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(54) **Title**: EXTRACTION SOLVENTS DERIVED FROM OIL FOR ALCOHOL REMOVAL IN EXTRACTIVE FERMENTATION

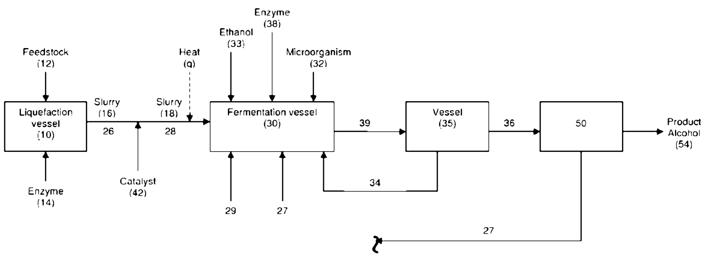

FIG. 1

(57) **Abstract**: In an alcohol fermentation process, oil derived from biomass is hydrolyzed into an extractant available for *in situ* removal of a product alcohol such as butanol from a fermentation broth. The glycerides in the oil can be catalytically (e.g., enzymatically) hydrolyzed into free fatty acids, which form a fermentation product extractant having a partition coefficient for a product alcohol greater than a partition coefficient of the oil of the biomass for the product alcohol. Oil derived from a feedstock of an alcohol fermentation process can be hydrolyzed by contacting the feedstock including the oil with one or more enzymes whereby at least a portion of the oil is hydrolyzed into free fatty acids forming a fermentation product extractant, or the oil can be separated from the feedstock prior to the feedstock being fed to a fermentation vessel, and the separated oil can be contacted with the enzymes to form the fermentation product extractant. The fermentation product extractant can be contacted with a fermentation broth for *in situ* removal of a product alcohol.

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EXTRACTION SOLVENTS DERIVED FROM OIL FOR ALCOHOL REMOVAL IN EXTRACTIVE FERMENTATION

[0001] This application claims the benefit of U.S. Provisional Application No. 61/356,290, filed on June 18, 2010; U.S. Provisional Application No. 61/368,451, filed on July 28, 2010; U.S. Provisional Application No. 61/368,436, filed on July 28, 2010; U.S. Provisional Application No. 61/368,444, filed on July 28, 2010; U.S. Provisional Application No. 61/368,429, filed on July 28, 2010; U.S. Provisional Application No. 61/379,546, filed on September 2, 2010; and U.S. Provisional Application No. 61/440,034, filed on February 7, 2011; U.S. Patent Application No. 13/160,766, filed on June 15, 2011; the entire contents of which are all herein incorporated by reference.

[0002] The Sequence Listing associated with this application is filed in electronic form via EFS-Web and hereby incorporated by reference into the specification in its entirety.

FIELD OF THE INVENTION

[0003] The present invention relates the production of fermentative alcohols such as butanol, and in particular to extraction solvents for extractive fermentation and processes for converting oil derived from biomass into the extraction solvents.

BACKGROUND OF THE INVENTION

[0004] Alcohols have a variety of applications in industry and science such as a beverage (i.e., ethanol), fuel, reagents, solvents, and antiseptics. For example, butanol is an alcohol that is an important industrial chemical with a variety of applications including use as a fuel additive, as a feedstock chemical in the plastics industry, and as a food-grade extractant in the food and flavor industry. Accordingly, there is a high demand for alcohols such as butanol, as well as for efficient and environmentally-friendly production methods.

[0005] Production of alcohol utilizing fermentation by microorganisms is one such environmentally-friendly production method. In the production of butanol, in particular, some microorganisms that produce butanol in high yields also have low butanol toxicity thresholds. Removal of butanol from the fermentation vessel as it is being produced is a means to manage these low butanol toxicity thresholds.

[0006] *In situ* product removal (ISPR) (also referred to as extractive fermentation) can be used to remove butanol (or other fermentative alcohol) from the fermentation vessel as it is produced, thereby allowing the microorganism to produce butanol at high yields. One ISPR method for removing fermentative alcohol that has been described in the art is liquid-liquid extraction (U.S. Patent Application Publication No. 2009/0305370). In order to be technically and economically viable, liquid-liquid extraction calls for good contact between the extractant and the fermentation broth for efficient mass transfer of the product alcohol into the extractant; good phase separation of the extractant from the fermentation broth (during and/or after fermentation); efficient recovery and recycle of the extractant; minimal degradation of the ability of the extractant to extract the product alcohol (e.g., by preventing the lowering of the partition coefficient for the product alcohol into the extractant); and minimal contamination of the extractant by lipids that lower the partition coefficient over a long-term operation.

[0007] The partition coefficient of the extractant can be degraded over time with each recycle, for example, by the build-up of lipids present in the biomass that is fed to the fermentation vessel as feedstock of hydrolysable starch. As an example, a liquefied corn mash loaded to a fermentation vessel at 30 wt% dry corn solids can result in a fermentation broth that contains about 1.2 wt% corn oil during conversion of glucose to butanol by simultaneous saccharification and fermentation (SSF) (with saccharification of the liquefied mash occurring during fermentation by the addition of glucoamylase to produce glucose). The dissolution of the corn oil lipids in oleyl alcohol (OA) serving as an extractant during ISPR can result in build-up of lipid concentration with each OA recycle, decreasing the partition coefficient for the product alcohol in OA as the lipid concentration in OA increases with each recycle of OA.

[0008] In addition, the presence of the undissolved solids during extractive fermentation can negatively affect the efficiency of alcohol production. For example, the presence of the undissolved solids may lower the mass transfer coefficient inside the fermentation vessel, impede phase separation in the fermentation vessel, result in the accumulation of corn oil from the undissolved solids in the extractant leading to reduced extraction efficiency over time, increase the loss of solvent because it becomes trapped in solids and ultimately removed as Dried Distillers' Grains with Solubles (DDGS), slow the disengagement of extractant drops from the fermentation broth, and/or result in a lower fermentation vessel volume efficiency.

[0009] Several approaches for reducing the degradation of the extractant used in extractive fermentation with lipid have included biomass wet milling, fractionation, and removal of solids. Wet milling is an expensive, multi-step process that separates a biomass (e.g., corn) into its key components (germ, pericarp fiber, starch, and gluten) in order to capture value from each co-product separately. This process gives a purified starch stream; however, it is costly and includes the separation of the biomass into its non-starch components which is unnecessary for fermentative alcohol production. Fractionation removes fiber and germ which contains a majority of the lipids present in ground whole grain such as corn, resulting in corn that has a higher starch (endosperm) content. Dry fractionation does not separate the germ and fiber and therefore, it is less expensive than wet milling. However, fractionation does not remove the entirety of the fiber or germ, and does not result in total elimination of solids. Furthermore, there is some loss of starch in fractionation. Wet milling of corn is more expensive than dry fractionation, but dry fractionation is more expensive than dry grinding of unfractionated corn. Removal of solids including germ containing lipids, from liquefied mash prior to use in fermentation can substantially eliminate undissolved solids as described, for example, in co-pending, commonly owned U.S. Provisional Application Serial No. 61/356,290, filed June 18, 2010. However, it would be advantageous if the degradation of the partition coefficient of the extractant can be reduced even without fractionation or removal of undissolved solids. Thus, there is a continuing need to develop more efficient methods and systems for producing product alcohols, such as butanol, through extractive fermentation in which the degradation of the partition coefficient of the extractant is reduced.

[0010] Moreover, the extractant (e.g., oleyl alcohol) is typically added to the fermentation process, rather than produced at a step in the process and therefore, the extractant is a raw material expense. Since extractant can be lost by adsorption on non-fermentable solids and/or diluted by lipids introduced into the fermentation process, the economics of an alcohol production process can be affected by the efficiency of the extractant recovery and recycle. Thus, there exists a continuing need for alternative extractants for ISPR that can result in a more economical process by reducing capital and/or operating costs.

BRIEF SUMMARY OF THE INVENTION

[0011] The present invention satisfies the above needs by providing methods for producing product alcohols such as butanol, in which the lipids in a biomass are converted into an extractant that can be used in ISPR, and in which the amount of lipids that are fed to the fermentation vessel with the feedstock and/or upon extractant recycle, are decreased. The present invention offers a solution to the degradation of the ability of the extractant to extract a product alcohol (e.g., butanol) by preventing the lowering of the partition coefficient for the product alcohol into the extractant. The application offers a solution to the contamination of the extractant by triglycerides that lower the partition coefficient of the extractant for a product alcohol. The present invention provides further related advantages as will be made apparent by the description of the embodiments that follow.

[0012] Catalytic (e.g., enzymatic) hydrolysis of lipids derived from biomass into fatty acids can decrease the rate of undesirable build-up of lipids in the ISPR extractant. The fatty acids can be obtained from hydrolysis of lipids found in the biomass which supplies the fermentable carbon for fermentation. Fatty acids would not be expected to decrease the partition coefficient of the product alcohol such as a butanol into the extractant phase as much as the lipids, as the partition coefficient for butanol from water to fatty acids has been determined to be significantly greater than the partition coefficient for butanol from water to fatty acid esters or triglycerides. Moreover, the fatty acids can be used as an ISPR extractant which can be produced at a step in the alcohol production process and can be used in place of, or in addition to, a supplied, exogenous ISPR extractant that is not produced in the process (such as, but not limited to, oleyl alcohol or oleic acid), thereby reducing the raw material expense for the ISPR extractant.

[0013] In one embodiment, the present invention is directed to a method comprising contacting biomass comprising water, fermentable carbon source, and oil with one or more catalyst whereby at least a portion of the oil is hydrolyzed by one or more catalyst to form an extractant, wherein the fermentable carbon source and the oil are both derived from the biomass. The biomass may comprises corn grain, corn cobs, crop residues, corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, sugar cane, soy, grains, cellulosic material, lignocellulosic material, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof. In a further embodiment, the oil may comprise glycerides and one or more catalysts may hydrolyze the glycerides to form fatty acids. In another embodiment, the one or more catalysts may be selected from esterase, lipase, phospholipase, and lysophospholipase.

[0014] In another embodiment, the extractant may comprise fatty acids, fatty amides, fatty alcohols, fatty esters, triglycerides, or mixtures thereof. In a further embodiment, the extractant may comprise a mixture of fatty acids or a mixture of fatty acids and fatty amides. In a further embodiment, a partition coefficient of the extractant for the product alcohol may be greater than a partition coefficient of the oil of the biomass for the product alcohol.

[0015] The method of the present invention may further comprise the step of inactivating the catalyst after at least a portion of the oil is hydrolyzed. In another embodiment, the method may further comprise the step of separating the oil from the biomass prior to hydrolysis by one or more catalyst. The claimed method may also further comprise the steps of contacting the biomass with a fermentation broth in a fermentation vessel; fermenting the carbon source of the biomass to produce a product alcohol; and removing *in situ* the product alcohol from the fermentation broth by contacting the broth with the extractant. The product alcohol may be butanol.

[0016] In another embodiment, the present invention is directed to a method for producing an alcohol comprising (a) providing biomass comprising water, fermentable carbon source, and oil; (b) liquefying the biomass to produce a liquefied biomass; (c) contacting the liquefied biomass with one or more catalysts whereby at least a portion of the oil is hydrolyzed to form an extractant; (d) contacting the liquefied biomass with a saccharification enzyme capable of converting oligosaccharides into fermentable sugar; (e) contacting the liquefied biomass with a fermentation broth in a fermentation vessel; (f) fermenting the carbon source of the liquefied biomass to produce a product alcohol; (g) removing *in situ* the product alcohol from the fermentation broth by contacting the broth with the extractant; and optionally steps (c) and (d) occur concurrently. The biomass may comprises corn grain, corn cobs, crop residues, corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, sugar cane, soy, grains, cellulosic material, lignocellulosic material, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof. In a further embodiment, the oil may comprise glycerides and one or more catalysts may hydrolyze the glycerides to form fatty acids. In another embodiment, the one or more catalysts may be selected from esterase, lipase, phospholipase, and lysophospholipase. In another embodiment, the extractant may comprise fatty acids, fatty amides, fatty alcohols, fatty esters, triglycerides, or mixtures thereof. In a further embodiment, the extractant may comprise a mixture of fatty acids or a mixture of fatty acids or fatty amides. In a further embodiment, a partition coefficient of the extractant for the product alcohol may be greater than a partition coefficient of the oil of the biomass for the product alcohol. The method of the present invention may further comprise the step of inactivating the catalyst after at least a portion of the oil is hydrolyzed. The product alcohol may be butanol.

[0017] The present invention is also directed to a composition comprising a recombinant microorganism capable of producing an alcohol; fermentable carbon source; one or more catalysts capable of hydrolyzing glycerides into fatty acids; oil comprising glycerides; and fatty acids. The one or more catalysts may be selected from esterase, lipase, phospholipase, and lysophospholipase, and the oil may be corn, tallow, canola, capric/caprylic triglycerides, castor, coconut, cottonseed, fish, jojoba, lard, linseed, neetsfoot, oiticica, palm, peanut, rapeseed, rice, safflower, soya, sunflower, tung, jatropha and vegetable oil blends. In a further embodiment, the fermentable carbon source and the oil are derived from biomass. The biomass may comprise corn grain, corn cobs, crop residues, corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, sugar cane, soy, grains, cellulosic material, lignocellulosic material, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof. The composition may further comprise a saccharification enzyme and/or undissolved solids. The composition may also comprise at least one or more of monoglycerides, diglycerides, triglycerides, glycerol, monosaccharides, oligosaccharides, or alcohol. In addition, the alcohol may be butanol.

[0018] In some embodiments, a method of removing oil derived from biomass from a fermentation process includes contacting an aqueous biomass feedstream with a catalyst. The feedstream includes water, fermentable carbon and an amount of oil, and the fermentable carbon and the oil are both derived from the biomass. At least a portion of the oil is hydrolyzed according to methods described in the present invention into fatty acids to form a catalyst-treated biomass feedstream including the fatty acids.

[0019] In some embodiments, a method of producing an extractant for *in situ* removal of a product alcohol includes providing biomass which includes sugar and oil, the oil having an amount of triglycerides, and contacting the oil with a composition including one or more enzymes capable of hydrolyzing the triglycerides into fatty acids. The triglycerides in the oil are hydrolyzed to form a fermentation product extractant having a partition coefficient for the product alcohol greater than a partition coefficient of the oil of the biomass for the product alcohol.

[0020] In some embodiments, a method for producing butanol includes (a) providing biomass having starch and oil, the oil including an amount of glycerides; (b) liquefying the biomass to produce a liquefied biomass, the liquefied biomass including oligosaccharides hydrolyzed from the starch; (c) contacting the biomass of step (a) or the liquefied biomass of step (b) with a composition having one or more enzymes capable of converting the glycerides into free fatty acids whereby the free fatty acids form a fermentation product extractant; (d) contacting the liquefied biomass with a saccharification enzyme capable of converting oligosaccharides into fermentable sugar including monomeric glucose; (e) contacting the liquefied biomass with a biocatalyst capable of converting the fermentable sugar to butanol whereby a fermentation product comprising butanol is produced; and (f) contacting the fermentation product with the fermentation product extractant whereby the butanol is separated from the fermentation product, the fermentation product extractant having a partition coefficient for the butanol greater than a partition coefficient of the oil of the biomass for the butanol.

[0021] In some embodiments, a method includes, at a step during a process to produce a product alcohol from a feedstock, contacting the product alcohol with an extractant comprising free fatty acids obtained from enzymatic hydrolysis of a native oil wherein the oil comprises glycerides. The extractant has a partition coefficient for the product alcohol greater than a partition coefficient of the native oil for the product alcohol.

[0022] In some embodiments, the process to produce a product alcohol from a feedstock includes (a) liquefying the feedstock to create a feedstock slurry; (b) centrifuging the feedstock slurry of (a) to produce a centrifuge product including (i) an aqueous layer comprising sugar, (ii) a plant-derived oil layer, and (iii) a solids layer; (c) feeding the aqueous layer of (b) to a fermentation vessel; and (d) fermenting the sugar of the aqueous layer to produce the product alcohol.

[0023] In some embodiments, the process to produce a product alcohol from a feedstock further includes adding the extractant to the fermentation vessel to form a two-phase mixture comprising an aqueous phase and a product alcohol-containing organic phase.

[0024] In some embodiments, the native oil is a plant-derived oil, and in some embodiments, the process to produce a product alcohol from a feedstock further includes obtaining the plant-derived oil from the plant-derived oil layer; and converting the plant-derived oil into the extractant by contacting the oil with one or more enzymes that hydrolyze the glycerides into free fatty acids.

[0025] In some embodiments, the process to produce a product alcohol from a feedstock further includes inactivating the one or more enzymes after at least a portion of the glycerides have been hydrolyzed into free fatty acids.

[0026] In some embodiments, the process to produce a product alcohol from a feedstock further includes feeding the plant-derived oil to the fermentation vessel prior to the step of converting the plant-derived oil into the extractant.

[0027] In some embodiments, the process to produce a product alcohol from a feedstock further includes adding a second extractant to the fermentation vessel to form a two-phase mixture comprising an aqueous phase and a product alcohol-containing organic phase.

[0028] In some embodiments, the plant-derived oil is converted to the extractant after the step of adding a second extractant.

[0029] In some embodiments, a method of removing oil derived from biomass from a fermentation process, includes (a) providing a fermentation broth comprising a product alcohol and oil derived from biomass, the oil including glycerides; (b) contacting the fermentation broth with a first extractant to form a two-phase mixture comprising an aqueous phase and an organic phase, wherein the product alcohol and the oil partition into the organic phase to form a product alcohol-containing organic phase; (c) separating the product alcohol-containing organic phase from the aqueous phase; (d) separating the product alcohol from the organic phase to produce a lean organic phase; and (e) contacting the lean organic phase with a composition comprising one or more catalysts capable of hydrolyzing the glycerides into free fatty acids to produce a second extractant comprising at least a portion of the first extractant and free fatty acids.

[0030] In some embodiments, the method further includes repeating step (b) by contacting the fermentation broth with the second extractant of step (e).

[0031] In some embodiments, an *in situ* fermentation extractant-forming composition includes (a) mash formed from biomass and including water, starch and oil, (b) a catalyst capable of hydrolyzing at least a portion of the triglycerides into free fatty acids, and (c) free fatty acids. The starch and the oil are both derived from the biomass, and the oil includes an amount of triglycerides.

