobacteria	Acinetobacter baumannii
Bacteria	Proteobacteria	Aeromonas hydrophila
Bacteria	Proteobacteria	Aeromonas hydrophila subsp. hydrophila
Bacteria	Proteobacteria	Aeromonas punctata
Bacteria	Proteobacteria	Aeromonas salmonicida subsp. salmonicida
Bacteria	Proteobacteria	Agrobacterium tumefaciens
Bacteria	Proteobacteria	Alcaligenes sp.
Bacteria	Proteobacteria	Anaeromyxobacter dehalogenans

Bacteria	Proteobacteria	Anaeromyxobacter sp.
Bacteria	Proteobacteria	Asaia bogorensis
	Division	Organism
Bacteria	Proteobacteria	Azoarcus sp.
Bacteria	Proteobacteria	Azorhizobium caulinodans
Bacteria	Proteobacteria	Beijerinckia indica subsp. indica
Bacteria	Proteobacteria	Bordetella avium
Bacteria	Proteobacteria	Bradyrhizobium japonicum
Bacteria	Proteobacteria	Brucella abortus
Bacteria	Proteobacteria	Brucella canis
Bacteria	Proteobacteria	Brucella melitensis
Bacteria	Proteobacteria	Brucella ovis
Bacteria	Proteobacteria	Brucella suis
Bacteria	Proteobacteria	Burkholderia ambifaria
Bacteria	Proteobacteria	Burkholderia ambifaria
Bacteria	Proteobacteria	Burkholderia cenocepacia
Bacteria	Proteobacteria	Burkholderia cepacia
Bacteria	Proteobacteria	Burkholderia mallei
Bacteria	Proteobacteria	Burkholderia multivorans
Bacteria	Proteobacteria	Burkholderia phymatum
Bacteria	Proteobacteria	Burkholderia phytofirmans
Bacteria	Proteobacteria	Burkholderia pseudomallei
Bacteria	Proteobacteria	Burkholderia sp.
Bacteria	Proteobacteria	Burkholderia sp.
Bacteria	Proteobacteria	Burkholderia thailandensis
Bacteria	Proteobacteria	Burkholderia vietnamiensis
Bacteria	Proteobacteria	Burkholderia xenovorans
Bacteria	Proteobacteria	Caulobacter crescentus
Bacteria	Proteobacteria	Caulobacter sp.
Bacteria	Proteobacteria	Cellvibrio japonicus (formerly Pseudomonas cellulosa)
Bacteria	Proteobacteria	Cellvibrio mixtus
Bacteria	Proteobacteria	Chromobacterium violaceum
Bacteria	Proteobacteria	Citrobacter koseri
Bacteria	Proteobacteria	Colwellia psychrerythraea
Bacteria	Proteobacteria	Enterobacter cloacae
Bacteria	Proteobacteria	Enterobacter cloacae
Bacteria	Proteobacteria	Enterobacter sakazakii
Bacteria	Proteobacteria	Enterobacter sp.
Bacteria	Proteobacteria	Erwinia carotovora
Bacteria	Proteobacteria	Erwinia carotovora subsp. Atroseptica
Bacteria	Proteobacteria	Erwinia chrysanthemi
Bacteria	Proteobacteria	Erwinia rhapontici
Bacteria	Proteobacteria	Erwinia tasmaniensis
Bacteria	Proteobacteria	Escherichia coli

Bacteria	Proteobacteria	Gluconacetobacter diazotrophicus
Bacteria	Proteobacteria	Gluconacetobacter xylinus
Bacteria	Proteobacteria	Hahella chejuensis
	Division	Organism
Bacteria	Proteobacteria	Halorhodospira halophila
Bacteria	Proteobacteria	Klebsiella pneumoniae
Bacteria	Proteobacteria	Klebsiella pneumoniae subsp. pneumoniae
Bacteria	Proteobacteria	Legionella pneumophila Lens
Bacteria	Proteobacteria	Legionella pneumophila Paris
Bacteria	Proteobacteria	Legionella pneumophila str. Corby
Bacteria	Proteobacteria	Legionella pneumophila subsp. Pneumophila
Bacteria	Proteobacteria	Leptothrix cholodnii
Bacteria	Proteobacteria	Leptothrix cholodnii
Bacteria	Proteobacteria	Lysobacter sp.
Bacteria	Proteobacteria	Maricaulis maris
Bacteria	Proteobacteria	Marinomonas sp.
Bacteria	Proteobacteria	Mesorhizobium loti
Bacteria	Proteobacteria	Methylobacillus flagellatus
Bacteria	Proteobacteria	Methylobacterium extorquens
Bacteria	Proteobacteria	Methylobacterium radiotolerans
Bacteria	Proteobacteria	Methylobacterium sp.
Bacteria	Proteobacteria	Myxococcus xanthus
Bacteria	Proteobacteria	Nitrosospira multiformis
Bacteria	Proteobacteria	Parvibaculum lavamentivorans
Bacteria	Proteobacteria	Pectobacterium carotovorum
Bacteria	Proteobacteria	Pectobacterium carotovorum atroseptica
Bacteria	Proteobacteria	Pectobacterium carotovorum subsp. carotovorum
Bacteria	Proteobacteria	Photobacterium profundum
Bacteria	Proteobacteria	Polaromonas sp.
Bacteria	Proteobacteria	Polynucleobacter sp.
Bacteria	Proteobacteria	Proteus mirabilis
Bacteria	Proteobacteria	Pseudoalteromonas atlantica
Bacteria	Proteobacteria	Pseudoalteromonas atlantica
Bacteria	Proteobacteria	Pseudoalteromonas haloplanktis
Bacteria	Proteobacteria	Pseudoalteromonas sp.
Bacteria	Proteobacteria	Pseudomonas entomophila
Bacteria	Proteobacteria	Pseudomonas fluorescens
Bacteria	Proteobacteria	Pseudomonas putida
Bacteria	Proteobacteria	Pseudomonas sp.
Bacteria	Proteobacteria	Pseudomonas stutzeri
Bacteria	Proteobacteria	Pseudomonas syringae pv. mori
Bacteria	Proteobacteria	Pseudomonas syringae pv. phaseolicola

Bacteria	Proteobacteria	Pseudomonas syringae pv. syringae
Bacteria	Proteobacteria	Pseudomonas syringae pv. Tomato
Bacteria	Proteobacteria	Psychromonas ingrahamii
	Division	Organism
Bacteria	Proteobacteria	Ralstonia eutropha
Bacteria	Proteobacteria	Ralstonia metallidurans
Bacteria	Proteobacteria	Ralstonia solanacearum
Bacteria	Proteobacteria	Ralstonia syzygii
Bacteria	Proteobacteria	Rhizobium etli
Bacteria	Proteobacteria	Rhizobium leguminosarum bv. trifolii
Bacteria	Proteobacteria	Rhizobium sp.
Bacteria	Proteobacteria	Rhodobacter sphaeroides
Bacteria	Proteobacteria	Rhodoferax ferrireducens
Bacteria	Proteobacteria	Rhodopseudomonas palustris
Bacteria	Proteobacteria	Saccharophagus degradans
Bacteria	Proteobacteria	Salmonella enterica subsp. arizonae
Bacteria	Proteobacteria	Salmonella typhimurium
Bacteria	Proteobacteria	Serratia proteamaculans
Bacteria	Proteobacteria	Shigella boydii
Bacteria	Proteobacteria	Shigella flexneri
Bacteria	Proteobacteria	Shigella sonnei
Bacteria	Proteobacteria	Sinorhizobium medicae
Bacteria	Proteobacteria	Sinorhizobium meliloti
Bacteria	Proteobacteria	Sorangium cellulosum
Bacteria	Proteobacteria	Stigmatella aurantiaca
Bacteria	Proteobacteria	Teredinibacter turnerae
Bacteria	Proteobacteria	Thiobacillus denitrificans
Bacteria	Proteobacteria	Vibrio cholerae
Bacteria	Proteobacteria	Vibrio fischeri
Bacteria	Proteobacteria	Vibrio harveyi
Bacteria	Proteobacteria	Vibrio parahaemolyticus
Bacteria	Proteobacteria	Vibrio sp.
Bacteria	Proteobacteria	Vibrio vulnificus
Bacteria	Proteobacteria	Xanthomonas albilineans
Bacteria	Proteobacteria	Xanthomonas axonopodis pv. citri str.
Bacteria	Proteobacteria	Xanthomonas campestris pv. campestris
Bacteria	Proteobacteria	Xanthomonas campestris pv. vesicatoria
Bacteria	Proteobacteria	Xanthomonas oryzae pv. oryzae
Bacteria	Proteobacteria	Xylella fastidiosa
Bacteria	Proteobacteria	Yersinia enterocolitica subsp. enterocolitica
Bacteria	Proteobacteria	Yersinia enterocolitica subsp. enterocolitica
Bacteria	Proteobacteria	Yersinia pestis
Bacteria	Proteobacteria	Yersinia pestis