[0032] In some embodiments, a fermentation broth includes (a) a recombinant microorganism capable of producing butanol, (b) oligosaccharides, (c) a catalyst for hydrolyzing glycerides into free fatty acids, (d) glycerides, and (e) free fatty acids.

BRIEF DESCRIPTION OF THE DRAWINGS/FIGURES

[0033] The accompanying drawings, which are incorporated herein and form a part of the specification, illustrate the present invention and, together with the description, further serve to explain the principles of the invention and to enable a person skilled in the pertinent art to make and use the invention.

[0034] FIG. 1 schematically illustrates an exemplary method and system of the present invention, in which a liquefied biomass is contacted with a catalyst for lipid hydrolysis before fermentation.

[0035] FIG. 2 schematically illustrates an exemplary method and system of the present invention, in which a liquefied and saccharified biomass is contacted with a catalyst for lipid hydrolysis before fermentation.

[0036] FIG. 3 schematically illustrates an exemplary method and system of the present invention, in which lipids in a biomass feedstream are contacted with a catalyst for lipid hydrolysis before or during liquefaction.

[0037] FIG. 4 schematically illustrates an exemplary method and system of the present invention, in which undissolved solids and lipids are removed from a liquefied biomass before fermentation, and in which the removed lipids are hydrolyzed into free fatty acids using a catalyst, and the free fatty acids are supplied to the fermentation vessel.

[0038] FIG. 5 schematically illustrates an exemplary method and system of the present invention, in which lipids derived from native oil are hydrolyzed into free fatty acids using a catalyst, and the free fatty acids are supplied to the fermentation vessel.

[0039] FIG. 6 schematically illustrates an exemplary method and system of the present invention, in which biomass lipids present in a first extractant exiting a fermentation vessel are converted into free fatty acids that are supplied to a fermentation vessel as a second extractant.

[0040] FIG. 7 is a chart illustrating the effect that the presence of fatty acids in a fermentation vessel has on glucose consumption for butanologen strain NGCI-047.

[0041] FIG. 8 is a chart illustrating the effect that the presence of fatty acids in a fermentation vessel has on glucose consumption for butanologen strain NGCI-049.

[0042] FIG. 9 is a chart illustrating the effect that the presence of fatty acids in a fermentation vessel has on glucose consumption for butanologen strain NYLA84.

DETAILED DESCRIPTION OF THE INVENTION

[0043] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In case of conflict, the present application including the definitions will control. Also, unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. All publications, patents and other references mentioned herein are incorporated by reference in their entireties for all purposes.

[0044] In order to further define this invention, the following terms and definitions are herein provided.

[0045] As used herein, the terms "comprises," "comprising," "includes," "including," "has," "having," "contains," or "containing," or any other variation thereof, will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. For example, a composition, a mixture, a process, a method, an article, or an apparatus that comprises a list of elements is not necessarily limited to only those elements but can include other elements not expressly listed or inherent to such composition, mixture, process, method, article, or apparatus. Further, unless expressly stated to the contrary, "or" refers to an inclusive or and not to an exclusive or. For example, a condition A or B is satisfied by any one of the following: A is true (or present) and B is false (or not present), A is false (or not present) and B is true (or present), and both A and B are true (or present).

[0046] Also, the indefinite articles "a" and "an" preceding an element or component of the invention are intended to be nonrestrictive regarding the number of instances, that is, occurrences of the element or component. Therefore "a" or "an" should be read to include one or at least one, and the singular word form of the element or component also includes the plural unless the number is obviously meant to be singular.

[0047] The term "invention" or "present invention" as used herein is a non-limiting term and is not intended to refer to any single embodiment of the particular invention but encompasses all possible embodiments as described in the application.

[0048] As used herein, the term "about" modifying the quantity of an ingredient or reactant of the invention employed refers to variation in the numerical quantity that can occur, for example, through typical measuring and liquid handling procedures used for making concentrates or solutions in the real world; through inadvertent error in these procedures; through differences in the manufacture, source, or purity of the ingredients employed to make the compositions or to carry out the methods; and the like. The term "about" also encompasses amounts that differ due to different equilibrium conditions for a composition resulting from a particular initial mixture. Whether or not modified by the term "about," the claims include equivalents to the quantities. In one embodiment, the term "about" means within 10% of the reported numerical value, alternatively within 5% of the reported numerical value.

[0049] "Biomass" as used herein refers to a natural product containing hydrolyzable polysaccharides that provide fermentable sugars including any sugars and starch derived from natural resources such as corn, cane, wheat, cellulosic or lignocellulosic material and materials comprising cellulose, hemicellulose, lignin, starch, oligosaccharides, disaccharides and/or monosaccharides, and mixtures thereof. Biomass may also comprise additional components such as protein and/or lipids. Biomass may be derived from a single source or biomass can comprise a mixture derived from more than one source. For example, biomass may comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, sugar cane, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof. For example, mash, juice, molasses, or hydrolysate may be formed from biomass by any processing known in the art for processing the biomass for purposes of fermentation such as by milling, treating, and/or liquefying and comprises fermentable sugar and may comprise water. For example, cellulosic and/or lignocellulosic biomass may be processed to obtain a hydrolysate containing fermentable sugars by any method known to one skilled in the art. A low ammonia pretreatment is disclosed in U.S. Patent Application Publication No. 2007/0031918A1, which is herein incorporated by reference. Enzymatic saccharification of cellulosic and/or lignocellulosic biomass typically makes use of an enzyme consortium for breaking down cellulose and hemicellulose to produce a hydrolysate containing sugars including glucose, xylose, and arabinose. (Saccharification enzymes suitable for cellulosic and/or lignocellulosic biomass are reviewed in Lynd, et al. (Microbiol. Mol. Biol. Rev. 66:506-577, 2002).

[0050] Mash, juice, molasses, or hydrolysate may include feedstock 12 and feedstock slurry 16 as described herein. An aqueous feedstream may be derived or formed from biomass by any processing known in the art for processing the biomass for purposes of fermentation such as by milling, treating, and/or liquefying and comprises fermentable carbon substrate (e.g., sugar) and may comprise water. An aqueous feedstream may include feedstock 12 and feedstock slurry 16 as described herein.

[0051] "Feedstock" as used herein means a feed in a fermentation process, the feed containing a fermentable carbon source with or without undissolved solids, and where applicable, the feed containing the fermentable carbon source before or after the fermentable carbon source has been liberated from starch or obtained from the break down of complex sugars by further processing such as by liquefaction, saccharification, or other process. Feedstock includes or is derived from a biomass. Suitable feedstocks include, but are not limited to, rye, wheat, corn, cane, barley, cellulosic material, lignocellulosic material, or mixtures thereof.

[0052] "Fermentation broth" as used herein means the mixture of water, sugars, dissolved solids, optionally microorganisms producing alcohol, product alcohol, and all other constituents of the material held in the fermentation vessel in which product alcohol is being made by the reaction of sugars to alcohol, water, and carbon dioxide (CO_2) by the microorganisms present. From time to time, as used herein the term "fermentation medium" and "fermented mixture" can be used synonymously with "fermentation broth."

[0053] "Fermentable carbon source" or “fermentable carbon substrate” as used herein means a carbon source capable of being metabolized by the microorganisms disclosed herein for the production of fermentative alcohol. Suitable fermentable carbon sources include, but are not limited to, monosaccharides such as glucose or fructose; disaccharides such as lactose or sucrose; oligosaccharides; polysaccharides such as starch or cellulose; C5 sugars such as xylose and arabinose; one carbon substrates including methane; and mixtures thereof.

[0054] "Fermentable sugar" as used herein refers to one or more sugars capable of being metabolized by the microorganisms disclosed herein for the production of fermentative alcohol.

[0055] "Fermentation vessel" as used herein means the vessel in which the fermentation reaction is carried out whereby product alcohol such as butanol is made from sugars.

[0056] "Liquefaction vessel" as used herein means the vessel in which liquefaction is carried out. Liquefaction is the process in which oligosaccharides are liberated from the feedstock. In some embodiments where the feedstock is corn, oligosaccharides are liberated from the corn starch content during liquefaction.

[0057] "Saccharification vessel" as used herein means the vessel in which saccharification (i.e., the break down of oligosaccharides into monosaccharides) is carried out. Where fermentation and saccharification occur simultaneously, the saccharification vessel and the fermentation vessel may be one in the same vessel.

[0058] "Sugar" as used herein refers to oligosaccharides, disaccharides, monosaccharides, and/or mixtures thereof. The term "saccharide" also includes carbohydrates including starches, dextrans, glycogens, cellulose, pentosans, as well as sugars.

[0059] As used herein, "saccharification enzyme" means one or more enzymes that are capable of hydrolyzing polysaccharides and/or oligosaccharides, for example, alpha-1,4-glucosidic bonds of glycogen, or starch. Saccharification enzymes may include enzymes capable of hydrolyzing cellulosic or lignocellulosic materials as well.

[0060] "Undissolved solids" as used herein means non-fermentable portions of feedstock, for example, germ, fiber, and gluten.

[0061] "Product alcohol" as used herein refers to any alcohol that can be produced by a microorganism in a fermentation process that utilizes biomass as a source of fermentable carbon substrate. Product alcohols include, but are not limited to, C_1 to C_8 alkyl alcohols. In some embodiments, the product alcohols are C_2 to C_8 alkyl alcohols. In other embodiments, the product alcohols are C_2 to C_5 alkyl alcohols. It will be appreciated that C_1 to C_8 alkyl alcohols include, but are not limited to, methanol, ethanol, propanol, butanol, and pentanol. Likewise C_2 to C_8 alkyl alcohols include, but are not limited to, ethanol, propanol, butanol, and pentanol. "Alcohol" is also used herein with reference to a product alcohol.

[0062] "Butanol" as used herein refers with specificity to the butanol isomers 1-butanol (1-BuOH), 2-butanol (2-BuOH), *tert*-butanol, and/or isobutanol (iBuOH or *i*-BuOH or I-BUOH, also known as 2-methyl-1-propanol), either individually or as mixtures thereof. From time to time, when referring to esters of butanol, the terms "butyl esters" and "butanol esters" may be used interchangeably.

[0063] “Propanol” as used herein refers to the propanol isomers isopropanol or 1-propanol.

[0064] “Pentanol” as used herein refers to the pentanol isomers 1-pentanol, 3-methyl-1-butanol, 2-methyl-1-butanol, 2,2-dimethyl-1-propanol, 3-pentanol, 2-pentanol, 3-methyl-2-butanol, or 2-methyl-2-butanol.

[0065] The term "alcohol equivalent" as used herein refers to the weight of alcohol that would be obtained by a perfect hydrolysis of an alcohol ester and the subsequent recovery of the alcohol from an amount of alcohol ester.

[0066] The term "aqueous phase titer" as used herein refers to the concentration of a particular alcohol (e.g., butanol) in the fermentation broth.

[0067] The term "effective titer" as used herein refers to the total amount of a particular alcohol (e.g., butanol) produced by fermentation or alcohol equivalent of the alcohol ester produced by alcohol esterification per liter of fermentation medium. For example, the effective titer of butanol in a unit volume of a fermentation includes: (i) the amount of butanol in the fermentation medium; (ii) the amount of butanol recovered from the organic extractant; (iii) the amount of butanol recovered from the gas phase, if gas stripping is used; and (iv) the alcohol equivalent of the butanol ester in either the organic or aqueous phase.

[0068] "*In Situ* Product Removal (ISPR)" as used herein means the selective removal of a specific fermentation product from a biological process such as fermentation, to control the product concentration in the biological process as the product is produced.

[0069] "Extractant" or "ISPR extractant" as used herein means an organic solvent used to extract any product alcohol such as butanol or used to extract any product alcohol ester produced by a catalyst from a product alcohol and a carboxylic acid or lipid. From time to time, as used herein the term "solvent" may be used synonymously with "extractant." For the processes described herein, extractants are water-immiscible.

[0070] The terms "water-immiscible" or “insoluble” refer to a chemical component such as an extractant or solvent, which is incapable of mixing with an aqueous solution such as a fermentation broth, in such a manner as to form one liquid phase.

[0071] The term "aqueous phase" as used herein refers to the aqueous phase of a biphasic mixture obtained by contacting a fermentation broth with a water-immiscible organic extractant. In an embodiment of a process described herein that includes fermentative extraction, the term "fermentation broth" then specifically refers to the aqueous phase in biphasic fermentative extraction.

[0072] The term "organic phase" as used herein refers to the non-aqueous phase of a biphasic mixture obtained by contacting a fermentation broth with a water-immiscible organic extractant.

[0073] The term "carboxylic acid" as used herein refers to any organic compound with the general chemical formula −COOH in which a carbon atom is bonded to an oxygen atom by a double bond to make a carbonyl group (−C=O) and to a hydroxyl group (−OH) by a single bond. A carboxylic acid may be in the form of the protonated carboxylic acid, in the form of a salt of a carboxylic acid (e.g., an ammonium, sodium, or potassium salt), or as a mixture of protonated carboxylic acid and salt of a carboxylic acid. The term carboxylic acid may describe a single chemical species (e.g., oleic acid) or a mixture of carboxylic acids as can be produced, for example, by the hydrolysis of biomass-derived fatty acid esters or triglycerides, diglycerides, monoglycerides, and phospholipids.

[0074] The term "fatty acid" as used herein refers to a carboxylic acid (e.g., aliphatic monocarboxylic acid) having C_4 to C_{28} carbon atoms (most commonly C_{12} to C_{24} carbon atoms), which is either saturated or unsaturated. Fatty acids may also be branched or unbranched. Fatty acids may be derived from, or contained in esterified form, in an animal or vegetable fat, oil, or wax. Fatty acids may occur naturally in the form of glycerides in fats and fatty oils or may be obtained by hydrolysis of fats or by synthesis. The term fatty acid may describe a single chemical species or a mixture of fatty acids. In addition, the term fatty acid also encompasses free fatty acids.

[0075] The term "fatty alcohol" as used herein refers to an alcohol having an aliphatic chain of C_4 to C_{22} carbon atoms, which is either saturated or unsaturated.

[0076] The term "fatty aldehyde" as used herein refers to an aldehyde having an aliphatic chain of C_4 to C_{22} carbon atoms, which is either saturated or unsaturated.

[0077] The term "fatty amide" as used herein refers to an amide having a long, aliphatic chain of C_4 to C_{22} carbon atoms, which is either saturated or unsaturated

[0078] The term "fatty ester" as used herein refers to an ester having a long aliphatic chain of C_4 to C_{22} carbon atoms, which is either saturated or unsaturated.

[0079] "Native oil" as used herein refers to lipids obtained from plants (e.g., biomass) or animals. "Plant-derived oil" as used herein refers to lipids obtain from plants in particular. From time to time, "lipids" may be used synonymously with "oil" and "acyl glycerides." Native oils include, but are not limited to, tallow, corn, canola, capric/caprylic triglycerides, castor, coconut, cottonseed, fish, jojoba, lard, linseed, neetsfoot, oiticica, palm, peanut, rapeseed, rice, safflower, soya, sunflower, tung, jatropha, and vegetable oil blends.

[0080] The term "separation" as used herein is synonymous with “recovery” and refers to removing a chemical compound from an initial mixture to obtain the compound in greater purity or at a higher concentration than the purity or concentration of the compound in the initial mixture.

[0081] As used herein, "recombinant microorganism" refers to microorganisms such as bacteria or yeast, that are modified by use of recombinant DNA techniques, for example, by engineering a host cell to comprise a biosynthetic pathway such as a biosynthetic pathway to produce an alcohol such as butanol.

[0082] The present invention provides extractants obtained by catalytic hydrolysis of oil glycerides derived from biomass and methods of producing the extractants. In particular, the glycerides in biomass oil can be catalytically hydrolyzed into fatty acids using a catalyst such as an enzyme. The fatty acids can serve as extractants for *in situ* removal of a product alcohol such as butanol from a fermentation broth. Thus, the present invention also provides methods for producing a product alcohol such as butanol through extractive fermentation using the extractants that were produced from the biomass oil. The present invention also provides methods for catalytically hydrolyzing the oil present in a feedstock slurry into fatty acids prior to fermentation, whereby the oil is converted to fatty acids and the degradation of the partition coefficient of the ISPR extractant over time that is attributable to the presence of the oil in the fermentation vessel can be reduced. Moreover, the fatty acids obtained by hydrolysis of the feedstock oil can serve as an ISPR extractant having a partition coefficient for a fermentative alcohol greater than a partition coefficient of the feedstock oil for the fermentative alcohol. The feedstock oil can be separated from the feedstock slurry prior to hydrolysis and used as an ISPR extractant, or the oil can be hydrolyzed into fatty acids while in the feedstock slurry. Further, fatty acids as ISPR extractant can be used in place of or in addition to a conventional exogenous extractant, such as oleyl alcohol or oleic acid, thereby reducing the raw material expense associated with the exogenous extractant.

[0083] The present invention will be described with reference to the Figures. FIG. 1 illustrates an exemplary process flow diagram for production of fermentative alcohol according to an embodiment of the present invention. As shown, a feedstock 12 can be introduced to an inlet in a liquefaction vessel 10 and liquefied to produce a feedstock slurry 16. Feedstock 12 contains hydrolysable starch that supplies a fermentable carbon source (e.g., fermentable sugar such as glucose), and can be a biomass such as, but not limited to, rye, wheat, corn, cane, barley, cellulosic material, lignocellulosic material, or mixtures thereof, or can otherwise be derived from a biomass. In some embodiments, feedstock 12 can be one or more components of a fractionated biomass and in other embodiments, feedstock 12 can be a milled, unfractionated biomass. In some embodiments, feedstock 12 can be corn such as dry milled, unfractionated corn kernels, and the undissolved solids can include germ, fiber, and gluten. The undissolved solids are non-fermentable portions of feedstock 12. For purposes of the discussion herein with reference to the embodiments shown in the Figures, feedstock 12 will often be described as constituting milled, unfractionated corn in which the undissolved solids have not been separated therefrom. However, it should be understood that the exemplary methods and systems described herein can be modified for different feedstocks whether fractionated or not, as apparent to one of skill in the art. In some embodiments, feedstock 12 can be high-oleic corn, such that corn oil derived therefrom is a high-oleic corn oil having an oleic acid content of at least about 55 wt% oleic acid. In some embodiments, the oleic acid content in high-oleic corn oil can be up to about 65 wt%, as compared with the oleic acid content in normal corn oil which is about 24 wt%. High-oleic oil can provide some advantages for use in the methods of the present invention, as hydrolysis of the oil provides fatty acids having a high oleic acid content for contacting with a fermentation broth. In some embodiments, the fatty acids or mixtures thereof comprise unsaturated fatty acids. The presence of unsaturated fatty acids decreases the melting point, providing advantages for handling. Of the unsaturated fatty acids, those which are monounsaturated, that is, possessing a single carbon-carbon double bond, may provide advantages with respect to melting point without sacrificing suitable thermal and oxidative stability for process considerations.

[0084] The process of liquefying feedstock 12 involves hydrolysis of starch in feedstock 12 into sugars including, for example, dextrins and oligosaccharides, and is a conventional process. Any known liquefying processes, as well as the corresponding liquefaction vessel, normally utilized by the industry can be used including, but not limited to, the acid process, the acid-enzyme process, or the enzyme process. Such processes can be used alone or in combination. In some embodiments, the enzyme process can be utilized and an appropriate enzyme 14, for example, alpha-amylase, is introduced to an inlet in liquefaction vessel 10. Water can also be introduced to liquefaction vessel 10. In some embodiments, a saccharification enzyme, for example, glucoamylase, may also be introduced to liquefaction vessel 10. In additional embodiments, a lipase may also be introduced to liquefaction vessel 10 to catalyze the conversion of one or more components of the oil to fatty acids.

[0085] Feedstock slurry 16 produced from liquefying feedstock 12 includes sugar, oil 26, and undissolved solids derived from the biomass from which feedstock 12 was formed. In some embodiments, the oil is in an amount of about 0 wt% to at least about 2 wt% of the fermentation broth composition. In some embodiments, the oil is in an amount of at least about 0.5 wt% of the feedstock. Feedstock slurry 16 can be discharged from an outlet of liquefaction vessel 10. In some embodiments, feedstock 12 is corn or corn kernels and therefore, feedstock slurry 16 is a corn mash slurry.

[0086] A catalyst 42 can be added to feedstock slurry 16. Catalyst 42 is capable of hydrolyzing glycerides in oil 26 to free fatty acids (FFA) 28. For example, when feedstock 12 is corn, then oil 26 is the feedstock's constituent corn oil, and the free fatty acids 28 are corn oil fatty acids (COFA). Thus, after introduction of catalyst 42 to feedstock slurry 16, at least a portion of the glycerides in oil 26 are hydrolyzed to FFA 28, resulting in a feedstock slurry 18 having FFA 28 and catalyst 42. The resulting acid/oil composition from hydrolyzing oil 26 is typically at least about 17 wt% FFA. In some embodiments, the resulting acid/oil composition from hydrolyzing oil 26 is at least about 20 wt% FFA, at least about 25 wt% FFA, at least about 30 wt% FFA, at least about 35 wt% FFA, at least about 40 wt% FFA, at least about 45 wt% FFA, at least about 50 wt% FFA, at least about 55 wt% FFA, at least about 60 wt% FFA, at least about 65 wt% FFA, at least about 70 wt% FFA, at least about 75 wt% FFA, at least about 80 wt% FFA, at least about 85 wt% FFA, at least about 90 wt% FFA, at least about 95 wt% FFA, or at least about 99 wt% FFA. In some embodiments, the concentration of the fatty acid (such as carboxylic acid) in the fermentation vessel exceeds the solubility limit in the aqueous phase and results in the production a two-phase fermentation mixture comprising an organic phase and an aqueous phase. In some embodiments, the concentration of carboxylic acid (or fatty acid) in the fermentation broth is typically not greater than about 0.8 g/L and is limited by the solubility of the carboxylic acid (or fatty acid) in the broth.

[0087] In some embodiments, catalyst 42 can be one or more enzymes, for example, hydrolase enzymes such as lipase enzymes. Lipase enzymes used may be derived from any source including, for example, *Absidia, Achromobacter, Aeromonas, Alcaligenes, Alternaria, Aspergillus, Achromobacter, Aureobasidium, Bacillus, Beauveria, Brochothrix, Candida, Chromobacter, Coprinus, Fusarium,* *Geotricum, Hansenula, Humicola, Hyphozyma, Lactobacillus, Metarhizium, Mucor, Nectria, Neurospora*, *Paecilomyces, Penicillium, Pseudomonas, Rhizoctonia, Rhizomucor, Rhizopus, Rhodosporidium, Rhodotorula, Saccharomyces, Sus, Sporobolomyces, Thermomyces, Thiarosporella, Trichoderma, Verticillium,* and/or a strain of *Yarrowia.* In a preferred aspect, the source of the lipase is selected from the group consisting of *Absidia blakesleena*, *Absidia corymbifera, Achromobacter iophagus, Alcaligenes* sp., *Alternaria brassiciola, Aspergillus flavus, Aspergillus niger*, *Aspergillus tubingensis*, Aureo*basidium pullulans, Bacillus pumilus, Bacillus strearothermophilus*, *Bacillus subtilis, Brochothrix thermosohata, Candida cylindracea (Candida rugosa), Candida paralipolytica, Candida Antarctica* lipase A, *Candida antartica* lipase B, *Candida ernobii, Candida deformans, Chromobacter viscosum, Coprinus cinerius, Fusarium oxysporum, Fusarium solani, Fusarium solani pisi, Fusarium roseum culmorum, Geotricum penicillatum, Hansenula anomala, Humicola brevispora, Humicola brevis* var. thermoidea, *Humicola insolens, Lactobacillus curvatus, Rhizopus oryzae*, *Penicillium cyclopium, Penicillium crustosum, Penicillium expansum, Penicillium* sp. I, *Penicillium* sp. II, *Pseudomonas aeruginosa, Pseudomonas alcaligenes, Pseudomonas cepacia* (syn. *Burkholderia cepacia), Pseudomonas fluorescens, Pseudomonas fragi, Pseudomonas maltophilia, Pseudomonas mendocina, Pseudomonas mephitica lipolytica, Pseudomonas alcaligenes, Pseudomonas plantari, Pseudomonas pseudoalcaligenes, Pseudomonas putida, Pseudomonas stutzeri,* and *Pseudomonas wisconsinensis, Rhizoctonia solani, Rhizomucor miehei, Rhizopus japonicus, Rhizopus microsporus*, *Rhizopus nodosus, Rhodosporidium toruloides, Rhodotorula glutinis, Saccharomyces cerevisiae, Sporobolomyces shibatanus, Sus scrofa*, *Thermomyces lanuginosus* (formerly *Humicola lanuginose*), *Thiarosporella phaseolina, Trichoderma harzianum, Trichoderma reesei,* and *Yarrowia lipolytica.* In a further preferred aspect, the lipase is selected from the group consisting of *Thermomcyces lanuginosus lipase*, *Aspergillus* sp. lipase, *Aspergillus niger* lipase, *Aspergillus tubingensis* lipase, *Candida antartica lipase* B, *Pseudomonas sp*. lipase, *Penicillium roqueforti* lipase, *Penicillium camembertii* lipase, *Mucor javanicus* lipase, *Burkholderia* *cepacia* lipase, *Alcaligenes sp.* lipase, *Candida rugosa* lipase, *Candida parapsilosis* lipase, *Candida deformans* lipase, lipases A and B from *Geotrichum candidum*, *Neurospora crassa* lipase, *Nectria haematococca* lipase, *Fusarium heterosporum* lipase *Rhizopus delemar* lipase, *Rhizomucor miehei* lipase, *Rhizopus arrhizus* lipase, and *Rhizopus oryzae* lipase. Suitable commercial lipase preparations suitable as enzyme catalyst 42 include, but are not limited to Lipolase® 100 L, Lipex® 100L, Lipoclean® 2000T, Lipozyme® CALB L, Novozyme® CALA L, and Palatase 20000L, available from Novozymes, or from *Pseudomonas fluorescens, Pseudomonas cepacia, Mucor miehei, hog pancreas, Candida cylindracea, Rhizopus niveus, Candida antarctica, Rhizopus arrhizus or Aspergillus* available from SigmaAldrich.

[0088] Phospholipases are enzymes that hydrolyze the ester bonds of phospholipids, but many phospholipases also can hydrolyze triglycerides, diglycerides, and monoglycerides (lipid acyl hydrolase (LAH) activity). As used herein, the term "phospholipase" encompasses enzymes having any phospholipase activity, for example, cleaving a glycerolphosphate ester linkage (catalyzing hydrolysis of a glycerolphosphate ester linkage), for example, in an oil, such as a crude oil or a vegetable oil. The phospholipase activity of the invention can generate a water extractable phosphorylated base and a diglyceride. The phospholipase activity can comprise a phospholipase C (PLC) activity; a PI-PLC activity, a phospholipase A (PLA) activity such as a phospholipase AI or phospholipase A2 activity; a phospholipase B (PLB) activity such as a phospholipase B1 or phospholipase B2 activity, including lysophospholipase (LPL) activity and/or lysophospholipase- transacylase (LPT A) activity; a phospholipase D (PLD) activity such as a phospholipase DI or a phospholipase D2 activity; and/or a patatin activity or any combination thereof. The term "phospholipase" also encompasses enzymes having lysophospholipase activity, where the two substrates of this enzyme are 2-lysophosphatidylcholine and H_2O, and where its two products are glycerophosphocholine and carboxylate. Phospholipase AI (PLA1) enzymes remove the 1-position fatty acid to produce free fatty acid and 1-lyso-2-acylphospholipid. Phospholipase A2 (PLA2) enzymes remove the 2-position fatty acid to produce free fatty acid and l-acyl-2-

lysophospholipid. PLA1 and PLA2 enzymes can be intra- or extra-cellular, membrane-bound or soluble. Phospholipase C (PLC) enzymes remove the phosphate moiety to produce 1 ,2 diacylglycerol and a phosphate ester. Phospholipase D (PLD) enzymes produce 1 ,2-diacylglycerophosphate and base group. A phospholipase useful in the present invention may be obtained from a variety of biological sources, for example, but not limited to, filamentous fungal species within the genus *Fusarium*, such as a strain of *F. culmorum*, *F. heterosporum*, *F. solani*, or *F. oxysporum*; or a filamentous fungal species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or *Aspergillus oryzae*. Also useful in the present invention are *Thermomyces lanuginosus* phospholipase variants such as the commercial product Lecitase® Ultra (Novozymes A'S, Denmark). One or more phospholipases may be applied as lyophilized powder, immobilized or in aqueous solution.

[0089] After at least a portion of the glycerides are hydrolyzed, in some embodiments, catalyst 42 can be inactivated. Any method known in the art can be used to render catalyst 42 inactive. For example, in some embodiments, catalyst 42 can be inactivated by the application of heat, by adjusting the pH of the reaction mass to a pH where catalyst 42 is irreversibly inactivated, and/or by adding a chemical or biochemical species capable of selectively inactivating the catalyst activity. As shown, for example, in the embodiment of FIG. 1, heat *q* is applied to feedstock slurry 18, whereby catalyst 42 becomes inactive. The application of heat *q* can be applied to feedstock slurry 18 before feedstock slurry 18 is fed to a fermentation vessel 30. Heat-treated feedstock slurry 18 (with inactive catalyst 42) is then introduced into a fermentation vessel 30 along with a microorganism 32 to be included in a fermentation broth held in fermentation vessel 30. Alternatively, feedstock slurry 18 can be fed to fermentation vessel 30 and subjected to heat *q* while in the fermentation vessel, before fermentation vessel inoculation of microorganism 32. For example, in some embodiments, catalyst inactivation treatment can be achieved by heating feedstock slurry 18 with heat *q* to temperature of at least about 75ºC for at least about 5 minutes, at least about 75ºC for at least about 10 minutes, at least about 75ºC for at least about 15 minutes, at least about 80ºC for at least about 5 minutes, at least about 80ºC for at least about 10 minutes, at least about 80ºC for at least about 15 minutes, at least about 85ºC for at least about 5 minutes, at least about 85ºC for at least about 10 minutes, or at least about 85ºC for at least about 15 minutes. In some embodiments, after being subject to heat *q*, feedstock slurry 18 is cooled to an appropriate temperature for fermentation prior to introduction to fermentation vessel 30 (or prior to fermentation vessel inoculation in the case that the application of heat *q* is conducted in the fermentation vessel). For example, in some embodiments, the temperature of feedstock slurry 18 is about 30ºC prior to contacting with a fermentation broth.

[0090] Inactivation of catalyst 42 is preferred when it is desirable to prevent catalyst 42 from esterifying alcohol with fatty acids 28 in the fermentation vessel. In some embodiments, production of an alcohol ester by esterification of product alcohol in a fermentation medium with an organic acid (e.g., fatty acid) and a catalyst (e.g., lipase) is desirable, as further described in co-pending, commonly owned U.S. Provisional Application Serial No. 61/368,429, filed on July 28, 2010; U.S. Provisional Application Serial No. 61/379,546, filed on September 2, 2010; and U.S. Provisional Application Serial No. 61/440,034, filed on February 7, 2011, all incorporated herein in its entirety by reference thereto. For example, for butanol production, active catalyst 42 in fermentation vessel (introduced via slurry 18) can catalyze the esterification of the butanol with fatty acids 28 (introduced via slurry 18) to form fatty acid butyl esters (FABE) in situ.

[0091] Fermentation vessel 30 is configured to ferment slurry 18 to produce a product alcohol such as butanol. In particular, microorganism 32 metabolizes the fermentable sugar in slurry 18 and excretes a product alcohol. Microorganism 32 is selected from the group of bacteria, cyanobacteria, filamentous fungi, and yeasts. In some embodiments, microorganism 32 can be a bacteria such as *E.coli*. In some embodiments, microorganism 32 can be a fermentative recombinant microorganism. The slurry can include sugar, for example, in the form of oligosaccharides, and water, and can comprise less than about 20 g/L of monomeric glucose, more preferably less than about 10 g/L or less than about 5 g/L of monomeric glucose. Suitable methodology to determine the amount of monomeric glucose is well known in the art. Such suitable methods known in the art include HPLC.

[0092] In some embodiments, slurry 18 is subjected to a saccharification process in order to break the complex sugars (e.g., oligosaccharides) in slurry 18 into monosaccharides that can be readily metabolized by microorganism 32. Any known saccharification process that is routinely utilized by the industry can be used including, but not limited to, the acid process, the acid-enzyme process, or the enzyme process. In some embodiments, simultaneous saccharification and fermentation (SSF) can occur inside fermentation vessel 30, as shown in FIG. 1. In some embodiments, an enzyme 38, such as glucoamylase, can be introduced to an inlet in fermentation vessel 30 in order to breakdown the starch or oligosaccharides to glucose capable of being metabolized by microorganism 32.

[0093] Optionally, ethanol 33 may be supplied to fermentation vessel 30 to be included in the fermentation broth. In some embodiments, when a recombinant microorganism having a butanol biosynthetic pathway is used as microorganism 32 for butanol production, microorganism 32 may require supplementation of a 2-carbon substrate (e.g., ethanol) to survive and grow. Thus, in some embodiments, ethanol 33 may be supplied to fermentation vessel 30.

[0094] However, it has been surprisingly found that methods of the present invention, in which free fatty acid (e.g., FFA 28) is present in the fermentation vessel, can allow reduction of the amount of ethanol 33 typically supplied for a given recombinant microorganism without detriment to the vitality of the recombinant microorganism. Further, in some embodiments, the methods of the present invention provide that the alcohol (e.g., butanol) production rate without ethanol supplementation to be comparable with the production rate that can be realized when ethanol 33 is supplemented. As further demonstrated by the comparative examples presented in Examples 1-14 below, the butanol production rate when fatty acid but not ethanol is in the fermentation vessel can be greater than the butanol production rate when neither fatty acid nor ethanol is in the fermentation vessel. Thus, in some embodiments, the amount of ethanol 33 supplementation is reduced compared to conventional processes. For example, a typical amount of ethanol added to a fermentation vessel for microorganisms requiring supplementation of a 2-carbon substrate is about 5 g/L anhydrous ethanol (i.e., 5 g anhydrous ethanol per liter of fermentation medium). In some embodiments, the butanol fermentation is not supplemented with any ethanol 33. In the latter case, the stream of ethanol 33 is entirely omitted from the fermentation vessel. Thus, in some embodiments of the present invention, it is possible to reduce or eliminate the cost associated with supplemental ethanol 33, as well as the inconvenience associated with storing vats of ethanol 33 and supplying it to the fermentation vessel during butanol fermentation.

[0095] Moreover, regardless of ethanol supplementation, in some embodiments, the methods of the present invention can provide a higher rate of glucose uptake by microorganism 32 by virtue of the presence of fatty acids during the fermentation. The fatty acids can be introduced into fermentation vessel 30 as carboxylic acid 28, hydrolyzed from supplied oil 26, and/or derived from hydrolysis of constituent biomass oil of slurry 16. Methods for producing a product alcohol from a fermentation process in which fatty acids are produced at a step in the process and are contacted with microorganism cultures in a fermentation vessel for improving microorganism growth rate and glucose consumption are described in co-pending, commonly owned U.S. Provisional Application Serial No. 61/368,451, filed on July 28, 2010, which is incorporated herein in its entirety by reference thereto.

[0096] In fermentation vessel 30, alcohol is produced by microorganism 32. *In situ* product removal (ISPR) can be utilized to remove the product alcohol from the fermentation broth. In some embodiments, ISPR includes liquid-liquid extraction. Liquid-liquid extraction can be performed according to the processes described in U.S. Patent Application Publication No. 2009/0305370, the disclosure of which is hereby incorporated in its entirety. U.S. Patent Application Publication No. 2009/0305370 describes methods for producing and recovering butanol from a fermentation broth using liquid-liquid extraction, the methods comprising the step of contacting the fermentation broth with a water-immiscible extractant to form a two-phase mixture comprising an aqueous phase and an organic phase. Typically, the extractant can be an organic extractant selected from the group consisting of saturated, mono-unsaturated, poly-unsaturated (and mixtures thereof) C_{12} to C_{22} fatty alcohols, C_{12} to C_{22} fatty acids, esters of C_{12} to C_{22} fatty acids, C_{12} to C_{22} fatty aldehydes, C_{12} to C_{22} fatty amides, triglycerides, and mixtures thereof, which contacts a fermentation broth and to form a two-phase mixture comprising an aqueous phase and an organic phase. The extractant may also be an organic extractant selected from the group consisting of saturated, mono-unsaturated, poly-unsaturated (and mixtures thereof) C_4 to C_{22} fatty alcohols, C_4 to C_{28} fatty acids, esters of C_4 to C_{28} fatty acids, C_4 to C_{22} fatty aldehydes, C_4 to C_{22} fatty amides, and mixtures thereof, which contacts a fermentation broth and to form a two-phase mixture comprising an aqueous phase and an organic phase. Free fatty acids 28 from slurry 18 can also serve as an ISPR extractant 28. For example, when free fatty acids 28 are corn oil fatty acids (COFA), ISPR extractant 28 is COFA. ISPR extractant (FFA) 28 contacts the fermentation broth and forms a two-phase mixture comprising an aqueous phase 34 and an organic phase. The product alcohol present in the fermentation broth preferentially partitions into the organic phase to form an alcohol-containing organic phase 36. In some embodiments, fermentation vessel 30 has one or more inlets for receiving one or more additional ISPR extractants 29 which form a two-phase mixture comprising an aqueous phase and an organic phase, with the product alcohol partitioning into the organic phase.