Bacteria	Proteobacteria	Yersinia pestis Antiqua
Bacteria	Proteobacteria	Yersinia pestis biovar Medievalis
Bacteria	Proteobacteria	Yersinia pseudotuberculosis
	Division	Organism
Bacteria	Proteobacteria	Yersinia pseudotuberculosis
Bacteria	Proteobacteria	Zymomonas mobilis subsp. mobilis
Bacteria	Spirochaetes	Leptospira biflexa
Bacteria	Spirochaetes	Leptospira borgpetersenii
Bacteria	Spirochaetes	Leptospira interrogans
Bacteria	Thermotogae	Fervidobacterium nodosum
Bacteria	Thermotogae	Petrotoga mobilis
Bacteria	Thermotogae	Thermotoga lettingae
Bacteria	Thermotogae	Thermotoga maritima
Bacteria	Thermotogae	Thermotoga neapolitana
Bacteria	Thermotogae	Thermotoga petrophila
Bacteria	Thermotogae	Thermotoga sp.
Bacteria	Verrucomicrobia	Opitutus terrae
Eukaryota	Ascomycota	Acremonium cellulolyticus
Eukaryota	Ascomycota	Acremonium sp.
Eukaryota	Ascomycota	Acremonium thermophilum
Eukaryota	Ascomycota	Alternaria alternata
Eukaryota	Ascomycota	Aspergillus aculeatus
Eukaryota	Ascomycota	Aspergillus flavus
Eukaryota	Ascomycota	Aspergillus fumigatus
Eukaryota	Ascomycota	Aspergillus kawachii
Eukaryota	Ascomycota	Aspergillus nidulans
Eukaryota	Ascomycota	Aspergillus niger
Eukaryota	Ascomycota	Aspergillus oryzae
Eukaryota	Ascomycota	Aspergillus sojae
Eukaryota	Ascomycota	Aspergillus sp.
Eukaryota	Ascomycota	Aspergillus sulphureus
Eukaryota	Ascomycota	Aspergillus terreus
Eukaryota	Ascomycota	Aspergillus tubingensis
Eukaryota	Ascomycota	Aspergillus versicolor
Eukaryota	Ascomycota	Aureobasidium pullulans var. melanigenum
Eukaryota	Ascomycota	Beltraniella portoricensis
Eukaryota	Ascomycota	Bionectria ochroleuca
Eukaryota	Ascomycota	Blumeria graminis
Eukaryota	Ascomycota	Botryosphaeria rhodina
Eukaryota	Ascomycota	Botryotinia fuckeliana
Eukaryota	Ascomycota	Candida albicans
Eukaryota	Ascomycota	Candida glabrata
Eukaryota	Ascomycota	Candida oleophila
Eukaryota	Ascomycota	Chaetomidium pingtungium

Eukaryota	Ascomycota	Chaetomium brasiliense
Eukaryota	Ascomycota	Chaetomium thermophilum
Eukaryota	Ascomycota	Chaetomium thermophilum var. thermophilum
	Division	Organism
Eukaryota	Ascomycota	Chrysosporium lucknowense
Eukaryota	Ascomycota	Claviceps purpurea
Eukaryota	Ascomycota	Coccidioides posadasii
Eukaryota	Ascomycota	Cochliobolus heterostrophus
Eukaryota	Ascomycota	Coniothyrium minitans
Eukaryota	Ascomycota	Corynascus heterothallicus
Eukaryota	Ascomycota	Cryphonectria parasitica
Eukaryota	Ascomycota	Cryptovalsa sp.
Eukaryota	Ascomycota	Cylindrocarpon sp.
Eukaryota	Ascomycota	Daldinia eschscholzii
Eukaryota	Ascomycota	Debaryomyces hansenii
Eukaryota	Ascomycota	Debaryomyces occidentalis
Eukaryota	Ascomycota	Emericella desertorum
Eukaryota	Ascomycota	Emericella nidulans
Eukaryota	Ascomycota	Epichloe festucae
Eukaryota	Ascomycota	Eremothecium gossypii
Eukaryota	Ascomycota	Fusarium anguioides
Eukaryota	Ascomycota	Fusarium chlamydosporum
Eukaryota	Ascomycota	Fusarium culmorum
Eukaryota	Ascomycota	Fusarium equiseti
Eukaryota	Ascomycota	Fusarium lateritium
Eukaryota	Ascomycota	Fusarium oxysporum
Eukaryota	Ascomycota	Fusarium poae
Eukaryota	Ascomycota	Fusarium proliferatum
Eukaryota	Ascomycota	Fusarium sp.
Eukaryota	Ascomycota	Fusarium tricinctum
Eukaryota	Ascomycota	Fusarium udum
Eukaryota	Ascomycota	Fusarium venenatum
Eukaryota	Ascomycota	Fusicoccum sp.
Eukaryota	Ascomycota	Geotrichum sp.
Eukaryota	Ascomycota	Gibberella avenacea
Eukaryota	Ascomycota	Gibberella moniliformis
Eukaryota	Ascomycota	Gibberella pulicaris
Eukaryota	Ascomycota	Gibberella zeae
Eukaryota	Ascomycota	Gliocladium catenulatum
Eukaryota	Ascomycota	Humicola grisea
Eukaryota	Ascomycota	Humicola grisea var. thermoidea
Eukaryota	Ascomycota	Humicola insolens
Eukaryota	Ascomycota	Humicola nigrescens
Eukaryota	Ascomycota	Hypocrea jecorina

Eukaryota	Ascomycota	Hypocrea koningii
Eukaryota	Ascomycota	Hypocrea lixii
Eukaryota	Ascomycota	Hypocrea pseudokoningii
Eukaryota	Ascomycota	Hypocrea schweinitzii
Eukaryota	Ascomycota	Hypocrea virens
	Division	Organism
Eukaryota	Ascomycota	Kluyveromyces lactis
Eukaryota	Ascomycota	Lacazia loboi
Eukaryota	Ascomycota	Leptosphaeria maculans
Eukaryota	Ascomycota	Macrophomina phaseolina
Eukaryota	Ascomycota	Magnaporthe grisea
Eukaryota	Ascomycota	Malbranchea cinnamomea
Eukaryota	Ascomycota	Melanocarpus
Eukaryota	Ascomycota	Melanocarpus albomyces
Eukaryota	Ascomycota	Nectria haematococca
Eukaryota	Ascomycota	Nectria ipomoeae
Eukaryota	Ascomycota	Neotyphodium lolii
Eukaryota	Ascomycota	Neotyphodium sp.
Eukaryota	Ascomycota	Neurospora crassa
Eukaryota	Ascomycota	Nigrospora sp.
Eukaryota	Ascomycota	Paecilomyces lilacinus
Eukaryota	Ascomycota	Paracoccidioides brasiliensis (various strains)
Eukaryota	Ascomycota	Penicillium canescens
Eukaryota	Ascomycota	Penicillium chrysogenum
Eukaryota	Ascomycota	Penicillium citrinum
Eukaryota	Ascomycota	Penicillium decumbens
Eukaryota	Ascomycota	Penicillium funiculosum
Eukaryota	Ascomycota	Penicillium janthinellum
Eukaryota	Ascomycota	Penicillium occitanis
Eukaryota	Ascomycota	Penicillium oxalicum
Eukaryota	Ascomycota	Penicillium purpurogenum
Eukaryota	Ascomycota	Penicillium simplicissimum
Eukaryota	Ascomycota	Pichia angusta
Eukaryota	Ascomycota	Pichia anomala
Eukaryota	Ascomycota	Pichia guilliermondii
Eukaryota	Ascomycota	Pichia pastoris
Eukaryota	Ascomycota	Pichia stipitis
Eukaryota	Ascomycota	Pseudoplectania nigrella
Eukaryota	Ascomycota	Robillarda sp.
Eukaryota	Ascomycota	Saccharomyces bayanus
Eukaryota	Ascomycota	Saccharomyces castellii
Eukaryota	Ascomycota	Saccharomyces cerevisiae
Eukaryota	Ascomycota	Saccharomyces kluyveri
Eukaryota	Ascomycota	Saccobolus dilutellus

Eukaryota	Ascomycota	Sarcoscypha occidentalis
Eukaryota	Ascomycota	Schizosaccharomyces pombe
Eukaryota	Ascomycota	Scopulariopsis brevicaulis
Eukaryota	Ascomycota	Scytalidium thermophilum
Eukaryota	Ascomycota	Stachybotrys chartarum
Eukaryota	Ascomycota	Stachybotrys echinata
	Division	Organism
Eukaryota	Ascomycota	Staphylotrichum coccosporum
Eukaryota	Ascomycota	Stilbella annulata
Eukaryota	Ascomycota	Talaromyces emersonii
Eukaryota	Ascomycota	Thermoascus aurantiacus
Eukaryota	Ascomycota	Thermoascus aurantiacus var. levisporus
Eukaryota	Ascomycota	Thermomyces lanuginosus
Eukaryota	Ascomycota	Thermomyces verrucosus
Eukaryota	Ascomycota	Thielavia australiensis
Eukaryota	Ascomycota	Thielavia microspora
Eukaryota	Ascomycota	Thielavia terrestris
Eukaryota	Ascomycota	Trichoderma asperellum
Eukaryota	Ascomycota	Trichoderma longibrachiatum
Eukaryota	Ascomycota	Trichoderma parceramosum
Eukaryota	Ascomycota	Trichoderma sp.
Eukaryota	Ascomycota	Trichoderma viride
Eukaryota	Ascomycota	Trichophaea saccata
Eukaryota	Ascomycota	Trichothecium roseum
Eukaryota	Ascomycota	Verticillium dahliae
Eukaryota	Ascomycota	Verticillium fungicola
Eukaryota	Ascomycota	Verticillium tenerum
Eukaryota	Ascomycota	Volutella colletotrichoides
Eukaryota	Ascomycota	Xylaria polymorpha
Eukaryota	Ascomycota	Yarrowia lipolytica
Eukaryota	Basidiomycota	Agaricus bisporus
Eukaryota	Basidiomycota	Armillariella tabescens
Eukaryota	Basidiomycota	Athelia rolfsii
Eukaryota	Basidiomycota	Chlorophyllum molybdites
Eukaryota	Basidiomycota	Clitocybe nuda
Eukaryota	Basidiomycota	Clitopilus prunulus
Eukaryota	Basidiomycota	Coprinopsis cinerea
Eukaryota	Basidiomycota	Crinipellis stipitaria
Eukaryota	Basidiomycota	Cryptococcus adeliensis
Eukaryota	Basidiomycota	Cryptococcus flavus
Eukaryota	Basidiomycota	Cryptococcus neoformans
Eukaryota	Basidiomycota	Cryptococcus neoformans var. neoformans
	Division	Organism
Eukaryota	Basidiomycota	Cryptococcus sp.

Eukaryota	Basidiomycota	Exidia glandulosa
Eukaryota	Basidiomycota	Filobasidium floriforme (Cryptococcus albidus)
Eukaryota	Basidiomycota	Fomitopsis palustris
Eukaryota	Basidiomycota	Gloeophyllum sepiarium
Eukaryota	Basidiomycota	Gloeophyllum trabeum
	Division	Organism
Eukaryota	Basidiomycota	Infundibulicybe gibba
Eukaryota	Basidiomycota	Irpex lacteus
Eukaryota	Basidiomycota	Lentinula edodes
Eukaryota	Basidiomycota	Meripilus giganteus
Eukaryota	Basidiomycota	Phanerochaete chrysosporium
Eukaryota	Basidiomycota	Pleurotus sajor-caju
Eukaryota	Basidiomycota	Pleurotus sp.
Eukaryota	Basidiomycota	Polyporus arcularius
Eukaryota	Basidiomycota	Schizophyllum commune
Eukaryota	Basidiomycota	Trametes hirsuta
Eukaryota	Basidiomycota	Trametes versicolor
Eukaryota	Basidiomycota	Ustilago maydis
Eukaryota	Basidiomycota	Volvariella volvacea
Eukaryota	Basidiomycota	Xylaria hypoxylon
Eukaryota	Chlorophyta	Chlorella vulgaris
Eukaryota	Chytridiomycota	Anaeromyces sp.
Eukaryota	Chytridiomycota	Neocallimastix frontalis
Eukaryota	Chytridiomycota	Neocallimastix patriciarum
Eukaryota	Chytridiomycota	Neocallimastix sp.
Eukaryota	Chytridiomycota	Orpinomyces joyonii
Eukaryota	Chytridiomycota	Orpinomyces sp.
Eukaryota	Cnidaria	Hydra magnipapillata
Eukaryota	Mycetozoa	Dictyostelium discoideum
Eukaryota	Ochrophyta	Eisenia andrei
Eukaryota	Oomycota	Phytophthora cinnamomi
Eukaryota	Oomycota	Phytophthora infestans
Eukaryota	Oomycota	Phytophthora ramorum
Eukaryota	Oomycota	Phytophthora sojae
Eukaryota	Prasinophyta	Ostreococcus lucimarinus
Eukaryota	Prasinophyta	Ostreococcus tauri
Eukaryota	Zygomycota	Mucor circinelloides
Eukaryota	Zygomycota	Phycomyces nitens
Eukaryota	Zygomycota	Poitrasia circinans
Eukaryota	Zygomycota	Rhizopus oryzae
Eukaryota	Zygomycota	Syncephalastrum racemosum