[0097] The biphasic mixture can be removed from fermentation vessel 30 as stream 39 and introduced into a vessel 35, in which the alcohol-containing organic phase 36 is separated from the aqueous phase 34. The alcohol-containing organic phase 36 is separated from the aqueous phase 34 of the biphasic mixture stream 39 using methods known in the art including, but not limited to, siphoning, aspiration, decantation, centrifugation, using a gravity settler, membrane-assisted phase splitting, and the like. All or part of the aqueous phase 34 can be recycled into fermentation vessel 30 as fermentation medium (as shown), or otherwise discarded and replaced with fresh medium, or treated for the removal of any remaining product alcohol and then recycled to fermentation vessel 30. Then, the alcohol-containing organic phase 36 is treated in a separator 50 to recover product alcohol 54, and the resulting alcohol-lean extractant 27 can then be recycled back into fermentation vessel 30, usually in combination with fresh FFA 28 from slurry 18 and/or with fresh extractant 29 for further extraction of the product alcohol. Alternatively, fresh FFA 28 (from slurry 18) and/or extractant 29 can be continuously added to the fermentation vessel to replace the ISPR extractant(s) removed in biphasic mixture stream 39.

[0098] In some embodiments, any additional ISPR extractant 29 can be an exogenous organic extractant such as oleyl alcohol, behenyl alcohol, cetyl alcohol, lauryl alcohol, myristyl alcohol, stearyl alcohol, 1-undecanol, oleic acid, lauric acid, myristic acid, stearic acid, methyl myristate, methyl oleate, undecanal, lauric aldehyde, 20-methylundecanal, and mixtures thereof. In some embodiments, ISPR extractant 29 can be a carboxylic acid and in some embodiments, ISPR extractant 29 can be a fatty acid. In some embodiments, the carboxylic acid or fatty acid can have 4 to 28 carbons, 4 to 22 carbons in other embodiments, 8 to 22 carbons in other embodiments, 10 to 28 carbons in other embodiments, 7 to 22 carbons in other embodiments, 12 to 22 carbons in other embodiments, 4 to 18 carbons in other embodiments, 12 to 22 carbons in other embodiments, and 12 to 18 carbons in still other embodiments. In some embodiments, ISPR extractant 29 is one or more of the following fatty acids: azaleic, capric, caprylic, castor, coconut (i.e., as a naturally-occurring combination of fatty acids, including lauric, myrisitic, palmitic, caprylic, capric, stearic, caproic, arachidic, oleic, and linoleic, for example), dimer, isostearic, lauric, linseed, myristic, oleic, olive, palm oil, palmitic, palm kernel, peanut, pelargonic, ricinoleic, sebacic, soya, stearic acid, tall oil, tallow, #12 hydroxy stearic, or any seed oil. In some embodiments, ISPR extractant 29 is one or more of diacids, azelaic, dimer and sebacic acid. Thus, in some embodiments, ISPR extractant 29 can be a mixture of two or more different fatty acids. In some embodiments, ISPR extractant 29 can be a fatty acid derived from chemical or enzymatic hydrolysis of glycerides derived from native oil. For example, in some embodiments, ISPR extractant 29 can be free fatty acids 28' obtained by enzymatic hydrolysis of native oil such as biomass lipids as later described with reference to the embodiment of FIG. 5. In some embodiments, ISPR extractant 29 can be a fatty acid extractant selected from the group consisting of fatty acids, fatty alcohols, fatty amides, fatty acid methyl esters, lower alcohol esters of fatty acids, fatty acid glycol esters, hydroxylated triglycerides, and mixtures thereof, obtained from chemical conversion of native oil such as biomass lipids as described for example in co-pending, commonly owned U.S. Provisional Application Serial No. 61/368,436, filed on July 28, 2010. In such embodiments, the biomass lipids for producing extractant 29 can be from a same or different biomass source from which feedstock 12 is obtained. For example, in some embodiments, the biomass lipids for producing extractant 29 can be derived from soya, whereas the biomass source of feedstock 12 is corn. Any possible combination of different biomass sources for extractant 29 versus feedstock 12 can be used, as should be apparent to one of skill in the art. In some embodiments, additional ISPR extractant 29 includes COFA.

[0099] *In situ* extractive fermentation can be carried out in a batch mode or a continuous mode in fermentation vessel 30. For *in situ* extractive fermentation, the organic extractant can contact the fermentation medium at the start of the fermentation forming a biphasic fermentation medium. Alternatively, the organic extractant can contact the fermentation medium after the microorganism has achieved a desired amount of growth, which can be determined by measuring the optical density of the culture. Further, the organic extractant can contact the fermentation medium at a time at which the product alcohol level in the fermentation medium reaches a preselected level. In the case of butanol production, for example, the ISPR extractant can contact the fermentation medium at a time before the butanol concentration reaches a level which would be toxic to the microorganism. After contacting the fermentation medium with the ISPR extractant, the butanol product partitions into the extractant, decreasing the concentration in the aqueous phase containing the microorganism, thereby limiting the exposure of the production microorganism to the inhibitory butanol product.

[00100] The volume of the ISPR extractant to be used depends on a number of factors including the volume of the fermentation medium, the size of the fermentation vessel, the partition coefficient of the extractant for the butanol product, and the fermentation mode chosen, as described below. The volume of the extractant can be about 3% to about 60% of the fermentation vessel working volume. Depending on the efficiency of the extraction, the aqueous phase titer of butanol in the fermentation medium can be, for example, from about 1 g/L to about 85 g/L, from about 10 g/L to about 40 g/L, from about 10 g/L to about 20 g/L, from about 15 g/L to about 50 g/L or from about 20 g/L to about 60 g/L. In some embodiments, the resulting fermentation broth after alcohol esterification can comprise free (i.e., unesterified) alcohol and in some embodiments, the concentration of free alcohol in the fermentation broth after alcohol esterification is not greater than 1, 3, 6, 10, 15, 20, 25, 30 25, 40, 45, 50, 55, or 60 g/L when the product alcohol is butanol, or when the product alcohol is ethanol, the concentration of free alcohol in the fermentation broth after alcohol esterification is not greater than 15, 20, 25, 30 25, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 g/L. Without being held to theory, it is believed that higher butanol titer may obtained with the extractive fermentation method, in part, from the removal of the toxic butanol product from the fermentation medium, thereby keeping the level below that which is toxic to the microorganism.

[00101] In a batchwise mode of *in situ* extractive fermentation, a volume of organic extractant is added to the fermentation vessel and the extractant is not removed during the process. This mode requires a larger volume of organic extractant to minimize the concentration of the inhibitory butanol product in the fermentation medium. Consequently, the volume of the fermentation medium is less and the amount of product produced is less than that obtained using the continuous mode. For example, the volume of the extractant in the batchwise mode can be 20% to about 60% of the fermentation vessel working volume in one embodiment, and about 30% to about 60% in another embodiment.

[00102] Gas stripping (not shown) can be used concurrently with the ISPR extractant to remove the product alcohol from the fermentation medium.

[00103] In the embodiment of FIG. 1, the product alcohol is extracted from the fermentation broth *in situ*, with the separation of the biphasic mixture 39 occurring in a separate vessel 35. In some embodiments, separation of the biphasic mixture 39 can occur in the fermentation vessel, as shown in the embodiments of later described FIGs. 2 and 3 in which the alcohol-containing organic phase stream 36 exits directly from fermentation vessel 30. Aqueous phase stream 34 can also exit directly from fermentation vessel 30, be treated for the removal of any remaining product alcohol and recycled, or discarded and replaced with fresh fermentation medium. The extraction of the product alcohol by the organic extractant(s) can be done with or without the removal of the microorganism from the fermentation broth. The microorganism can be removed from the fermentation broth by means known in the art including, but not limited to, filtration or centrifugation. For example, aqueous phase stream 34 can include microorganism 32 such as yeast. Microorganism 32 can be easily separated from the aqueous phase stream, for example, in a centrifuge (not shown). Microorganism 32 can then be recycled to fermentation vessel 30 which over time can increase the production rate of alcohol production, thereby resulting in an increase in the efficiency of the alcohol production.

[00104] In a continuous mode of *in situ* extractive fermentation, the volume of the extractant can be about 3% to about 50% of the fermentation vessel working volume in one embodiment, about 3% to about 30% in another embodiment, 3% to about 20% in another embodiment; and 3% to about 10% in another embodiment. Because the product is continually removed from the reactor, a smaller volume of extractant is required enabling a larger volume of the fermentation medium to be used.

[00105] As an alternative to *in situ* extractive fermentation, the product alcohol can be extracted from the fermentation broth downstream of fermentation vessel 30. In such an instance, the fermentation broth can be removed from fermentation vessel 30 and introduced into vessel 35. Extractant 28 can then be introduced into vessel 35 and contacted with the fermentation broth to obtain biphasic mixture 39 in vessel 35, which is then separated into the organic and aqueous phases 36 and 34. Alternatively, extractant 28 can be added to the fermentation broth in a separate vessel (not shown) prior to introduction to vessel 35.

[00106] As a non-limiting prophetic example, with reference to the embodiment of FIG. 1, an aqueous suspension of ground whole corn (as feedstock 12), which can nominally contain about 4 wt% corn oil, can be treated with amylase (as liquefaction enzyme 14) at about 85ºC to 120ºC for 30 minutes to 2 hours, and the resulting liquefied mash 16 cooled to between 65ºC and 30ºC and treated with 0.1 ppm to 10 ppm (in some embodiments, 0.5 ppm to 1.0 ppm) of lipase (as catalyst 42) at pH 4.5 to 7.5 (in some embodiments, between pH 5.5 and 6.5) for sufficient time to produce from at least 30% to as high as at least 99% conversion of the available fatty acid content in lipids to free fatty acids. Optionally, the liquefied and lipase-treated mash 18 can be heated to inactivate lipase 42 prior to fermentation. Mash 18 can be cooled to about 30ºC (e.g., using a heat-exchanger) and loaded to fermentation vessel 30 at about 25% to 30 wt% dry corn solids. Saccharification of the liquefied mash 18 during fermentation by the addition of glucoamylase (as saccharification enzyme 38) can result in the production of glucose. The resulting fermentation broth can contain significantly less than the amount of corn oil (e.g., about 1.2 wt% corn oil) that can be present in a fermentation broth using a liquefied mash that has not been treated with lipase 42. In particular, the lipase 42 treatment can result in the conversion of corn oil lipids 26 (triglycerides (TG)) into COFA as FFA 28 (and some diglycerides (DG) or monoglycerides (MG)), decreasing the rate of build-up of lipids 26 in any ISPR extractant 29 (e.g., oleyl alcohol), and dissolution of COFA 28 into organic phase 36 during ISPR should not decrease the partition coefficient of butanol in organic phase 36 as much as would the dissolution of lipids (TG) into the organic phase 36.

[00107] In some embodiments, the system and processes of FIG. 1 can be modified such that feedstock slurry 16 (having oil 26) and catalyst 42 are introduced and contacted in fermentation vessel 30 so as to produce slurry 18 (having FFA 28). The fermentation vessel temperature can then be raised to heat inactivate catalyst 42. The fermentation vessel temperature can then be reduced, and the fermentation vessel can be inoculated with microorganism 32, whereby the sugars of slurry 18 can be fermented to produce a product alcohol.

[00108] In some embodiments, the system and processes of FIG. 1 can be modified such that simultaneous saccharification and fermentation (SSF) in fermentation vessel 30 is replaced with a separate saccharification vessel 60 (see FIG. 2) prior to fermentation vessel 30, as should be apparent to one of skill in the art. Thus, slurry 18 can be saccharified either before fermentation or during fermentation in an SSF process. It should also be apparent that catalyst

42 for hydrolysis of feedstock oil 26 can be introduced before, after, or contemporaneously with saccharification enzyme 38. Thus, in some embodiments, addition of enzyme 38 and catalyst 42 can be stepwise (e.g., catalyst 42, then enzyme 38, or vice versa), or substantially simultaneous (i.e., at exactly the same time as in the time it takes for a person or a machine to perform the addition in one stroke, or one enzyme/catalyst immediately following the other catalyst/enzyme as in the time it takes for a person or a machine to perform the addition in two strokes).

[00109] For example, as shown in the embodiment of FIG. 2, the system and processes of FIG. 1 can be modified such that simultaneous saccharification and fermentation (SSF) in fermentation vessel 30 is replaced with a separate saccharification vessel 60 prior to fermentation vessel 30. FIG. 2 is substantially identical to FIG. 1 except for the inclusion of a separate saccharification vessel 60 receiving enzyme 38, with catalyst 42 being introduced to a liquefied, saccharified feedstock stream 62. Feedstock slurry 16 is introduced into saccharification vessel 60 along with enzyme 38 such as glucoamylase, whereby sugars in the form of oligosaccharides in slurry 16 can be broken down into monosaccharides. A liquefied, saccharified feedstock stream 62 exits saccharification vessel 60 to which catalyst 42 is introduced. Feedstock stream 62 includes monosaccharides, oil 26, and undissolved solids derived from the feedstock. Oil 26 is hydrolyzed by the introduction of catalyst 42 resulting in a liquefied, saccharified feedstock slurry 64 having free fatty acids 28 and catalyst 42.

[00110] Alternatively, in some embodiments, catalyst 42 can be added with saccharification enzyme 38 to simultaneously produce glucose and hydrolyze oil lipids 26 to free fatty acids 28. The addition of enzyme 38 and catalyst 42 can be stepwise (e.g., catalyst 42, then enzyme 38, or vice versa) or simultaneous. Alternatively, in some embodiments, slurry 62 can be introduced to fermentation vessel with catalyst 42 being added directly to the fermentation vessel 30.

[00111] In the embodiment of FIG. 2, heat q is applied to feedstock slurry 64, whereby catalyst 42 becomes inactive, and heat-treated slurry 64 is then introduced to fermentation vessel 30 along with alcohol-producing microorganism

32, which metabolizes the monosaccharides to produce a product alcohol (e.g., butanol). Alternatively, slurry 64 can be fed to fermentation vessel 30 and subjected to heat *q* while in the fermentation vessel, before inoculation of microorganism 32.

[00112] As described above with reference to FIG. 1, free fatty acids 28 can also serve as an ISPR extractant for preferentially partitioning the product alcohol from the aqueous phase. In some embodiments, one or more additional ISPR extractants 29 can also be introduced into fermentation vessel 30. Separation of the biphasic mixture occurs in fermentation vessel 30, whereby alcohol-containing organic phase stream 36 and aqueous phase stream 34 exit directly from fermentation vessel 30. Alternatively, separation of the biphasic mixture can be conducted in a separate vessel 35 as provided in the embodiments of FIG. 1. The remaining process operations of the embodiment of FIG. 2 are identical to FIG. 1 and therefore, will not be described in detail again.

[00113] In still other embodiments of the present invention, oil 26 derived from feedstock 12 can be catalytically hydrolyzed into FFA 28 either prior to or during liquefaction. For example, in the embodiment of FIG. 3, feedstock 12 having oil 26 is fed to liquefaction vessel 10, along with catalyst 42 for hydrolysis of at least a portion of the glycerides in oil 26 into FFA 28. Enzyme 14 (e.g., alpha-amylase) for hydrolyzing the starch in feedstock 12 can also be introduced to vessel 10 to produce a liquefied feedstock. The addition of enzyme 14 and catalyst 42 can be stepwise or simultaneous. For example, catalyst 42 can be introduced, and then enzyme 14 can be introduced after at least a portion of oil 26 has been hydrolyzed. Alternatively, enzyme 14 can be introduced, and then catalyst 42 can be introduced. The liquefaction process can involve the application of heat *q.* In such embodiments, it is preferred that catalyst 42 is introduced prior to or during liquefaction when the process temperature is below that which inactivates catalyst 42, so that oil 26 can be hydrolyzed. Thereafter, application of heat *q* can provide a two-fold purpose of liquefaction and inactivation of catalyst 42.

[00114] In any case, oil 26 in feedstock 12 is converted to FFA 28 in liquefaction vessel 10, such that biphasic feedstock slurry 18 exits liquefaction vessel 10.

Biphasic slurry 18 includes both an organic phase of FFA 28 as well as sugar, water, and undissolved solids of an aqueous phase. In some embodiments, the aqueous phase can include glycerol (glycerin) from converting the glycerides in the oil to fatty acids. In some embodiments, such glycerol, if present, can be removed from the stream 18 prior to introduction into fermentation vessel 30.

[00115] With reference to FIG. 3, biphasic stream 18 is contacted with the fermentation broth in fermentation vessel 30 to form a biphasic mixture. In fermentation vessel 30, product alcohol produced by SSF partitions into the organic phase including FFA 28. Alternatively, in some embodiments, the process can be modified to include a separate saccharification vessel as discussed in connection with FIG. 2. Separation of the biphasic mixture occurs in fermentation vessel 30, whereby alcohol-containing organic phase stream 36 and aqueous phase stream 34 exit directly from fermentation vessel 30. Alternatively, separation of the biphasic mixture can be conducted in a separate vessel 35 as provided in the embodiments of FIG. 1. Optionally, one or more additional extractants 29 can be introduced into fermentation vessel 30 to form an organic phase that preferentially partitions the product alcohol from the aqueous phase. Alcohol-containing organic phase 36 can be introduced to separator 50 for recovery of product alcohol 54 and optional recycle of recovered extractant 27 as shown in FIG. 1. The remaining process operations of the embodiment of FIG. 3 can be identical to the previously described figures and therefore, will not be described in detail again.