TABLES 2 – EXAMPLES OF MICRO-ORGANISMS PRODUCING EXTRA- AND/OR INTRA-CELLULAR LACCASE ENZYMES

	Division	Organism
Eukaryota	Ascomycota	Alternaria alternata
Eukaryota	Ascomycota	Arxula adeninivorans
Eukaryota	Ascomycota	Ashbya gossypii
Eukaryota	Ascomycota	Aspergillus fumigatus
Eukaryota	Ascomycota	Aspergillus niger
Eukaryota	Ascomycota	Aspergillus oryzae
Eukaryota	Ascomycota	Aspergillus terreus
Eukaryota	Ascomycota	Botryotinia fuckeliana
Eukaryota	Ascomycota	Buergenerula spartinae
Eukaryota	Ascomycota	Candida albicans
Eukaryota	Ascomycota	Candida glabrata
Eukaryota	Ascomycota	Chaetomium globosum
Eukaryota	Ascomycota	Chaetomium thermophilum var. thermophilum
Eukaryota	Ascomycota	Claviceps purpurea
Eukaryota	Ascomycota	Coccidioides immitis
Eukaryota	Ascomycota	Colletotrichum lagenarium
Eukaryota	Ascomycota	Corynascus heterothallicus
Eukaryota	Ascomycota	Cryphonectria parasitica
Eukaryota	Ascomycota	Cryptococcus bacillisporus
Eukaryota	Ascomycota	Cryptococcus gattii
Eukaryota	Ascomycota	Cryptococcus neoformans
Eukaryota	Ascomycota	Cryptococcus neoformans var. neoformans
Eukaryota	Ascomycota	Davidiella tassiana
Eukaryota	Ascomycota	Debaryomyces hansenii
Eukaryota	Ascomycota	Emericella nidulans
Eukaryota	Ascomycota	Fusarium oxysporum
Eukaryota	Ascomycota	Fusarium oxysporum f. sp. lycopersici
Eukaryota	Ascomycota	Fusarium proliferatum
Eukaryota	Ascomycota	Gaeumannomyces graminis
Eukaryota	Ascomycota	Gaeumannomyces graminis var. graminis
Eukaryota	Ascomycota	Gaeumannomyces graminis var. tritici
Eukaryota	Ascomycota	Gibberella zeae
Eukaryota	Ascomycota	Glomerella cingulata
Eukaryota	Ascomycota	Hortaea acidophila

Eukaryota	Ascomycota	Humicola insolens
	Division	Organism
Eukaryota	Ascomycota	Hypomyces rosellus
Eukaryota	Ascomycota	Hypoxylon sp.
Eukaryota	Ascomycota	Kluyveromyces lactis
Eukaryota	Ascomycota	Lachnum spartinae
Eukaryota	Ascomycota	Lactarius blennius
Eukaryota	Ascomycota	Lactarius subdulcis
Eukaryota	Ascomycota	Melanocarpus albomyces
Eukaryota	Ascomycota	Morchella conica
Eukaryota	Ascomycota	Morchella crassipes
Eukaryota	Ascomycota	Morchella elata
Eukaryota	Ascomycota	Morchella esculenta
Eukaryota	Ascomycota	Morchella sp.
Eukaryota	Ascomycota	Morchella spongiola
Eukaryota	Ascomycota	Mycosphaerella sp.
Eukaryota	Ascomycota	Neurospora crassa
Eukaryota	Ascomycota	Paracoccidioides brasiliensis
Eukaryota	Ascomycota	Penicillium adametzii
Eukaryota	Ascomycota	Penicillium amagasakiense
Eukaryota	Ascomycota	Penicillium expansum
Eukaryota	Ascomycota	Penicillium simplissimum
Eukaryota	Ascomycota	Penicillium variabile
Eukaryota	Ascomycota	Phaeosphaeria halima
Eukaryota	Ascomycota	Phaeosphaeria spartinicola
Eukaryota	Ascomycota	Pichia pastoris
Eukaryota	Ascomycota	Pleospora spartinae
Eukaryota	Ascomycota	Podospora anserina
Eukaryota	Ascomycota	Saccharomyces cerevisiae
Eukaryota	Ascomycota	Saccharomyces pastorianus
Eukaryota	Ascomycota	Schizosaccharomyces pombe
Eukaryota	Ascomycota	Stagonospora sp.
Eukaryota	Ascomycota	Talaromyces flavus
Eukaryota	Ascomycota	Verpa conica
Eukaryota	Ascomycota	Yarrowia lipolytica
Eukaryota	Basidiomycota	Agaricus bisporus
Eukaryota	Basidiomycota	Amanita citrina
Eukaryota	Basidiomycota	Amylostereum areolatum
Eukaryota	Basidiomycota	Amylostereum chailletii
Eukaryota	Basidiomycota	Amylostereum ferreum
Eukaryota	Basidiomycota	Amylostereum laevigatum
Eukaryota	Basidiomycota	Amylostereum sp.
Eukaryota	Basidiomycota	Athelia rolfsii
Eukaryota	Basidiomycota	Auricularia auricula-judae

Eukaryota	Basidiomycota	Auricularia polytricha
	Division	Organism
Eukaryota	Basidiomycota	Bjerkandera adusta
Eukaryota	Basidiomycota	Bjerkandera sp.
Eukaryota	Basidiomycota	Bondarzewia montana
Eukaryota	Basidiomycota	Ceriporiopsis rivulosa
Eukaryota	Basidiomycota	Ceriporiopsis subvermispora
Eukaryota	Basidiomycota	Cerrena unicolor
Eukaryota	Basidiomycota	Climacocystis borealis
Eukaryota	Basidiomycota	Clitocybe nebularis
Eukaryota	Basidiomycota	Clitocybe quercina
Eukaryota	Basidiomycota	Collybia butyracea
Eukaryota	Basidiomycota	Coniophora puteana
Eukaryota	Basidiomycota	Coprinellus congregatus
Eukaryota	Basidiomycota	Coprinellus disseminatus
Eukaryota	Basidiomycota	Coprinopsis cinerea
Eukaryota	Basidiomycota	Coprinopsis cinerea okayama
Eukaryota	Basidiomycota	Coriolopsis gallica
Eukaryota	Basidiomycota	Cortinarius flexipes
Eukaryota	Basidiomycota	Crinipellis sp.
Eukaryota	Basidiomycota	Cyathus bulleri
Eukaryota	Basidiomycota	Cyathus sp.
Eukaryota	Basidiomycota	Daedalea quercina
Eukaryota	Basidiomycota	Dichomitus squalens
Eukaryota	Basidiomycota	Echinodontium japonicum
Eukaryota	Basidiomycota	Echinodontium tinctorium
Eukaryota	Basidiomycota	Echinodontium tsugicola
Eukaryota	Basidiomycota	Filobasidiella neoformans
Eukaryota	Basidiomycota	Flammulina velutipes
Eukaryota	Basidiomycota	Funalia trogii
Eukaryota	Basidiomycota	Ganoderma applanatum
Eukaryota	Basidiomycota	Ganoderma australe
Eukaryota	Basidiomycota	Ganoderma formosanum
Eukaryota	Basidiomycota	Ganoderma lucidum
Eukaryota	Basidiomycota	Ganoderma sp.
Eukaryota	Basidiomycota	Ganoderma tsunodae
Eukaryota	Basidiomycota	Gloeophyllum trabeum
Eukaryota	Basidiomycota	Grifola frondosa
Eukaryota	Basidiomycota	Gymnopus fusipes
Eukaryota	Basidiomycota	Gymnopus peronatus
Eukaryota	Basidiomycota	Gyromitra esculenta
Eukaryota	Basidiomycota	Halocyphina villosa
Eukaryota	Basidiomycota	Hebeloma radicosum
Eukaryota	Basidiomycota	Heterobasidion abietinum
Eukaryota	Basidiomycota	Heterobasidion annosum