[00116] In some embodiments, including any of the earlier described embodiments with respect to FIGs. 1-3, undissolved solids can be removed from the feedstock slurry prior to introduction into fermentation vessel 30. For example, as shown in the embodiment of FIG. 4, feedstock slurry 16 is introduced into an inlet of a separator 20 which is configured to discharge the undissolved solids as a solid phase or wet cake 24. For example, in some embodiments, separator 20 may include a filter press, vacuum filtration, or a centrifuge for separating the undissolved solids from feedstock slurry 16. Optionally, in some embodiments, separator 20 can also be configured to remove some, or substantially all, of oil 26 present in feedstock slurry 16. In such embodiments, separator 20 can be any suitable separator known in the art for removing oil from an aqueous feedstream including, but not limited to, siphoning, decantation, aspiration, centrifugation, using a gravity settler, membrane-assisted phase splitting, and the like. The remaining feedstock including the sugar and water is discharged as an aqueous stream 22 to fermentation vessel 30.

[00117] In some embodiments, separator 20 removes oil 26 but not undissolved solids. Thus, aqueous stream 22 fed to fermentation vessel 30 includes undissolved solids. For example, in some embodiments, separator 20 includes a tricanter centrifuge 20 that agitates or spins feedstock slurry 16 to produces a centrifuge product comprising an aqueous layer containing the sugar and water (i.e., stream 22), a solids layer containing the undissolved solids (i.e., wet cake 24), and an oil layer (i.e., oil stream 26). In such a case, catalyst 42 can be contacted with the removed oil 26 to produce a stream of FFA 28 including catalyst 42, as shown in FIG. 4. Heat *q* can then be applied to the stream of FFA 28, whereby catalyst 42 becomes inactive. The stream of FFA 28 and inactive catalyst 42 can then be introduced into fermentation vessel 30, along with stream 22 and microorganism 32. Alternatively, FFA 28 and active catalyst 42 can be fed to fermentation vessel 30 from vessel 40, and active catalyst 42 can thereafter be subjected to heat *q* and inactivated while in the fermentation vessel, before inoculation of microorganism 32.

[00118] FFA 28 can serve as ISPR extractant 28 and forms a biphasic mixture in fermentation vessel 30. Product alcohol produced by SSF partitions into organic phase 36 constituted by FFA 28. In some embodiments, one or more additional ISPR extractants 29 can also be introduced into fermentation vessel 30. Thus, oil 26 (e.g., from feedstock) can be catalytically hydrolyzed to FFA 28, thereby decreasing the rate of build-up of lipids in an ISPR extractant while also producing an ISPR extractant. The organic phase 36 can be separated from the aqueous phase 34 of the biphasic mixture 39 at vessel 35. In some embodiments, separation of the biphasic mixture 39 can occur in the fermentation vessel, as shown in the embodiments described in FIGs. 2 and 3 in which the alcohol-containing organic phase stream 36 exits directly from fermentation vessel 30. Organic phase 36 can be introduced to separator 50 for recovery of product alcohol 54 and optional recycle of recovered extractant 27 as shown in FIG. 1. The remaining process operations of the embodiment of FIG. 4 are identical to FIG. 1 and therefore, will not be described in detail again.

[00119] When wet cake 24 is removed via centrifuge 20, in some embodiments, a portion of the oil from feedstock 12, such as corn oil when the feedstock is corn, remains in wet cake 24. Wet cake 24 can be washed with additional water in the centrifuge once aqueous solution 22 has been discharged from the centrifuge 20. Washing wet cake 24 will recover the sugar (e.g., oligosaccharides) present in the wet cake and the recovered sugar and water can be recycled to the liquefaction vessel 10. After washing, wet cake 20 can be dried to form Dried Distillers' Grains with Solubles (DDGS) through any suitable known process. The formation of the DDGS from wet cake 24 formed in centrifuge 20 has several benefits. Since the undissolved solids do not go to the fermentation vessel, DDGS does not have trapped extractant and/or product alcohol such as butanol, it is not subjected to the conditions of the fermentation vessel, and it does not contact the microorganisms present in the fermentation vessel. All these benefits make it easier to process and sell DDGS, for example, as animal feed. In some embodiments, oil 26 is not discharged separately from wet cake 24, but rather oil 26 is included as part of wet cake 24 and is ultimately present in the DDGS. In such instances, the oil can be separated from the DDGS and converted to an ISPR extractant 29 for subsequent use in the same or different alcohol fermentation process. Methods and systems for removing undissolved solids from feedstock 16 via centrifugation are described in detail in co-pending, commonly owned U.S. Patent Application No. 61/356,290, filed June 18, 2010, which is incorporated herein in its entirety by reference thereto.

[00120] In still other embodiments (not shown), saccharification can occur in a separate saccharification vessel 60 (see FIG. 2) which is located between separator 20 and liquefaction vessel 10, as should be apparent to one of skill in the art.

[00121] In still other embodiments, as shown, for example, in the embodiment of FIG. 5, a native oil 26' is supplied to a vessel 40 to which catalyst 42 is also supplied, whereby at least a portion of glycerides in oil 26' are hydrolyzed to form

FFA 28'. Catalyst 42 can be subsequently inactivated, such as by the application of heat *q*. A product stream from vessel 40 containing FFA 28' and inactive catalyst 42 are then introduced into fermentation vessel 30, along with aqueous feedstock stream 22 in which feedstock oil 26, and in some embodiments, the undissolved solids have been previously removed by means of separator 20 (see, e.g., the embodiment of FIG. 4). Saccharification enzyme 38 and microorganism 32 are also introduced into fermentation vessel 30, whereby a product alcohol is produced by SSF.

[00122] Alternatively, oil 26' and catalyst 42 can be fed directly to fermentation vessel 30 in which oil 26' is hydrolyzed to FFA 28' rather than using vessel 40. Thereafter, active catalyst 42 can be subjected to heat *q* and inactivated while in the fermentation vessel before inoculation of microorganism 32. Alternatively, FFA 28' and active catalyst 42 can be fed to fermentation vessel 30 from vessel 40, and active catalyst 42 can thereafter be subjected to heat *q* and inactivated while in the fermentation vessel before inoculation of microorganism 32. In such embodiments, feedstock slurry 16 including oil 26, rather than stream 22 in which oil 26 was removed, can be fed to fermentation vessel 30 and contacted with active catalyst 42. Active catalyst 42 can therefore be used to hydrolyze oil 26 into FFA 28, thereby reducing the loss and/or degradation of the partition coefficient of the extractant over time that is attributable to the presence of the oil in the fermentation vessel.

[00123] In some embodiments, the system and processes of FIG. 5 can be modified such that simultaneous saccharification and fermentation in fermentation vessel 30 is replaced with a separate saccharification vessel 60 prior to fermentation vessel 30, as should be apparent to one of skill in the art (see, e.g., the embodiment of FIG. 2).

[00124] In some embodiments, native oil 26' can be tallow, corn, canola, capric/caprylic triglycerides, castor, coconut, cottonseed, fish, jojoba, lard, linseed, neetsfoot, oiticica, palm, peanut, rapeseed, rice, safflower, soya, sunflower, tung, jatropha, vegetable oil blends, and mixtures thereof. In some embodiments, native oil 26' is a mixture of two or more native oils, for example, a mixture of palm and soybean oils. In some embodiments, native oil 26' is a plant-derived oil. In some embodiments, the plant-derived oil can be, though not necessarily, derived from biomass that can be used in a fermentation process. The biomass can be the same or different source from which feedstock 12 (shown in FIG. 5 as stream 22) is obtained. Thus, for example, in some embodiments, oil 26' can be derived from corn, whereas feedstock 12 can be cane. For example, in some embodiments, oil 26' can be derived from corn, and the biomass source of feedstock 12 is also corn. Any possible combination of different biomass sources for oil 26' versus feedstock 12 can be used, as should be apparent to one of skill in the art.

[00125] FFA 28' can serve as an ISPR extractant 28' to form a two-phase mixture including an aqueous phase and an organic phase, with the product alcohol produced in the fermentation medium preferentially partitioning into the organic phase constituted by ISPR extractant 28'. In some embodiments, one or more additional ISPR extractants 29 can be introduced into fermentation vessel 30 as described above with reference to FIG. 1. The organic phase 36 can be separated from the aqueous phase 34 of the biphasic mixture 39 at vessel 35. In some embodiments, separation of the biphasic mixture 39 can occur in the fermentation vessel, as shown in the embodiments described in FIGs. 2 and 3 in which the alcohol-containing organic phase stream 36 exits directly from fermentation vessel 30. Organic phase 36 can be introduced in separator 50 for recovery of product alcohol 54 and optional recycle of recovered extractant 27 as shown in FIG. 1. The remaining process operations of the embodiment of FIG. 5 are identical to FIG. 1 and therefore, will not be described in detail again.

[00126] In some embodiments of the present invention, biomass oil present in feedstock 12 can be converted to FFA 28 at a step following alcoholic fermentation. FFA 28 can then be introduced as ISPR extractant 28 in the fermentation vessel. For example, in the embodiment of FIG. 6, feedstock 12 is liquefied to produced feedstock slurry 16 which includes oil 26 derived from the feedstock. Feedstock slurry 16 can also include undissolved solids from the feedstock. Alternatively, the undissolved solids can be separated from slurry 16 via a separator, such as a centrifuge (not shown). Feedstock slurry 16 containing oil 26 is introduced directly to fermentation vessel 30 containing a fermentation broth including saccharification enzyme 38 and microorganism 32. A product alcohol is produced by SSF in fermentation vessel 30. Alternatively, in some embodiments, the process can be modified to include a separate saccharification vessel as discussed in connection with FIG. 2.

[00127] ISPR extractant 29 is introduced to fermentation vessel 30 to form a biphasic mixture, and the product alcohol is removed by partitioning into the organic phase of the ISPR extractant 29. Oil 26 also partitions into the organic phase. Separation of the biphasic mixture occurs in fermentation vessel 30, whereby alcohol-containing organic phase stream 36 and aqueous phase stream 34 exit directly from fermentation vessel 30. Alternatively, separation of the biphasic mixture can be conducted in a separate vessel 35 as provided in the embodiments of FIG. 1. Organic phase stream 36 including oil 26 is introduced into separator 50 to recover product alcohol 54 from extractant 29. The resulting alcohol-lean extractant 27 includes recovered extractant 29 and oil 26. Extractant 27 is contacted with catalyst 42, whereby at least a portion of glycerides in oil 26 are hydrolyzed to form FFA 28. Heat *q* can then be applied to extractant 27 including FFA 28 so as to inactivate catalyst 42 before being recycled back into fermentation vessel 30. Such recycled extractant stream 27 can be a separate stream or a combined stream with fresh, make-up extractant stream 29. The subsequent withdrawal of alcohol-containing organic phase 36 from fermentation vessel 30 can then include FFA 28 and ISPR extractant 29 (as fresh extractant 29 and recycled extractant 27), in addition to the product alcohol and additional oil 26 from newly introduced feedstock slurry 16. Organic phase 36 can then be treated to recover the product alcohol, and recycled back into fermentation vessel 30 after contacting with catalyst 42 for hydrolysis of additional oil 26, in the same manner as just described. In some embodiments, use of make-up ISPR extractant 29 can be phased out as the fermentation process is operated over time because the process itself can produce FFA 28 as a make-up ISPR extractant for extracting the product alcohol. Thus, the ISPR extractant can be the stream of recycled extractant 27 with FFA 28.

[00128] Thus, FIGs. 1-5 provide various non-limiting embodiments of methods and systems involving fermentation processes and FFAs 28 produced from catalytic hydrolysis of biomass derived oil 26, and FFAs 28' produced from catalytic hydrolysis of native oil 26' such as plant-derived oil that can be used as ISPR extractants 28 and 28' to remove product alcohol in extractive fermentation.

[00129] In some embodiments, including any of the aforementioned embodiments described with reference to FIGs. 1-6, the fermentation broth in fermentation vessel 30 includes at least one recombinant microorganism 32 which is genetically modified (that is, genetically engineered) to produce butanol via a biosynthetic pathway from at least one fermentable carbon source. In particular, recombinant microorganisms can be grown in a fermentation broth which contains suitable carbon substrates. Additional carbon substrates may include, but are not limited to, monosaccharides such as fructose; oligosaccharides such as lactose, maltose, or sucrose; polysaccharides such as starch or cellulose; or mixtures thereof, and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. Other carbon substrates may include ethanol, lactate, succinate, or glycerol.

[00130] Additionally the carbon substrate may also be one-carbon substrates such as carbon dioxide or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated. In addition to one and two carbon substrates, methylotrophic organisms are also known to utilize a number of other carbon containing compounds such as methylamine, glucosamine, and a variety of amino acids for metabolic activity. For example, methylotrophic yeasts are known to utilize the carbon from methylamine to form trehalose or glycerol (Bellion, et al., *Microb. Growth C1 Compd.*, [Int. Symp.], 7th (1993), 415-32, Editor(s): Murrell, J. Collin; Kelly, Don P. Publisher: Intercept, Andover, UK). Similarly, various species of *Candida* will metabolize alanine or oleic acid (Sulter, et al., Arch. Microbiol. 153:485-489, 1990). Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon containing substrates and will only be limited by the choice of organism.

[00131] Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof are suitable, in some embodiments, the carbon substrates are glucose, fructose, and sucrose, or mixtures of these with C5 sugars such as xylose and/or arabinose for yeasts cells modified to use C5 sugars. Sucrose may be derived from renewable sugar sources such as sugar cane, sugar beets, cassava, sweet sorghum, and mixtures thereof. Glucose and dextrose may be derived from renewable grain sources through saccharification of starch based feedstocks including grains such as corn, wheat, rye, barley, oats, and mixtures thereof. In addition, fermentable sugars may be derived from renewable cellulosic or lignocellulosic biomass through processes of pretreatment and saccharification, as described in, for example, in U.S. Patent Application Publication No. 2007/0031918 A1, which is herein incorporated by reference. In addition to an appropriate carbon source (from aqueous stream 22), fermentation broth must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of an enzymatic pathway comprising a dihydroxyacid dehydratase (DHAD).

[00132] Recombinant microorganisms that produce butanol via a biosynthetic pathway can include a member of the genera *Clostridium, Zymomonas, Escherichia, Salmonella, Serratia, Erwinia, Klebsiella, Shigella, Rhodococcus, Pseudomonas, Bacillus, Lactobacillus, Enterococcus, Alcaligenes, Klebsiella, Paenibacillus, Arthrobacter, Corynebacterium, Brevibacterium, Schizosaccharomyces, Kluyveromyces, Yarrowia, Pichia, Candida, Hansenula, or Saccharomyces.* In one embodiment, recombinant microorganisms can be selected from the group consisting of *Escherichia coli, Lactobacillus plantarum,* and *Saccharomyces cerevisiae.* In one embodiment, the recombinant microorganism is a crabtree-positive yeast selected from *Saccharomyces, Zygosaccharomyces, Schizosaccharomyces, Dekkera, Torulopsis, Brettanomyces,* and some species of *Candida.* Species of crabtree-positive yeast include, but are not limited to*, Saccharomyces cerevisiae, Saccharomyces kluyveri, Schizosaccharomyces pombe, Saccharomyces bayanus, Saccharomyces mikitae, Saccharomyces paradoxus, Zygosaccharomyces rouxii, and Candida glabrata*. For example, the production of butanol utilizing fermentation with a microorganism, as well as which microorganisms produce butanol, is known and is disclosed, for example, in U.S. Patent Application

Publication No. 2009/0305370, herein incorporated by reference. In some embodiments, microorganisms comprise a butanol biosynthetic pathway. Suitable isobutanol biosynthetic pathways are known in the art (see, e.g., U.S. Patent Application Publication No. 2007/0092957, herein incorporated by reference). In some embodiments, at least one, at least two, at least three, or at least four polypeptides catalyzing substrate to product conversions of a pathway are encoded by heterologous polynucleotides in the microorganism. In some embodiments, all polypeptides catalyzing substrate to product conversions of a pathway are encoded by heterologous polynucleotides in the microorganism. In some embodiments, the microorganism comprises a reduction or elimination of pyruvate decarboxylase activity. Microorganisms substantially free of pyruvate decarboxylase activity are described in U.S. Patent Application Publication No. 2009/0305363, herein incorporated by reference.

[00133] Construction of certain strains, including those used in the Examples, is provided herein.

Construction of Saccharomyces cerevisiae strain BP1083 ("NGCI-070")

[00134] The strain BP1064 was derived from CEN.PK 113-7D (CBS 8340; Centraalbureau voor Schimmelcultures (CBS) Fungal Biodiversity Centre, Netherlands) and contains deletions of the following genes: URA3, HIS3, PDC1, PDC5, PDC6, and GPD2. BP1064 was transformed with plasmids pYZ090 (SEQ ID NO: 1, described in U.S. Provisional Application Serial No. 61/246,844) and pLH468 (SEQ ID NO: 2) to create strain NGCI-070 (BP1083, PNY1504).

[00135] Deletions, which completely removed the entire coding sequence, were created by homologous recombination with PCR fragments containing regions of homology upstream and downstream of the target gene and either a G418 resistance marker or URA3 gene for selection of transformants. The G418 resistance marker, flanked by loxP sites, was removed using Cre recombinase. The URA3 gene was removed by homologous recombination to create a scarless deletion or if flanked by loxP sites, was removed using Cre recombinase.

[0128] The scarless deletion procedure was adapted from Akada, et al., (Yeast 23:399-405, 2006). In general, the PCR cassette for each scarless deletion was made by combining four fragments, A-B-U-C, by overlapping PCR. The PCR cassette contained a selectable/counter-selectable marker, URA3 (Fragment U), consisting of the native CEN.PK 113-7D URA3 gene, along with the promoter (250 bp upstream of the URA3 gene) and terminator (150 bp downstream of the URA3 gene). Fragments A and C, each 500 bp long, corresponded to the 500 bp immediately upstream of the target gene (Fragment A) and the 3' 500 bp of the target gene (Fragment C). Fragments A and C were used for integration of the cassette into the chromosome by homologous recombination. Fragment B (500 bp long) corresponded to the 500 bp immediately downstream of the target gene and was used for excision of the URA3 marker and Fragment C from the chromosome by homologous recombination, as a direct repeat of the sequence corresponding to Fragment B was created upon integration of the cassette into the chromosome. Using the PCR product ABUC cassette, the URA3 marker was first integrated into and then excised from the chromosome by homologous recombination. The initial integration deleted the gene, excluding the 3' 500 bp. Upon excision, the 3' 500 bp region of the gene was also deleted. For integration of genes using this method, the gene to be integrated was included in the PCR cassette between fragments A and B.

<u>URA3 Deletion</u>

[0129] To delete the endogenous URA3 coding region, a ura3::loxP-kanMX-loxP cassette was PCR-amplified from pLA54 template DNA (SEQ ID NO: 3). pLA54 contains the K. lactis TEF1 promoter and kanMX marker, and is flanked by loxP sites to allow recombination with Cre recombinase and removal of the marker. PCR was done using Phusion® DNA polymerase (New England BioLabs Inc., Ipswich, MA) and primers BK505 and BK506 (SEQ ID NOs: 4 and 5). The URA3 portion of each primer was derived from the 5' region upstream of the URA3 promoter and 3' region downstream of the coding region such that integration of the loxP-kanMX-loxP marker resulted in replacement of the URA3 coding region. The PCR product was transformed into CEN.PK 113-7D using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on YPD containing G418 (100 µg/mL) at 30°C. Transformants were screened to verify correct integration by PCR using primers LA468 and LA492 (SEQ ID NOs: 6 and 7) and designated CEN.PK 113-7D Δura3::kanMX.

HIS3 Deletion

[0130] The four fragments for the PCR cassette for the scarless HIS3 deletion were amplified using Phusion® High Fidelity PCR Master Mix (New England BioLabs Inc., Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra® Puregene® Yeast/Bact, kit (Qiagen, Valencia, CA). HIS3 Fragment A was amplified with primer oBP452 (SEQ ID NO: 14) and primer oBP453 (SEQ ID NO: 15) containing a 5' tail with homology to the 5' end of HIS3 Fragment B. HIS3 Fragment B was amplified with primer oBP454 (SEQ ID NO: 16) containing a 5' tail with homology to the 3' end of HIS3 Fragment A, and primer oBP455 (SEQ ID NO: 17) containing a 5' tail with homology to the 5' end of HIS3 Fragment U. HIS3 Fragment U was amplified with primer oBP456 (SEQ ID NO: 18) containing a 5' tail with homology to the 3' end of HIS3 Fragment B, and primer oBP457 (SEQ ID NO: 19) containing a 5' tail with homology to the 5' end of HIS3 Fragment C. HIS3 Fragment C was amplified with primer oBP458 (SEQ ID NO: 20) containing a 5' tail with homology to the 3' end of HIS3 Fragment U, and primer oBP459 (SEQ ID NO: 21). PCR products were purified with a PCR Purification kit (Qiagen, Valencia, CA). HIS3 Fragment AB was created by overlapping PCR by mixing HIS3 Fragment A and HIS3 Fragment B and amplifying with primers oBP452 (SEQ ID NO: 14) and oBP455 (SEQ ID NO: 17). HIS3 Fragment UC was created by overlapping PCR by mixing HIS3 Fragment U and HIS3 Fragment C and amplifying with primers oBP456 (SEQ ID NO: 18) and oBP459 (SEQ ID NO: 21). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen, Valencia, CA). The HIS3 ABUC cassette was created by overlapping PCR by mixing HIS3 Fragment AB and HIS3 Fragment UC and amplifying with primers oBP452 (SEQ ID NO: 14) and oBP459 (SEQ ID NO: 21). The PCR product was purified with a PCR Purification kit (Qiagen, Valencia, CA).

[0131] Competent cells of CEN.PK 113-7D Δura3::kanMX were made and transformed with the HIS3 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II™ kit (Zymo Research Corporation, Irvine, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30°C. Transformants with a his3 knockout were screened for by PCR with primers oBP460 (SEQ ID NO: 22) and oBP461 (SEQ ID NO: 23) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). A correct transformant was selected as strain CEN.PK 113-7D Δura3::kanMX Δhis3::URA3.

KanMX Marker Removal from the Δura3 Site and URA3 Marker Removal from the Δhis3 Site

[0132] The KanMX marker was removed by transforming CEN.PK 113-7D Δura3::kanMX Δhis3::URA3 with pRS423::PGAL1-cre (SEQ ID NO: 66, described in U.S. Provisional Application No. 61/290,639) using a Frozen-EZ Yeast Transformation II™ kit (Zymo Research Corporation, Irvine, CA) and plating on synthetic complete medium lacking histidine and uracil supplemented with 2% glucose at 30°C. Transformants were grown in YP supplemented with 1% galactose at 30°C for ~6 hours to induce the Cre recombinase and KanMX marker excision and plated onto YPD (2% glucose) plates at 30°C for recovery. An isolate was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (5-FOA, 0.1%) at 30°C to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in and plated on YPD for removal of the pRS423::PGAL1-cre plasmid. Isolates were checked for loss of the KanMX marker, URA3 marker, and pRS423::PGAL1-cre plasmid by assaying growth on YPD+G418 plates, synthetic complete medium lacking uracil plates, and synthetic complete medium lacking histidine plates. A correct isolate that was sensitive to G418 and auxotrophic for uracil and histidine was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 and designated as BP857. The deletions and marker removal were confirmed by PCR and sequencing with primers oBP450 (SEQ ID NO: 24) and oBP451 (SEQ ID NO: 25) for Δura3 and primers oBP460 (SEQ ID NO: 22) and oBP461 (SEQ ID NO: 23) for Δhis3 using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA).

PDC6 Deletion

[0133] The four fragments for the PCR cassette for the scarless PDC6 deletion were amplified using Phusion® High Fidelity PCR Master Mix (New England BioLabs Inc., Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). PDC6 Fragment A was amplified with primer oBP440 (SEQ ID NO: 26) and primer oBP441 (SEQ ID NO: 27) containing a 5' tail with homology to the 5' end of PDC6 Fragment B. PDC6 Fragment B was amplified with primer oBP442 (SEQ ID NO: 28), containing a 5' tail with homology to the 3' end of PDC6 Fragment A, and primer oBP443 (SEQ ID NO: 29) containing a 5' tail with homology to the 5' end of PDC6 Fragment U. PDC6 Fragment U was amplified with primer oBP444 (SEQ ID NO: 30) containing a 5' tail with homology to the 3' end of PDC6 Fragment B, and primer oBP445 (SEQ ID NO: 31) containing a 5' tail with homology to the 5' end of PDC6 Fragment C. PDC6 Fragment C was amplified with primer oBP446 (SEQ ID NO: 32) containing a 5' tail with homology to the 3' end of PDC6 Fragment U, and primer oBP447 (SEQ ID NO: 33). PCR products were purified with a PCR Purification kit (Qiagen, Valencia, CA). PDC6 Fragment AB was created by overlapping PCR by mixing PDC6 Fragment A and PDC6 Fragment B and amplifying with primers oBP440 (SEQ ID NO: 26) and oBP443 (SEQ ID NO: 29). PDC6 Fragment UC was created by overlapping PCR by mixing PDC6 Fragment U and PDC6 Fragment C and amplifying with primers oBP444 (SEQ ID NO: 30) and oBP447 (SEQ ID NO: 33). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen, Valencia, CA). The PDC6 ABUC cassette was created by overlapping PCR by mixing PDC6 Fragment AB and PDC6 Fragment UC and amplifying with primers oBP440 (SEQ ID NO: 26) and oBP447 (SEQ ID NO: 33). The PCR product was purified with a PCR Purification kit (Qiagen, Valencia, CA).

[0134] Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 were made and transformed with the PDC6 ABUC PCR cassette using a Frozen-EZ Yeast Transformation II™ kit (Zymo Research Corporation, Irvine, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30°C. Transformants with a pdc6 knockout were screened for by PCR with primers oBP448 (SEQ ID NO: 34) and oBP449 (SEQ ID NO: 35) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6::URA3.

[0135] CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6::URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion and marker removal were confirmed by PCR and sequencing with primers oBP448 (SEQ ID NO: 34) and oBP449 (SEQ ID NO: 35) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). The absence of the PDC6 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC6, oBP554 (SEQ ID NO: 36) and oBP555 (SEQ ID NO: 37). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 and designated as BP891.

PDC1 Deletion ilvDSm Integration

[0136] The PDC1 gene was deleted and replaced with the ilvD coding region from Streptococcus mutans ATCC No. 700610. The A fragment followed by the ilvD coding region from Streptococcus mutans for the PCR cassette for the PDC1 deletion-ilvDSm integration was amplified using Phusion® High Fidelity PCR Master Mix (New England BioLabs Inc., Ipswich, MA) and NYLA83 (described herein and in U.S. Provisional Application No. 61/246,709) genomic DNA as template, prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). PDC1 Fragment A-ilvDSm (SEQ ID NO: 141) was amplified with primer oBP513 (SEQ ID NO: 38) and primer oBP515 (SEQ ID NO: 39) containing a 5' tail with homology to the 5' end of PDC1 Fragment B. The B, U, and C fragments for the PCR cassette for the PDC1 deletion-ilvDSm integration were amplified using Phusion® High Fidelity PCR Master Mix (New England BioLabs Inc., Ipswich, MA) and CEN.PK 113-7D genomic DNA as template, prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). PDC1 Fragment B was amplified with primer oBP516 (SEQ ID NO: 40) containing a 5' tail with homology to the 3' end of PDC1 Fragment A-ilvDSm, and primer oBP517 (SEQ ID NO: 41) containing a 5' tail with homology to the 5' end of PDC1 Fragment U.

PDC1 Fragment U was amplified with primer oBP518 (SEQ ID NO: 42) containing a 5' tail with homology to the 3' end of PDC1 Fragment B, and primer oBP519 (SEQ ID NO: 43) containing a 5' tail with homology to the 5' end of PDC1 Fragment C. PDC1 Fragment C was amplified with primer oBP520 (SEQ ID NO: 44), containing a 5' tail with homology to the 3' end of PDC1 Fragment U, and primer oBP521 (SEQ ID NO: 45). PCR products were purified with a PCR Purification kit (Qiagen, Valencia, CA. PDC1 Fragment A-ilvDSm-B was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm and PDC1 Fragment B and amplifying with primers oBP513 (SEQ ID NO: 38) and oBP517 (SEQ ID NO: 41). PDC1 Fragment UC was created by overlapping PCR by mixing PDC1 Fragment U and PDC1 Fragment C and amplifying with primers oBP518 (SEQ ID NO: 42) and oBP521 (SEQ ID NO: 45). The resulting PCR products were purified on an agarose gel followed by a Gel Extraction kit (Qiagen, Valencia, CA). The PDC1 A-ilvDSm-BUC cassette (SEQ ID NO: 142) was created by overlapping PCR by mixing PDC1 Fragment A-ilvDSm-B and PDC1 Fragment UC and amplifying with primers oBP513 (SEQ ID NO: 38) and oBP521 (SEQ ID NO: 45). The PCR product was purified with a PCR Purification kit (Qiagen, Valencia, CA).

[0137] Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 were made and transformed with the PDC1 A-ilvDSm-BUC PCR cassette using a Frozen-EZ Yeast Transformation II™ kit (Zymo Research Corporation, Irvine, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 2% glucose at 30°C. Transformants with a pdc1 knockout ilvDSm integration were screened for by PCR with primers oBP511 (SEQ ID NO: 46) and oBP512 (SEQ ID NO: 47) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). The absence of the PDC1 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC1, oBP550 (SEQ ID NO: 48) and oBP551 (SEQ ID NO: 49). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm-URA3.

[0138] CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm-URA3 was grown overnight in YPD and plated on synthetic complete medium containing 5-

fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion of PDC1, integration of ilvDSm, and marker removal were confirmed by PCR and sequencing with primers oBP511 (SEQ ID NO: 46) and oBP512 (SEQ ID NO: 47) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm and designated as BP907.

PDC5 Deletion sadB Integration

[0139] The PDC5 gene was deleted and replaced with the sadB coding region from *Achromobacter xylosoxidans*. A segment of the PCR cassette for the PDC5 deletion-sadB integration was first cloned into plasmid pUC19-URA3MCS.

[0140] pUC19-URA3MCS is pUC19 based and contains the sequence of the URA3 gene from *Saccaromyces cerevisiae* situated within a multiple cloning site (MCS). pUC19 contains the pMB1 replicon and a gene coding for beta-lactamase for replication and selection in *Escherichia coli*. In addition to the coding sequence for URA3, the sequences from upstream and downstream of this gene were included for expression of the URA3 gene in yeast. The vector can be used for cloning purposes and can be used as a yeast integration vector.

[0141] The DNA encompassing the URA3 coding region along with 250 bp upstream and 150 bp downstream of the URA3 coding region from *Saccaromyces cerevisiae* CEN.PK 113-7D genomic DNA was amplified with primers oBP438 (SEQ ID NO: 12) containing BamHI, AscI, PmeI, and FseI restriction sites, and oBP439 (SEQ ID NO: 13) containing XbaI, PacI, and NotI restriction sites, using Phusion® High Fidelity PCR Master Mix (New England BioLabs Inc., Ipswich, MA). Genomic DNA was prepared using a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). The PCR product and pUC19 (SEQ ID NO: 143) were ligated with T4 DNA ligase after digestion with BamHI and XbaI to create vector pUC19-URA3MCS. The vector was confirmed by PCR and sequencing with primers oBP264 (SEQ ID NO: 10) and oBP265 (SEQ ID NO: 11).

[0142] The coding sequence of sadB and PDC5 Fragment B were cloned into pUC19-URA3MCS to create the sadB-BU portion of the PDC5 A-sadB-BUC PCR cassette. The coding sequence of sadB was amplified using pLH468-sadB (SEQ ID NO: 67) as template with primer oBP530 (SEQ ID NO: 50) containing an AscI restriction site, and primer oBP531 (SEQ ID NO: 51) containing a 5' tail with homology to the 5' end of PDC5 Fragment B. PDC5 Fragment B was amplified with primer oBP532 (SEQ ID NO: 52) containing a 5' tail with homology to the 3' end of sadB, and primer oBP533 (SEQ ID NO: 53) containing a PmeI restriction site. PCR products were purified with a PCR Purification kit (Qiagen, Valencia, CA). sadB-PDC5 Fragment B was created by overlapping PCR by mixing the sadB and PDC5 Fragment B PCR products and amplifying with primers oBP530 (SEQ ID NO: 50) and oBP533 (SEQ ID NO: 53). The resulting PCR product was digested with AscI and PmeI and ligated with T4 DNA ligase into the corresponding sites of pUC19-URA3MCS after digestion with the appropriate enzymes. The resulting plasmid was used as a template for amplification of sadB-Fragment B-Fragment U using primers oBP536 (SEQ ID NO: 54) and oBP546 (SEQ ID NO: 55) containing a 5' tail with homology to the 5' end of PDC5 Fragment C. PDC5 Fragment C was amplified with primer oBP547 (SEQ ID NO: 56) containing a 5' tail with homology to the 3' end of PDC5 sadB-Fragment B-Fragment U, and primer oBP539 (SEQ ID NO: 57). PCR products were purified with a PCR Purification kit (Qiagen, Valencia, CA). PDC5 sadB-Fragment B-Fragment U-Fragment C was created by overlapping PCR by mixing PDC5 sadB-Fragment B-Fragment U and PDC5 Fragment C and amplifying with primers oBP536 (SEQ ID NO: 54) and oBP539 (SEQ ID NO: 57). The resulting PCR product was purified on an agarose gel followed by a Gel Extraction kit (Qiagen, Valencia, CA). The PDC5 A-sadB-BUC cassette (SEQ ID NO: 144) was created by amplifying PDC5 sadB-Fragment B-Fragment U-Fragment C with primers oBP542 (SEQ ID NO: 58) containing a 5' tail with homology to the 50 nucleotides immediately upstream of the native PDC5 coding sequence, and oBP539 (SEQ ID NO: 57). The PCR product was purified with a PCR Purification kit (Qiagen, Valencia, CA).

[0143] Competent cells of CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm were made and transformed with the PDC5 A-sadB-BUC PCR cassette using a Frozen-EZ Yeast Transformation II™ kit (Zymo Research

Corporation, Irvine, CA). Transformation mixtures were plated on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose) at 30°C. Transformants with a pdc5 knockout sadB integration were screened for by PCR with primers oBP540 (SEQ ID NO: 59) and oBP541 (SEQ ID NO: 60) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). The absence of the PDC5 gene from the isolate was demonstrated by a negative PCR result using primers specific for the coding sequence of PDC5, oBP552 (SEQ ID NO: 61) and oBP553 (SEQ ID NO: 62). A correct transformant was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB-URA3.

[0144] CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB-URA3 was grown overnight in YPE (1% ethanol) and plated on synthetic complete medium supplemented with ethanol (no glucose) and containing 5-fluoro-orotic acid (0.1%) at 30°C to select for isolates that lost the URA3 marker. The deletion of PDC5, integration of sadB, and marker removal were confirmed by PCR with primers oBP540 (SEQ ID NO: 59) and oBP541 (SEQ ID NO: 60) using genomic DNA prepared with a Gentra® Puregene® Yeast/Bact. kit (Qiagen, Valencia, CA). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB and designated as BP913.

GPD2 Deletion

[0145] To delete the endogenous GPD2 coding region, a gpd2::loxP-URA3-loxP cassette (SEQ ID NO: 145) was PCR-amplified using loxP-URA3-loxP (SEQ ID NO: 68) as template DNA. loxP-URA3-loxP contains the URA3 marker from (ATCC No. 77107) flanked by loxP recombinase sites. PCR was done using Phusion® DNA polymerase (New England BioLabs Inc., Ipswich, MA) and primers LA512 and LA513 (SEQ ID NOs: 8 and 9). The GPD2 portion of each primer was derived from the 5' region upstream of the GPD2 coding region and 3' region downstream of the coding region such that integration of the loxP-URA3-loxP marker resulted in replacement of the GPD2 coding region. The PCR product was transformed into BP913 and transformants were selected on synthetic complete media lacking uracil supplemented with 1% ethanol (no glucose). Transformants were screened to verify correct integration by PCR using primers oBP582 and AA270 (SEQ ID NOs: 63 and 64).

[0146] The URA3 marker was recycled by transformation with pRS423::PGAL1-cre (SEQ ID NO: 66) and plating on synthetic complete media lacking histidine supplemented with 1% ethanol at 30°C. Transformants were streaked on synthetic complete medium supplemented with 1% ethanol and containing 5-fluoro-orotic acid (0.1%) and incubated at 30°C to select for isolates that lost the URA3 marker. 5-FOA resistant isolates were grown in YPE (1% ethanol) for removal of the pRS423::PGAL1-cre plasmid. The deletion and marker removal were confirmed by PCR with primers oBP582 (SEQ ID NO: 63) and oBP591 (SEQ ID NO: 65). The correct isolate was selected as strain CEN.PK 113-7D Δura3::loxP Δhis3 Δpdc6 Δpdc1::ilvDSm Δpdc5::sadB Δgpd2::loxP and designated as PNY1503 (BP1064).