Eukaryota	Basidiomycota	Heterobasidion araucariae
	Division	Organism
Eukaryota	Basidiomycota	Heterobasidion insulare
Eukaryota	Basidiomycota	Heterobasidion parviporum
Eukaryota	Basidiomycota	Hypholoma sp.
Eukaryota	Basidiomycota	Irpex lacteus
Eukaryota	Basidiomycota	Lentinula edodes
Eukaryota	Basidiomycota	Lentinus tigrinus
Eukaryota	Basidiomycota	Lepista flaccida
Eukaryota	Basidiomycota	Lepista irina
Eukaryota	Basidiomycota	Lepista nuda
Eukaryota	Basidiomycota	Lyophyllum shimeji
Eukaryota	Basidiomycota	Macrolepiota procera
Eukaryota	Basidiomycota	Macrotyphula juncea
Eukaryota	Basidiomycota	Malassezia sympodialis
Eukaryota	Basidiomycota	Marasmius alliaceus
Eukaryota	Basidiomycota	Megacollybia platyphylla
Eukaryota	Basidiomycota	Mycena cinerella
Eukaryota	Basidiomycota	Mycena crocata
Eukaryota	Basidiomycota	Mycena galopus
Eukaryota	Basidiomycota	Mycena rosea
Eukaryota	Basidiomycota	Mycena zephirus
Eukaryota	Basidiomycota	Panus rudis
Eukaryota	Basidiomycota	Panus sp.
Eukaryota	Basidiomycota	Paxillus involutus
Eukaryota	Basidiomycota	Peniophora sp.
Eukaryota	Basidiomycota	Phanerochaete chrysosporium
Eukaryota	Basidiomycota	Phanerochaete flavidoalba
Eukaryota	Basidiomycota	Phanerochaete sordida
Eukaryota	Basidiomycota	Phlebia radiata
Eukaryota	Basidiomycota	Phlebiopsis gigantea
Eukaryota	Basidiomycota	Piloderma byssinum
Eukaryota	Basidiomycota	Piriformospora indica
Eukaryota	Basidiomycota	Pleurotus cornucopiae
Eukaryota	Basidiomycota	Pleurotus eryngii
Eukaryota	Basidiomycota	Pleurotus ostreatus
Eukaryota	Basidiomycota	Pleurotus pulmonarius
Eukaryota	Basidiomycota	Pleurotus sajor-caju
Eukaryota	Basidiomycota	Pleurotus sapidus
Eukaryota	Basidiomycota	Pleurotus sp. 'Florida'
Eukaryota	Basidiomycota	Polyporus alveolaris
Eukaryota	Basidiomycota	Polyporus ciliatus
Eukaryota	Basidiomycota	Psathyrella corrugis
Eukaryota	Basidiomycota	Psathyrella dicrani
Eukaryota	Basidiomycota	Psathyrella murcida

Eukaryota	Basidiomycota	Pycnoporus cinnabarinus
Eukaryota	Basidiomycota	Pycnoporus coccineus
	Division	Organism
Eukaryota	Basidiomycota	Pycnoporus sanguineus
Eukaryota	Basidiomycota	Rigidoporus microporus
Eukaryota	Basidiomycota	Russula atropurpurea
Eukaryota	Basidiomycota	Russula mairei
Eukaryota	Basidiomycota	Russula nigricans
Eukaryota	Basidiomycota	Russula ochroleuca
Eukaryota	Basidiomycota	Schizopora paradoxa
Eukaryota	Basidiomycota	Schizophyllum commune
Eukaryota	Basidiomycota	Schizophyllum commune f. trop. radiatum
Eukaryota	Basidiomycota	Spongipellis sp.
Eukaryota	Basidiomycota	Stropharia squamosa
Eukaryota	Basidiomycota	Termitomyces sp.
Eukaryota	Basidiomycota	Thanatephorus cucumeris
Eukaryota	Basidiomycota	Trametes cervina
Eukaryota	Basidiomycota	Trametes hirsuta
Eukaryota	Basidiomycota	Trametes ochracea
Eukaryota	Basidiomycota	Trametes pubescens
Eukaryota	Basidiomycota	Trametes sp.
Eukaryota	Basidiomycota	Trametes versicolor
Eukaryota	Basidiomycota	Trametes villosa
Eukaryota	Basidiomycota	Ustilago maydis
Eukaryota	Basidiomycota	Volvariella volvacea
Eukaryota	Basidiomycota	Xerocomus chrysenteron
Eukaryota	Basidiomycota	Xylaria sp.

What we claim is:

1. A method of producing fatty acids, the method comprising:

(i) inoculating a mixture of at least one of cellulose, hemicellulose, and lignin with at least one microorganism strain that produces one or more cellulase, hemicellulase and laccase, that hydrolyze at least one of cellulose, hemicellulose and lignin, under conditions to produce at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars;

(ii) inhibiting growth of said at least one microorganism strain; and

(iii) inoculating the mixture of step (ii) with at least one algae strain that metabolizes said at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars, under conditions so that said at least one algae strain produces one or more fatty acids.

2. The method of claim 1, wherein the mixture in step (i) further comprises at least one of furfural, phenolics compounds and acetic acid.

3. The method of claim 1, wherein the mixture in step (i) is obtained from a biomass.

4. The method of claim 3, wherein said biomass comprises plant biomass.

5. The method of claim 4, wherein said biomass is obtained from plant or animal waste.

6. The method of claim 4, wherein said plant biomass undergoes pretreatment by acid hydrolysis and heat treatment to produce said mixture inoculated in step (i).

7. The method of claim 4, wherein said plant biomass comprises:

10-35% lignin;

15-35% hemicellulose; and

30-60% cellulose.

8. The method of claim 4, wherein said plant biomass is obtained from at least one selected from the group consisting of: switchgrass, corn stover, and mixed waste of plant.

9. The method of claim 1, wherein said at least one microorganism strain is an extracellular and/or intracellular cellulase, hemicellulase and laccase enzyme producer microorganism.

10. The method of claim 9, wherein said extracellular and/or intracellular cellulase producer microorganism is selected from the group consisting of: prokaryote, bacteria, archaea, and eukaryote, and fungi.

11. The method of claim 10, wherein said extracellular and/or intracellular cellulase producer microorganism is a fungus or bacteria selected from the group consisting of *Humicola*, *Trichoderma*, *Penicillium*, *Ruminococcus*, *Bacillus*, *Cytophaga* and *Sporocytophaga*, *Humicola grisea*, *Trichoderma harzianum*, *Trichoderma lignorum*, *Trichoderma reesei*, *Penicillium verruculosum*, *Ruminococcus albus*, *Bacillus subtilis*, *Bacillus thermoglucosidasius*, *Cytophaga spp.*, and *Sporocytophaga spp*.

12. The method of claim 11, wherein said at least one microorganism strain is a fungi.

13. The method of claim 12, wherein said at least one microorganism strain is *Trichoderma reesei (Hypocrea jecorina)*.

14. The method of claim 1, wherein said at least one microorganism strain is tolerant to one or more compounds produced by a pretreatment of the biomass, wherein said one or more compounds are selected from the group consisting of: furfural, acetic acid, and other impurities.

15. The method of claim 1, wherein said at least one microorganism strain has been evolutionarily modified to metabolize pretreated biomass targeted more efficiently and to better tolerate furfural, phenolics compounds and acetic acid as compared to the unmodified wild-type version of the microorganism.

16. The method of claim 15, wherein said at least one evolutionarily modified microorganism strain produces one or more cellulases, hemicellulases, and/or laccases so that said evolutionarily modified microorganism strain has greater capacity to metabolize cellulose and hemicelluloses with lignin as compared to the unmodified wild-type version of the microorganism.

17. The method of claim 1, wherein said at least one microorganism strain has been evolutionarily modified by at

least one method selected from the group consisting of serial transfer, serial dilution, genetic engine, continuous culture, and chemostat.

18. The method of claim 17, wherein said method is continuous culture.

19. The method of claim 18, wherein said at least one evolutionarily modified microorganism strain is an aerobic fungi.

20. The method of claim 16, wherein said at least one microorganism strain is *Trichoderma reesei* (Hypocrea jecorina) and has been evolutionarily modified by continuous culture.

21. The method of claim 1, wherein said at least one microorganism strain has been evolutionary modified for a specific biomass plant.

22. The method of claim 1, wherein said one or more cellulases is at least one selected from the group consisting of: endoglucanase, exoglucanase, and β-glucosidase, and hemicellulases and optionally laccase.

23. The method of claim 1, further comprising measuring cellulase and/or hemicellulase activity in step (i), and depending on the activity of the enzyme, proceeding to step (ii).

24. The method of claim 1, wherein said inhibition step (ii) is performed by one more methods selected from the group consisting of: heat shock, UV exposure, radiation exposure, gas injection, homogenization, and genetic modification of said at least one microorganism prior to step (i) so that growth of said at least one genetically modified microorganism is inhibited when temperature is increased to 45°C.