[0147] BP1064 was transformed with plasmids pYZ090 (SEQ ID NO: 1) and pLH468 (SEQ ID NO: 2) to create strain NGCI-070 (BP1083; PNY1504).

Construction of Strains NYLA74, NYLA83, and NYLA84

[0148] Insertion-inactivation of endogenous PDC1 and PDC6 genes of S. cerevisiae. PDC1, PDC5, and PDC6 genes encode the three major isozymes of pyruvate decarboxylase is described as follows:

<u>Construction of pRS425::GPM-sadB</u>

[0149] A DNA fragment encoding a butanol dehydrogenase (SEQ ID NO: 70) from *Achromobacter xylosoxidans* (disclosed in U.S. Patent Application Publication No. 2009/0269823) was cloned. The coding region of this gene called sadB for secondary alcohol dehydrogenase (SEQ ID NO: 69) was amplified using standard conditions from A. xylosoxidans genomic DNA, prepared using a Gentra® Puregene® kit (Qiagen, Valencia, CA) following the recommended protocol for gram negative organisms using forward and reverse primers N473 and N469 (SEQ ID NOs: 74 and 75), respectively. The PCR product was TOPO®-Blunt cloned into pCR®4 BLUNT (Invitrogen™, Carlsbad, CA) to produce pCR4Blunt::sadB, which was transformed into E. coli Mach-1 cells. Plasmid was subsequently isolated from four clones, and the sequence verified.

[0150] The sadB coding region was PCR amplified from pCR4Blunt::sadB. PCR primers contained additional 5' sequences that would overlap with the yeast GPM1 promoter and the ADH1 terminator (N583 and N584, provided as SEQ ID NOs: 76 and 77). The PCR product was then cloned using "gap repair" methodology in *Saccharomyces cerevisiae* (Ma, et al., Gene 58:201-216, 1987) as follows. The yeast-E. coli shuttle vector pRS425::GPM::kivD::ADH which contains the GPM1 promoter (SEQ ID NO: 72), kivD coding region from *Lactococcus lactis* (SEQ ID NO: 71), and ADH1 terminator (SEQ ID NO: 73) (described in U.S. Patent Application Publication No. 2007/0092957 A1, Example 17) was digested with BbvCI and PacI restriction enzymes to release the kivD coding region. Approximately 1 µg of the remaining vector fragment was transformed into S. cerevisiae strain BY4741 along with 1 µg of sadB PCR product. Transformants were selected on synthetic complete medium lacking leucine. The proper recombination event, generating pRS425::GPM-sadB, was confirmed by PCR using primers N142 and N459 (SEQ ID NOs: 108 and 109).

<u>Construction of pdc6:: PGPM1-sadB integration cassette and PDC6 deletion:</u>

[0151] A pdc6::PGPM1-sadB-ADH1t-URA3r integration cassette was made by joining the GPM-sadB-ADHt segment (SEQ ID NO: 79) from pRS425::GPM-sadB (SEQ ID NO: 78) to the URA3r gene from pUC19-URA3r. pUC19-URA3r (SEQ ID NO:80) contains the URA3 marker from pRS426 (ATCC No. 77107) flanked by 75 bp homologous repeat sequences to allow homologous recombination in vivo and removal of the URA3 marker. The two DNA segments were joined by SOE PCR (as described by Horton, et al., Gene 77:61-68, 1989) using as template pRS425::GPM-sadB and pUC19-URA3r plasmid DNAs, with Phusion® DNA polymerase (New England BioLabs Inc., Ipswich, MA) and primers 114117-11A through 114117-11D (SEQ ID NOs: 81, 82, 83, and 84), and 114117-13A and 114117-13B (SEQ ID NOs: 85 and 86).

[0152] The outer primers for the SOE PCR (114117-13A and 114117-13B) contained 5' and 3' ~50 bp regions homologous to regions upstream and downstream of the PDC6 promoter and terminator, respectively. The completed cassette PCR fragment was transformed into BY4700 (ATCC No. 200866) and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Transformants were screened by PCR using primers 112590-34G and 112590-34H (SEQ ID NOs: 87 and 88), and 112590-34F and 112590-49E (SEQ ID NOs: 89 and 90) to verify integration at the PDC6 locus with deletion of the PDC6 coding region. The URA3r marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain has the genotype: BY4700 pdc6::PGPM1-sadB-ADH1t.

Construction of pdc1:: PPDC1-ilvD integration cassette and PDC1 deletion:

[0153] A pdc1:: PPDC1-ilvD-FBA1t-URA3r integration cassette was made by joining the ilvD-FBA1t segment (SEQ ID NO: 91) from pLH468 (SEQ ID NO: 2) to the URA3r gene from pUC19-URA3r by SOE PCR (as described by Horton, et al., Gene 77:61-68, 1989) using as template pLH468 and pUC19-URA3r plasmid DNAs, with Phusion® DNA polymerase (New England BioLabs Inc., Ipswich, MA) and primers 114117-27A through 114117-27D (SEQ ID NOs: 111, 112, 113, and 114).

[0154] The outer primers for the SOE PCR (114117-27A and 114117-27D) contained 5' and 3' ~50 bp regions homologous to regions downstream of the PDC1 promoter and downstream of the PDC1 coding sequence. The completed cassette PCR fragment was transformed into BY4700 pdc6::PGPM1-sadB-ADH1t and transformants were maintained on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202). Transformants were screened by PCR using primers 114117-36D and 135 (SEQ ID NOs: 92 and 93), and primers 112590-49E and 112590-30F (SEQ ID NOs: 90 and 94) to verify integration at the PDC1 locus with deletion of the PDC1 coding sequence. The URA3r marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain "NYLA67" has the genotype: BY4700 pdc6:: PGPM1-sadB-ADH1t pdc1:: PPDC1-ilvD-FBA1t.

HIS3 deletion

[0155] To delete the endogenous HIS3 coding region, a his3::URA3r2 cassette was PCR-amplified from URA3r2 template DNA (SEQ ID NO: 95). URA3r2 contains the URA3 marker from pRS426 (ATCC No. 77107) flanked by 500 bp homologous repeat sequences to allow homologous recombination in vivo and removal of the URA3 marker. PCR was done using Phusion® DNA polymerase (New England BioLabs Inc., Ipswich, MA) and primers 114117-45A and 114117-45B (SEQ ID NOs: 96 and 97) which generated a ~2.3 kb PCR product. The HIS3 portion of each primer was derived from the 5' region upstream of the HIS3 promoter and 3' region downstream of the coding region such that integration of the URA3r2 marker results in replacement of the HIS3 coding region. The PCR product was transformed into NYLA67 using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C. Transformants were screened to verify correct integration by replica plating of transformants onto synthetic complete media lacking histidine and supplemented with 2% glucose at 30°C. The URA3r marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth. The resulting identified strain, called NYLA73, has the genotype: BY4700 pdc6:: PGPM1-sadB-ADH1t pdc1:: PPDC1-ilvD-FBA1t Δhis3.

Construction of pdc5::kanMX integration cassette and PDC5 deletion:

[0156] A pdc5::kanMX4 cassette was PCR-amplified from strain YLR134W chromosomal DNA (ATCC No. 4034091) using Phusion® DNA polymerase (New

England BioLabs Inc., Ipswich, MA) and primers PDC5::KanMXF and PDC5::KanMXR (SEQ ID NOs: 98 and 99) which generated a ~2.2 kb PCR product. The PDC5 portion of each primer was derived from the 5' region upstream of the PDC5 promoter and 3' region downstream of the coding region such that integration of the kanMX4 marker results in replacement of the PDC5 coding region. The PCR product was transformed into NYLA73 using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on YP media supplemented with 1% ethanol and geneticin (200 μg/mL) at 30°C. Transformants were screened by PCR to verify correct integration at the PDC locus with replacement of the PDC5 coding region using primers PDC5kofor and N175 (SEQ ID NOs: 100 and 101). The identified correct transformants have the genotype: BY4700 pdc6:: PGPM1-sadB-ADH1t pdc1:: PPDC1-ilvD-FBA1t Δhis3 pdc5::kanMX4. The strain was named NYLA74.

[0157] Plasmid vectors pRS423::CUP1-alsS+FBA-budA and pRS426::FBA-budC+GPM-sadB were transformed into NYLA74 to create a butanediol producing strain (NGCI-047).

[0158] Plasmid vectors pLH475-Z4B8 (SEQ ID NO: 140) and pLH468 were transformed into NYLA74 to create an isobutanol producing strain (NGCI-049).

<u>Deletion of HXK2 (hexokinase II):</u>

[0159] A hxk2::URA3r cassette was PCR-amplified from URA3r2 template (described above) using Phusion® DNA polymerase (New England BioLabs Inc., Ipswich, MA) and primers 384 and 385 (SEQ ID NOs: 102 and 103) which generated a ~2.3 kb PCR product. The HXK2 portion of each primer was derived from the 5' region upstream of the HXK2 promoter and 3' region downstream of the coding region such that integration of the URA3r2 marker results in replacement of the HXK2 coding region. The PCR product was transformed into NYLA73 using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 201-202) and transformants were selected on synthetic complete media lacking uracil and supplemented with 2% glucose at 30°C. Transformants were screened by PCR to verify correct integration at the HXK2 locus with replacement of the HXK2 coding region using primers N869 and N871 (SEQ ID NOs: 104 and 105). The URA3r2 marker was recycled by plating on synthetic complete media supplemented with 2% glucose and 5-FOA at 30°C following standard protocols. Marker removal was confirmed by patching colonies from the 5-FOA plates onto SD-URA media to verify the absence of growth, and by PCR to verify correct marker removal using primers N946 and N947 (SEQ ID NOs: 106 and 107). The resulting identified strain named NYLA83 has the genotype: BY4700 pdc6:: PGPM1-sadB-ADH1t pdc1:: PPDC1-ilvD-FBA1t Δhis3 Δhxk2.

Construction of pdc5::kanMX integration cassette and PDC5 deletion:

[0160] A pdc5::kanMX4 cassette was PCR-amplified as described above. The PCR fragment was transformed into NYLA83, and transformants were selected and screened as described above. The identified correct transformants named NYLA84 have the genotype: BY4700 pdc6:: PGPM1-sadB-ADH1t pdc1:: PPDC1-ilvD-FBA1t Δhis3 Δhxk2 pdc5::kanMX4.

[0161] Plasmid vectors pLH468 and pLH532 were simultaneously transformed into strain NYLA84 (BY4700 pdc6::PGPM1-sadB-ADH1t pdc1::PPDC1-ilvD-FBA1t Δhis3 Δhxk2 pdc5::kanMX4) using standard genetic techniques (Methods in Yeast Genetics, 2005, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and the resulting "butanologen NYLA84" was maintained on synthetic complete media lacking histidine and uracil, and supplemented with 1% ethanol at 30°C.

Expression Vector pLH468

[0162] The pLH468 plasmid (SEQ ID NO: 2) was constructed for expression of DHAD, KivD, and HADH in yeast and is described in U.S. Patent Application Publication No. 2009/0305363, herein incorporated by reference. pLH486 was constructed to contain: a chimeric gene having the coding region of the ilvD gene from *Streptococcus mutans* (nt position 3313-4849) expressed from the S. cerevisiae FBA1 promoter (nt 2109 - 3105) followed by the FBA1 terminator (nt 4858 - 5857) for expression of DHAD; a chimeric gene having the coding region of codon optimized horse liver alcohol dehydrogenase (nt 6286-7413) expressed from the S. cerevisiae GPM1 promoter (nt 7425-8181) followed by the ADH1 terminator (nt 5962-6277) for expression of ADH; and a chimeric gene having the coding region of the codon-optimized kivD gene from *Lactococcus lactis* (nt 9249-10895) expressed from the TDH3 promoter (nt 10896-11918) followed by the TDH3 terminator (nt 8237-9235) for expression of KivD.

[0163] Coding regions for *Lactococcus lactis* ketoisovalerate decarboxylase (KivD) and horse liver alcohol dehydrogenase (HADH) were synthesized by DNA2.0, Inc. (Menlo Park, CA) based on codons that were optimized for expression in *Saccharomyces cerevisiae* (SEQ ID NO: 71 and 118, respectively) and provided in plasmids pKivDy-DNA2.0 and pHadhy-DNA2.0. The encoded proteins are SEQ ID NOs: 117 and 119, respectively. Individual expression vectors for KivD and HADH were constructed. To assemble pLH467 (pRS426::PTDH3-kivDy-TDH3t), vector pNY8 (SEQ ID NO: 121; also named pRS426.GPD-ald-GPDt, described in U.S. Patent Application Publication No. 2008/0182308, Example 17, which is herein incorporated by reference) was digested with AscI and SfiI enzymes, thus excising the GPD promoter and the ald coding region. A TDH3 promoter fragment (SEQ ID NO: 122) from pNY8 was PCR amplified to add an AscI site at the 5' end and an SpeI site at the 3' end, using 5' primer OT1068 and 3' primer OT1067 (SEQ ID NOs: 123 and 124). The AscI/SfiI digested pNY8 vector fragment was ligated with the TDH3 promoter PCR product digested with AscI and SpeI, and the SpeI-SfiI fragment containing the codon optimized kivD coding region isolated from the vector pKivD-DNA2.0. The triple ligation generated vector pLH467 (pRS426::PTDH3-kivDy-TDH3t). pLH467 was verified by restriction mapping and sequencing.

[0164] pLH435 (pRS425::PGPM1-Hadhy-ADH1t) was derived from vector pRS425::GPM-sadB (SEQ ID NO: 78) which is described in U.S. Provisional Application Serial No. 61/058,970, Example 3, which is herein incorporated by reference. pRS425::GPM-sadB is the pRS425 vector (ATCC No. 77106) with a chimeric gene containing the GPM1 promoter (SEQ ID NO:72), coding region from a butanol dehydrogenase of *Achromobacter xylosoxidans* (sadB; DNA SEQ ID NO: 69; protein SEQ ID NO:70: disclosed in U.S. Patent Application Publication No. 2009/0269823), and ADH1 terminator (SEQ ID NO: 73). pRS425::GPMp-sadB contains BbvI and PacI sites at the 5' and 3' ends of the sadB coding region, respectively. A NheI site was added at the 5' end of the sadB coding region by site-directed mutagenesis using primers OT1074 and OT1075 (SEQ ID NOs: 126 and 127) to generate vector pRS425-GPMp-sadB-NheI, which was verified by sequencing. pRS425::PGPM1-sadB-NheI was digested with NheI and PacI to drop out the sadB coding region, and ligated with the NheI-PacI fragment containing the codon optimized HADH coding region from vector pHadhy-DNA2.0 to create pLH435.

[0165] To combine KivD and HADH expression cassettes in a single vector, yeast vector pRS411 (ATCC No. 87474) was digested with SacI and NotI, and ligated with the SacI-SalI fragment from pLH467 that contains the PTDH3-kivDy-TDH3t cassette together with the SalI-NotI fragment from pLH435 that contains the PGPM1-Hadhy-ADH1t cassette in a triple ligation reaction. This yielded the vector pRS411::PTDH3-kivDy-PGPM1-Hadhy (pLH441) which was verified by restriction mapping.

[0166] In order to generate a co-expression vector for all three genes in the lower isobutanol pathway: ilvD, kivDy, and Hadhy, pRS423 FBA ilvD(Strep) (SEQ ID NO: 128) which is described in U.S. Patent Application Publication No. 2010/0081154 as the source of the IlvD gene, was used. This shuttle vector contains an F1 origin of replication (nt 1423 to 1879) for maintenance in E. coli and a 2 micron origin (nt 8082 to 9426) for replication in yeast. The vector has an FBA1 promoter (nt 2111 to 3108; SEQ ID NO: 120) and FBA terminator (nt 4861 to 5860; SEQ ID NO: 129). In addition, it carries the His marker (nt 504 to 1163) for selection in yeast and ampicillin resistance marker (nt 7092 to 7949) for selection in E. coli. The ilvD coding region (nt 3116 to 4828; SEQ ID NO: 115; protein SEQ ID NO: 116) from *Streptococcus mutans* UA159 (ATCC No. 700610) is between the FBA promoter and FBA terminator forming a chimeric gene for expression. In addition, there is a lumio tag fused to the ilvD coding region (nt 4829-4849).

[0167] The first step was to linearize pRS423 FBA ilvD(Strep) (also called pRS423-FBA(SpeI)-IlvD(*Streptococcus mutans*)-Lumio) with SacI and SacII (with SacII site blunt ended using T4 DNA polymerase), to give a vector with total length of 9,482 bp. The second step was to isolate the kivDy-hADHy cassette from pLH441 with SacI and KpnI (with KpnI site blunt ended using T4 DNA polymerase), which gives a 6,063 bp fragment. This fragment was ligated with the 9,482 bp vector fragment from pRS423-FBA(SpeI)-IlvD(*Streptococcus mutans*)-Lumio. This generated vector pLH468 (pRS423::PFBA1-ilvD(Strep) Lumio-FBA1t-PTDH3-kivDy-TDH3t-PGPM1-hadhy-ADH1t) which was confirmed by restriction mapping and sequencing.

pLH532 construction

[0168] The pLH532 plasmid (SEQ ID NO: 130) was constructed for expression of ALS and KARI in yeast. pLH532 is a pHR81 vector (ATCC No. 87541) containing the following chimeric genes: 1) the CUP1 promoter (SEQ ID NO: 139), acetolactate synthase coding region from *Bacillus subtilis* (AlsS; SEQ ID NO: 137; protein SEQ ID NO: 138) and CYC1 terminator2 (SEQ ID NO: 133); 2) an ILV5 promoter (SEQ ID NO: 134), Pf5.IlvC coding region (SEQ ID NO: 132) and ILV5 terminator (SEQ ID NO: 135); and 3) the FBA1 promoter (SEQ ID NO: 136), S. cerevisiae KARI coding region (ILV5; SEQ ID NO: 131); and CYC1 terminator.

[0169] The Pf5.IlvC coding region is a sequence encoding KARI derived from *Pseudomonas fluorescens* that was described in U.S. Patent Application Publication No. 2009/0163376, which is herein incorporated by reference.