25. The method of claim 1, wherein said at least one algae strain in step (iii) is selected from the group consisting of green algae, red algae, blue-green algae, cyanobacteria and diatoms.

26. The method of claim 25, wherein said at least one algae strain in step (iii) is selected from the group consisting of *Monalanthus Salina*; *Botryococcus Braunii*; *Chlorella prototecoides*; *Outirococcus sp.*; *Scenedesmus obliquus*; *Nannochloris sp.*; *Dunaliella bardawil (D. Salina)*; *Navicula pelliculosa*; *Radiosphaera negevensis*; *Biddulphia aurita*;

Chlorella vulgaris; *Nitzschia palea*; *Ochromonas dannica*; *Chrorella pyrenoidosa*; *Peridinium cinctum*; *Neochloris oleabundans*; *Oocystis polymorpha*; *Chrysochromulina spp.*; *Scenedesmus acutus*; *Scenedesmus spp.*; *Chlorella minutissima*; *Prymnesium parvum*; *Navicula pelliculosa*; *Scenedesmus dimorphus*; *Scotiella sp.*; *Chorella spp.*; *Euglena gracilis*; and *Porphyridium cruentum*.

27. The method of claim 1, wherein growth of said at least one algae strain in step (iii) is not inhibited by the presence of one or more of lignin, furfural, phenolics compounds, salts and cellulases enzymes and/or hemicelluases and/or laccase.

28. The method of claim 1, wherein said at least one algae strain in step (iii) can grow in one or more conditions selected from the group consisting of: aerobic, anaerobic, phototrophic, and heterotrophic.

29. The method of claim 1, wherein said at least one algae strain in step (iii) has been evolutionarily modified by at least one method selected from the group consisting of serial transfer, serial dilution, genetic engine, continuous culture, and chemostat.

30. The method of claim 29, wherein said method is continuous culture.

31. The method of claim 29, wherein said at least one algae strain *is Chlorella protothecoides* which has been evolutionarily modified by the continuous culture method.

32. The method of claim 1, wherein said at least one algae strain in step (iii) metabolizes said at least one of glucose, cellobiose, xylose, mannose, galactose, rhamnose, arabinose or other hemicellulose sugars, and waste glycerol.

33. The method of claim 1, wherein said at least one algae strain in step (iii) uses acetic acid as a carbon source.

34. The method of claim 1, wherein when step (iii) is under aerobic and heterotrophic conditions, said at least one algae strain uses respiration.

35. The method of claim 1, wherein in step (iii), when the algae using the same amount of carbon source as an organism producing fermentation by-product producer, the method produces up to 10% carbon dioxide.

36. The method of claim 1, wherein said at least one algae strain in step (iii) produces no inhibitory by-product that inhibits growth of said algae.

37. The method of claim 1, further comprising (iv) recovering said one or more fatty acids from said at least one algae strain.

38. The method of claim 37, wherein said recovering step (iv) comprises at least one selected from the group consisting of filtration-centrifugation, flocculation, solvent extraction, acid extraction, base extraction, homogenization, ultrasonication, microwave, pressing, distillation, thermal evaporation, hydrocracking (fluid catalytic cracking), and drying of said at least one algae strain containing fatty acids.

39. The method of claim 37, wherein supernatant recovered in step (iv) can be reused.

40. The method of claim 1, wherein step (iii) further comprises culturing and growing said at least one algae strain under conditions for extracellular and/or intracellular production of at least one compound selected from the group consisting of fatty acids, hydrocarbons, proteins, pigments,

sugars, such as polysaccharides and monosaccharides, and glycerol.

41. The method of claim 40, wherein said at least one compound can be used for biofuel, cosmetic, alimentary, mechanical grease, pigmentation, and medical use production.

42. The method of claim 1, wherein said at least one algae strain produces hydrocarbon chains which can be used as feedstock for hydrocracking in an oil refinery to produce one or more compounds selected from the group consisting of octane, gasoline, petrol, kerosene, diesel and other petroleum product as solvent, plastic, oil, grease and fibers .

43. The method of claim 37, further comprising, after step (iv), direct transesterification of cells of said at least one algae strain to produce fatty acids for biodiesel fuel.

44. The method of claim 43, wherein the direct transesterification comprises breaking the algae cells, releasing fatty acids and transesterification through a base or acid method with methanol or ethanol to produce biodiesel fuel.

45. The method of claim 1, wherein said at least one algae strain is adapted to use waste glycerol, as carbon source, produced by the transesterification reaction without pretreatment or refinement to produce fatty acids for biodiesel production.

Bio-Ethanol current art

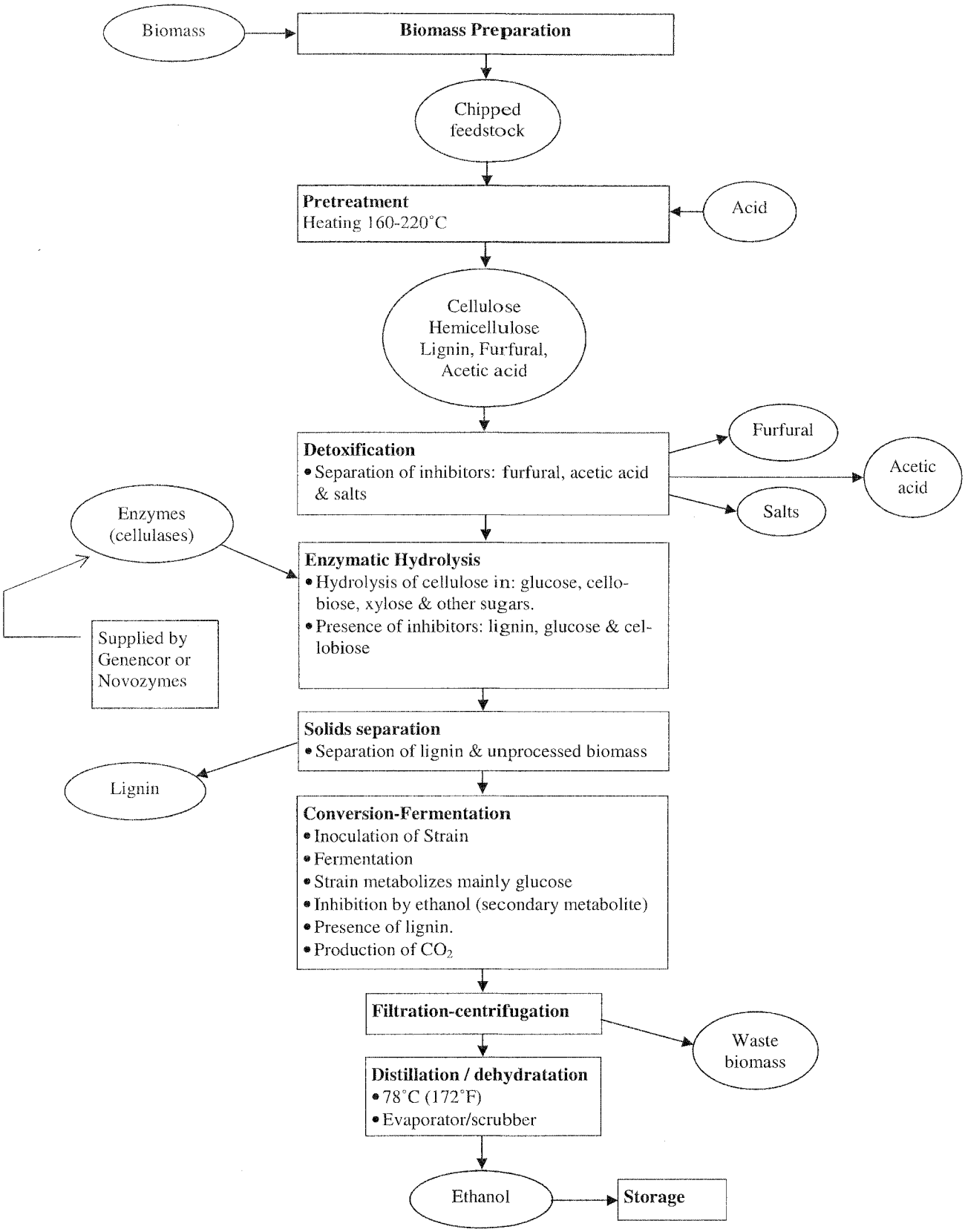

FIG. 1
PRIOR ART

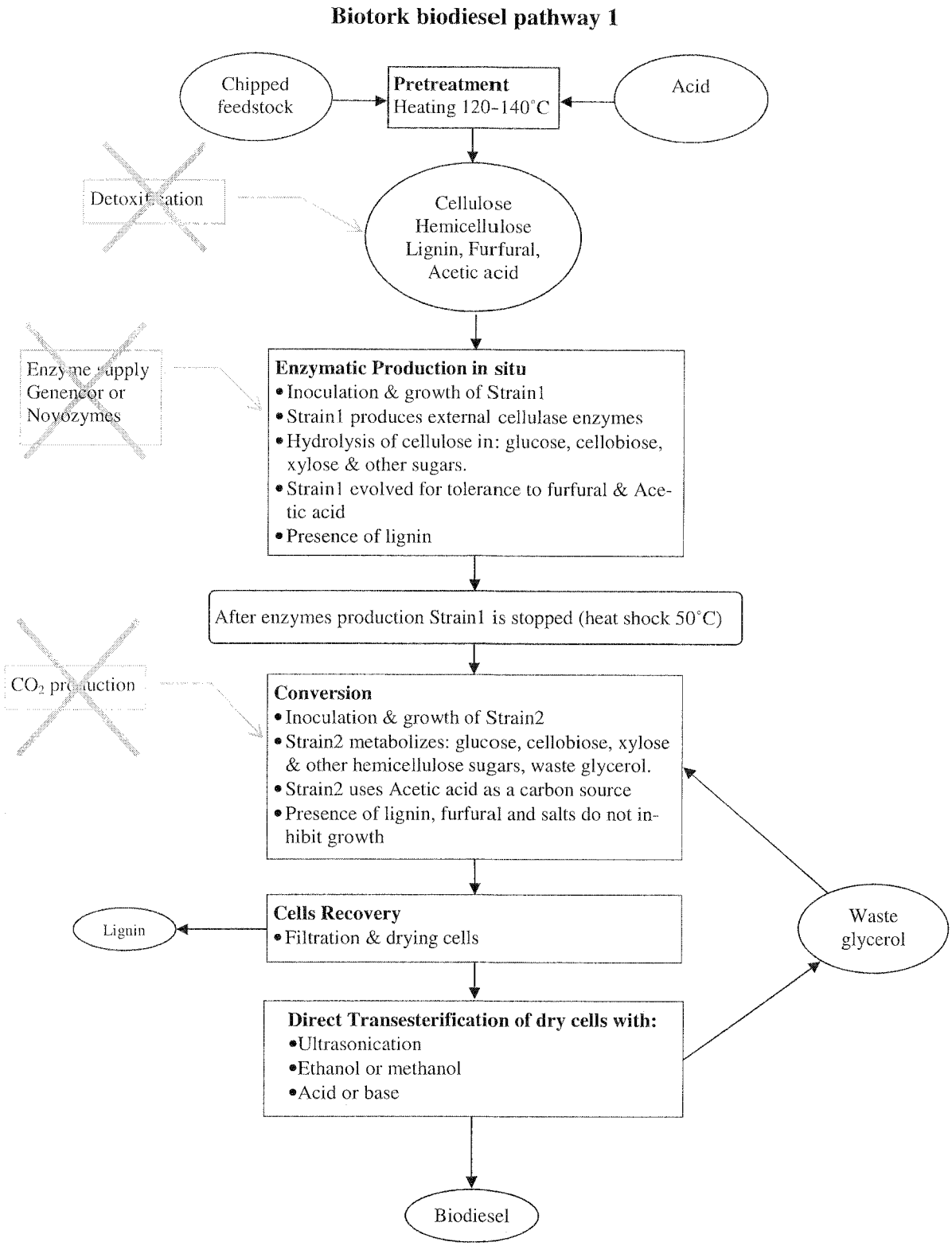
Biotork biodiesel pathway 1
Chipped feedstock
Pretreatment
Heating 120-140°C
Acid
Detoxification
Cellulose
Hemicellulose
Lignin, Furfural,
Acetic acid
Enzyme supply
Genencor or
Novozymes
Enzymatic Production in situ
• Inoculation & growth of Strain1
• Strain1 produces external cellulase enzymes
• Hydrolysis of cellulose in: glucose, cellobiose, xylose & other sugars.
• Strain1 evolved for tolerance to furfural & Acetic acid
• Presence of lignin
After enzymes production Strain1 is stopped (heat shock 50°C)
CO_2 production
Conversion
• Inoculation & growth of Strain2
• Strain2 metabolizes: glucose, cellobiose, xylose & other hemicellulose sugars, waste glycerol.
• Strain2 uses Acetic acid as a carbon source
• Presence of lignin, furfural and salts do not inhibit growth
Lignin
Cells Recovery
• Filtration & drying cells
Waste glycerol
Direct Transesterification of dry cells with:
•Ultrasonication
•Ethanol or methanol
•Acid or base
Biodiesel

FIG. 2

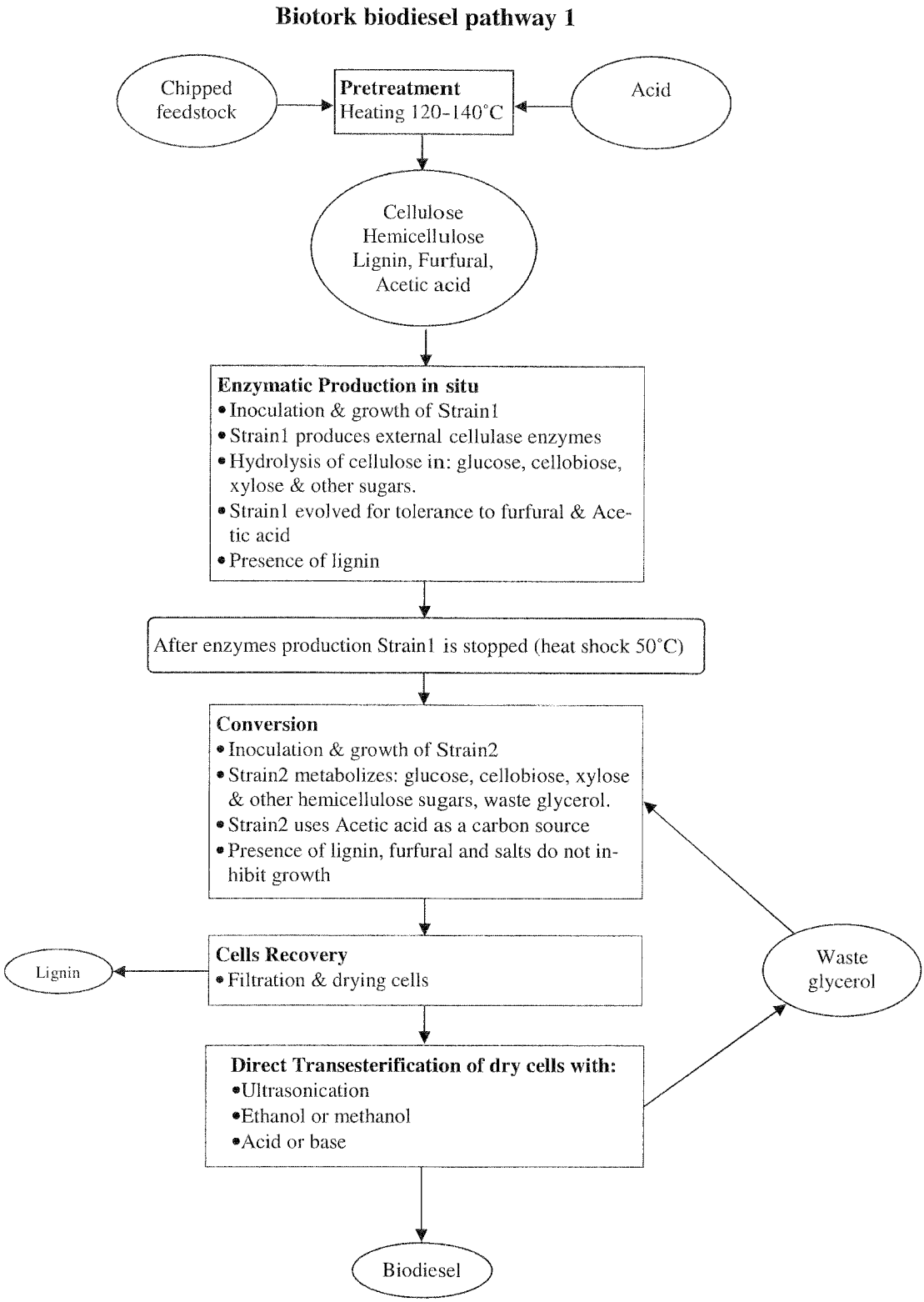
Biotork biodiesel pathway 1
Chipped feedstock
Pretreatment
Heating 120-140°C
Acid
Cellulose
Hemicellulose
Lignin, Furfural,
Acetic acid
Enzymatic Production in situ
• Inoculation & growth of Strain1
• Strain1 produces external cellulase enzymes
• Hydrolysis of cellulose in: glucose, cellobiose, xylose & other sugars.
• Strain1 evolved for tolerance to furfural & Acetic acid
• Presence of lignin
After enzymes production Strain1 is stopped (heat shock 50°C)
Conversion
• Inoculation & growth of Strain2
• Strain2 metabolizes: glucose, cellobiose, xylose & other hemicellulose sugars, waste glycerol.
• Strain2 uses Acetic acid as a carbon source
• Presence of lignin, furfural and salts do not inhibit growth
Lignin
Cells Recovery
• Filtration & drying cells
Waste glycerol
Direct Transesterification of dry cells with:
•Ultrasonication
•Ethanol or methanol
•Acid or base
Biodiesel

FIG. 3

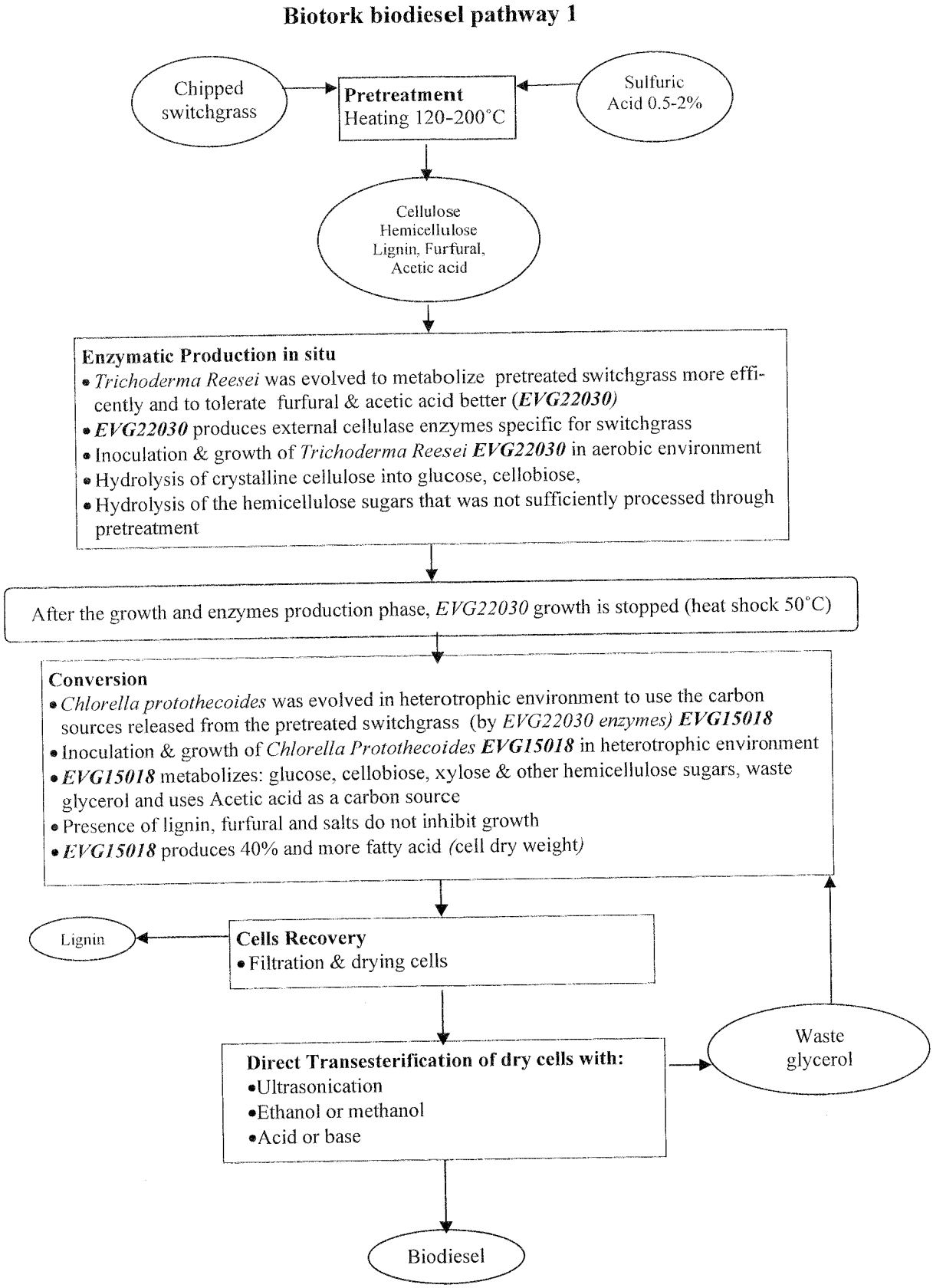
Biotork biodiesel pathway 1
Chipped switchgrass
Pretreatment
Heating 120-200°C
Sulfuric Acid 0.5-2%
Cellulose
Hemicellulose
Lignin, Furfural,
Acetic acid
Enzymatic Production in situ
• Trichoderma Reesei was evolved to metabolize pretreated switchgrass more efficently and to tolerate furfural & acetic acid better (EVG22030)
• EVG22030 produces external cellulase enzymes specific for switchgrass
• Inoculation & growth of Trichoderma Reesei EVG22030 in aerobic environment
• Hydrolysis of crystalline cellulose into glucose, cellobiose,
• Hydrolysis of the hemicellulose sugars that was not sufficiently processed through pretreatment
After the growth and enzymes production phase, EVG22030 growth is stopped (heat shock 50°C)
Conversion
• Chlorella protothecoides was evolved in heterotrophic environment to use the carbon sources released from the pretreated switchgrass (by EVG22030 enzymes) EVG15018
• Inoculation & growth of Chlorella Protothecoides EVG15018 in heterotrophic environment
• EVG15018 metabolizes: glucose, cellobiose, xylose & other hemicellulose sugars, waste glycerol and uses Acetic acid as a carbon source
• Presence of lignin, furfural and salts do not inhibit growth
• EVG15018 produces 40% and more fatty acid (cell dry weight)
Lignin
Cells Recovery
• Filtration & drying cells
Waste glycerol
Direct Transesterification of dry cells with:
•Ultrasonication
•Ethanol or methanol
•Acid or base
Biodiesel

FIG. 4