[0170] The Pf5.IlvC coding region was synthesized by DNA2.0, Inc. (Menlo Park, CA; SEQ ID NO: 132) based on codons that were optimized for expression in *Saccharomyces cerevisiae*.

pYZ090 construction

[0171] pYZ090 (SEQ ID NO: 1) is based on the pHR81 (ATCC No. 87541) backbone and was constructed to contain a chimeric gene having the coding region of the alsS gene from *Bacillus subtilis* (nt position 457-2172) expressed from the yeast CUP1 promoter (nt 2-449) and followed by the CYC1 terminator (nt 2181-2430) for expression of ALS, and a chimeric gene having the coding region of the ilvC gene from *Lactococcus lactis* (nt 3634-4656) expressed from the yeast ILV5 promoter (2433-3626) and followed by the ILV5 terminator (nt 4682-5304) for expression of KARI.

pYZ067 construction

[0172] pYZ067 was constructed to contain the following chimeric genes: 1) the coding region of the ilvD gene from S. mutans UA159 (nt position 2260-3971) expressed from the yeast FBA1 promoter (nt 1161-2250) followed by the FBA terminator (nt 4005-4317) for expression of dihydroxy acid dehydratase (DHAD), 2) the coding region for horse liver ADH (nt 4680-5807) expressed from the yeast GPM promoter (nt 5819-6575) followed by the ADH1 terminator (nt 4356-4671) for expression of alcohol dehydrogenase, and 3) the coding region of the KivD gene from *Lacrococcus lactis* (nt 7175-8821) expressed from the yeast TDH3 promoter (nt 8830-9493) followed by the TDH3 terminator (nt 5682-7161) for expression of ketoisovalerate decarboxylase.

pRS423::CUP1-alsS+FBA-budA and pRS426::FBA-budC+GPM-sadB and pLH475-Z4B8 construction

[0173] Construction of pRS423::CUP1-alsS+FBA-budA and pRS426::FBA-budC+GPM-sadB and pLH475-Z4B8 is described in U.S. Patent Application Publication No. 2009/0305363, incorporated herein by reference.

[0174] Further, while various embodiments of the present invention have been described above, it should be understood that they have been presented by way of example only, and not limitation. It will be apparent to persons skilled in the relevant art that various changes in form and detail can be made therein without departing from the spirit and scope of the invention. Thus, the breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the claims and their equivalents.

[0175] All publications, patents, and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

EXAMPLES

[0176] The following nonlimiting examples will further illustrate the invention. It should be understood that, while the following examples involve corn as feedstock and COFA as carboxylic acid, other biomass sources can be used for feedstock and acids other than COFA can serve as carboxylic acid, without departing from the present invention. Moreover, while the following examples involve butanol and butyl ester production, other alcohols including ethanol, and alcohol esters can be produced without departing from the present invention.

[0177] As used herein, the meaning of abbreviations used was as follows: “g” means gram(s), “kg” means kilogram(s), “L” means liter(s), “mL” means milliliter(s), "µL" means microliter(s), “mL/L” means milliliter(s) per liter, “mL/min” means milliliter(s) per min, “DI” means deionized, “uM” means micrometer(s), “nm” means nanometer(s), “w/v” means weight/volume, “OD” means optical density, “OD_{600}” means optical density at a wavelength of 600 nM, “dcw” means dry cell weight, “rpm” means revolutions per minute, “^{o}C” means degree(s) Celsius, “^{o}C/min” means degrees Celsius per minute, “slpm” means standard liter(s) per minute, “ppm” means part per million, “*pdc*” means pyruvate decarboxylase enzyme followed by the enzyme number.

GENERAL METHODS

<u>Seed Flask Growth</u>

[0178] A *Saccharomyces cerevisiae* strain that was engineered to produce isobutanol from a carbohydrate source, with *pdc1* deleted, *pdc5* deleted, and *pdc6* deleted, was grown to 0.55-1.1 g/L dcw (OD_{600} 1.3-2.6 – Thermo Helios α Thermo Fisher Scientific Inc., Waltham, Massachusetts) in seed flasks from a frozen culture. The culture was grown at 26^{o}C in an incubator rotating at 300 rpm. The frozen culture was previously stored at – 80^{o}C. The composition of the first seed flask medium was:

3.0 g/L dextrose

3.0 g/L ethanol, anhydrous

3.7 g/L ForMedium™ Synthetic Complete Amino Acid (Kaiser) Drop-Out: without HIS, without URA (Reference No. DSCK162CK)

6.7 g/L Difco Yeast Nitrogen Base without amino acids (No. 291920)

[0179] Twelve milliliters from the first seed flask culture was transferred to a 2 L flask and grown at 30°C in an incubator rotating at 300 rpm. The second seed flask has 220 mL of the following medium:

30.0 g/L dextrose
5.0 g/L ethanol, anhydrous
3.7 g/L ForMedium™ Synthetic Complete Amino Acid (Kaiser) Drop-Out: without HIS, without URA (Reference No. DSCK162CK)
6.7 g/L Difco Yeast Nitrogen Base without amino acids (No. 291920)
0.2 M MES Buffer titrated to pH 5.5-6.0

[0180] The culture was grown to 0.55-1.1 g/L dcw (OD_{600} 1.3-2.6). An addition of 30 mL of a solution containing 200 g/L peptone and 100 g/L yeast extract was added at this cell concentration. Then, an addition of 300 mL of 0.2 uM filter sterilized Cognis, 90-95% oleyl alcohol was added to the flask. The culture continues to grow to > 4 g/L dcw (OD_{600} > 10) before being harvested and added to the fermentation.

<u>Fermentation Preparation</u>

Initial Fermentation Vessel Preparation

[0181] A glass jacked, 2 L fermentation vessel (Sartorius AG, Goettingen, Germany) was charged with house water to 66% of the liquefaction weight. A pH probe (Hamilton Easyferm Plus K8, part number: 238627, Hamilton Bonaduz AG, Bonaduz, Switzerland) was calibrated through the Sartorius DCU-3 Control Tower Calibration menu. The zero was calibrated at pH=7. The span was calibrated at pH=4. The probe was then placed into the fermentation vessel through the stainless steel head plate. A dissolved oxygen probe (pO_2 probe) was also placed into the fermentation vessel through the head plate. Tubing used for delivering nutrients, seed culture, extracting solvent, and base were attached to the head plate and the ends were foiled. The entire fermentation vessel was placed into a Steris (Steris Corporation, Mentor, Ohio) autoclave and sterilized in a liquid cycle for 30 minutes.

[0182] The fermentation vessel was removed from the autoclave and placed on a load cell. The jacket water supply and return line was connected to the house water and clean drain, respectively. The condenser cooling water in and water out lines were connected to a 6-L recirculating temperature bath running at 7°C. The vent line that transfers the gas from the fermentation vessel was connected to a transfer line that was connected to a Thermo mass spectrometer (Prima dB, Thermo Fisher Scientific Inc., Waltham, Massachusetts). The sparger line was connected to the gas supply line. The tubing for adding nutrients, extract solvent, seed culture, and base was plumbed through pumps or clamped closed.

[0183] The fermentation vessel temperature was controlled at 55°C with a thermocouple and house water circulation loop. Wet corn kernels (#2 yellow dent) were ground using a hammer mill with a 1.0 mm screen, and the resulting ground whole corn kernels were then added to the fermentation vessel at a charge that was 29-30% (dry corn solids weight) of the liquefaction reaction mass.

Lipase Treatment Pre-Liquefaction

[0184] A lipase enzyme stock solution was added to the fermentation vessel to a final lipase concentration of 10 ppm. The fermentation vessel was held at 55°C, 300 rpm, and 0.3 slpm N_2 overlay for >6 hrs. After the lipase treatment was complete, liquefaction was performed as described below (*Liquefaction*).

Liquefaction

[0185] An alpha-amylase was added to the fermentation vessel per its specification sheet while the fermentation vessel was mixing at 300-1200 rpm, with sterile, house N_2 being added at 0.3 slpm through the sparger. The temperature set-point was changed from 55°C to 85°C. When the temperature was > 80°C, the liquefaction cook time was started and the liquefaction cycle was held at > 80°C for 90-120 minutes. The fermentation vessel temperature set-point was set to the fermentation temperature of 30°C after the liquefaction cycle was complete. N_2 was redirected from the sparger to the head space to prevent foaming without the addition of a chemical antifoaming agent.

Lipase Treatment Post-Liquefaction

[0186] The fermentation vessel temperature was set to 55°C instead of 30°C after the liquefaction cycle was complete (*Liquefaction*). The pH was manually controlled at pH=5.8 by making bolus additions of acid or base when needed. A lipase enzyme stock solution was added to the fermentation vessel to a final lipase concentration of 10 ppm. The fermentation vessel was held at 55°C, 300 rpm, and 0.3 slpm N_2 overlay for >6 hrs. After the Lipase Treatment was complete, the fermentation vessel temperature was set to 30°C.

Lipase Heat Inactivation Treatment (Heat Kill Treatment Method)

[0187] The fermentation vessel temperature was held at > 80 °C for > 15 minutes to inactivate the lipase. After the Heat Inactivation Treatment was complete, the fermentation vessel temperature was set to 30°C.

Nutrient Addition Prior to Inoculation

[0188] Ethanol (6.36 mL/L, post-inoculation volume, 200 proof, anhydrous) was added to the fermentation vessel just prior to inoculation. Thiamine was added to a final concentration of 20 mg/L and 100 mg/L nicotinic acid was also added just prior to inoculation.

Oleyl Alcohol or Corn Oil Fatty Acids Addition Prior to Inoculation

[0189] Added 1 L/L (post-inoculation volume) of oleyl alcohol or corn oil fatty acids immediately after inoculation.

Fermentation Vessel Inoculation

[0190] The fermentation vessels pO_2 probe was calibrated to zero while N_2 was being added to the fermentation vessel. The fermentation vessels pO_2 probe was calibrated to its span with sterile air sparging at 300 rpm. The fermentation vessel was inoculated after the second seed flask with > 4 g/L dcw. The shake flask was removed from the incubator/shaker for 5 minutes allowing a phase separation of the oleyl alcohol phase and the aqueous phase. The aqueous phase (110 mL) was transferred to a sterile, inoculation bottle. The inoculum was pumped into the fermentation vessel through a peristaltic pump.

Fermentation Vessel Operating Conditions

[0191] The fermentation vessel was operated at 30°C for the entire growth and production stages. The pH was allowed to drop from a pH between 5.7-5.9 to a control set-point of 5.2 without adding any acid. The pH was controlled for the remainder of the growth and production stage at a pH=5.2 with ammonium hydroxide. Sterile air was added to the fermentation vessel, through the sparger, at 0.3 slpm for the remainder of the growth and production stages. The pO_2 was set to be controlled at 3.0% by the Sartorius DCU-3 Control Box PID control loop, using stir control only, with the stirrer minimum being set to 300 rpm and the maximum being set to 2000 rpm. The glucose was supplied through simultaneous saccharification and fermentation of the liquified corn mash by adding a α-amylase (glucoamylase). The glucose was kept excess (1–50 g/L) for as long as starch was available for saccharification.

<u>Analytical</u>

Gas Analysis

[0192] Process air was analyzed on a Thermo Prima (Thermo Fisher Scientific Inc., Waltham, Massachusetts) mass spectrometer. This was the same process air that was sterilized and then added to each fermentation vessel. Each fermentation vessel's off-gas was analyzed on the same mass spectrometer. This Thermo Prima dB has a calibration check run every Monday morning at 6:00 am. The calibration check was scheduled through the Gas Works v1.0 (Thermo Fisher Scientific Inc., Waltham, Massachusetts) software associated with the mass spectrometer. The gas calibrated for were:

GAS	Calibration Concentration mole %	Cal Frequency
Nitrogen	78 %	weekly
Oxygen	21 %	weekly
Isobutanol	0.2 %	yearly
Argon	1 %	weekly
Carbon Dioxide	0.03 %	weekly

[0193] Carbon dioxide was checked at 5% and 15% during calibration cycle with other known bottled gases. Oxygen was checked at 15% with other known bottled gases. Based on the analysis of the off-gas of each fermentation vessel, the amount of isobutanol stripped, oxygen consumed, and carbon dioxide respired into the off-gas was measured by using the mass spectrometer's mole fraction analysis and gas flow rates (mass flow controller) into the fermentation vessel. Calculate the gassing rate per hour and then integrating that rate over the course of the fermentation.

Biomass Measurement

[0194] A 0.08% Trypan Blue solution was prepared from a 1:5 dilution of 0.4% Trypan Blue in NaCl (VWR BDH8721-0) with 1X PBS. A 1.0 mL sample was pulled from a fermentation vessel and placed in a 1.5 mL Eppendorf centrifuge tube and centrifuged in an Eppendorf, 5415C at 14,000 rpm for 5 minutes. After centrifugation, the top solvent layer was removed with an m200 Variable Channel BioHit pipette with 20–200 µL BioHit pipette tips. Care was made not to remove the layer between the solvent and aqueous layers. Once the solvent layer was removed, the sample was re-suspended using a Vortex-Genie® set at 2700 rpm.

[0195] A series of dilutions was required to prepare the ideal concentration for hemacytometer counts. If the OD was 10, a 1:20 dilution would be performed to achieve 0.5 OD which would give the ideal amount of cells to be counted per square, 20-30. In order to reduce inaccuracy in the dilution due to corn solids, multiple dilutions with cut 100–1000 µL BioHit pipette tips were required. Approximately, 1 cm was cut off the tips to increase the opening which prevented the tip from clogging. For a 1:20 final dilution, an initial 1:1 dilution of fermentation sample and 0.9% NaCl solution was prepared. Then, a 1:1 dilution of the previous solution (i.e., the initial 1:1 dilution) and 0.9% NaCl solution (the second dilution) was generated followed by a 1:5 dilution of the second dilution and Trypan Blue Solution. Samples were vortexed between each dilution and cut tips were rinsed into the 0.9% NaCl and Trypan Blue solutions.

[0196] The cover slip was carefully placed on top of the hemacytometer (Hausser Scientific Bright-Line 1492). An aliquot (10 µL) was drawn of the final Trypan Blue dilution with an m20 Variable Channel BioHit pipette with 2-20 µL BioHit pipette tips and injected into the hemacytometer. The hemacytometer was placed on the Zeis Axioskop 40 microscope at 40x magnification. The center quadrant was broken into 25 squares and the four corner and center squares in both chambers were then counted and recorded. After both chambers were counted, the average was taken and multiplied by the dilution factor (20), then by 25 for the number for squares in the quadrant in the hemacytometer, and then divided by 0.0001 mL which is the volume of the quadrant that was counted. The sum of this calculation is the number cells per mL.

LC Analysis of Fermentation Products in the Aqueous Phase

[0197] Samples were refrigerated until ready for processing. Samples were removed from refrigeration and allowed to reach room temperature (about one hour). Approximately 300 µL of sample was transferred with a m1000 Variable Channel BioHit pipette with 100–1000 µL BioHit pipette tips into a 0.2 um centrifuge filter (Nanosep® MF modified nylon centrifuge filter), then centrifuged using a Eppendorf, 5415C for five minutes at 14,000 rpm. Approximately 200 µL of filtered sample was transferred into a 1.8 auto sampler vial with a 250 µL glass vial insert with polymer feet. A screw cap with PTFE septa was used to cap the vial before vortexing the sample with a Vortex-Genie® set at 2700 rpm.

[0198] Sample was then run on Agilent 1200 series LC equipped with binary, isocratic pumps, vacuum degasser, heated column compartment, sampler cooling system, UV DAD detector and RI detector. The column used was an Aminex HPX-87H, 300 X 7.8 with a Bio-Rad Cation H refill, 30X4.6 guard column.

Column temperature was 40ºC, with a mobile phase of 0.01 N sulfuric acid, at a flow rate of 0.6 mL/min for 40 minutes. Results are shown in Table 1.

Table 1: Retention times of fermentation products in aqueous phase

HPLC 302/310 Normalized to 10 µL injections	FW	RID Retention Time, min	Range of Standards, g/L	UV Retention Time, min
citric acid	192.12	8.025	0.3-17	7.616
glucose	180.16	8.83	0.5-71	
pyruvic acid (Na)	110.04	9.388	0.1-5.2	8.5
A-Kiv (Na)	138.1	9.91	0.07-5.0	8.55
2,3-dihydroxyisovaleric acid (Na)	156.1	10.972	0.2-8.8	10.529
succinic acid	118.09	11.561	0.3-16	11.216
lactic acid (Li)	96.01	12.343	0.3-17	11.948
glycerol	92.09	12.974	0.8-39	
formic acid	46.03	13.686	0.2-13	13.232
acetate (Na)	82.03	14.914	0.5-16	14.563
meso-butanediol	90.12	17.583	0.1-19	
(+/-)-2,3-butanediol	90.12	18.4	0.2-19	
isobutyric acid	88.11	19.685	0.1-8.0	19.277
ethanol	46.07	21.401	0.5-34	
isobutyraldehyde	72.11	27.64	0.01-0.11	
isobutanol	74.12	32.276	0.2-15	
3-OH-2-butanone (acetoin)	88.11		0.1-11	17.151

GC Analysis of Fermentation Products in the Solvent Phase

[0199] Samples were refrigerated until ready for processing. Samples were removed from refrigeration and allowed to reach room temperature (about one hour). Approximately 150 µL of sample was transferred using a m1000 Variable Channel BioHit pipette with 100–1000 µL BioHit pipette tips into a 1.8 auto sampler vial with a 250 µL glass vial insert with polymer feet. A screw cap with PTFE septa was used to cap the vial.

[0200] Sample was then run on Agilent 7890A GC with a 7683B injector and a G2614A auto sampler. The column was a HP-InnoWax column (30 m x 0.32 mm ID, 0.25 µm film). The carrier gas was helium at a flow rate of 1.5 mL/min measured at 45ºC with constant head pressure; injector split was 1:50 at 225ºC; oven temperature was 45ºC for 1.5 minutes, 45ºC to 160ºC at 10ºC/min for 0 minutes, then 230ºC at 35ºC/min for 14 minutes for a run time of 29 minutes.

Flame ionization detection was used at 260ºC with 40 mL/min helium makeup gas. Results are shown in Table 2.

Table 2: Retention times of fermentation products in solvent phase.

GC 302/310 Normalized to 10 µL injections	FW	Solvent Retention Time, min	Range of Standards, g/L
isobutyraldehyde	72.11	2.75	0.7-10.4
ethanol	46.07	3.62	0.5-34
isobutanol	74.12	5.53	0.2-16
3-OH-2-butanone (acetoin)	88.11	8,29	0.1-11
(+/-)-2,3-butanediol	90,12	10.94	0.1-19
isobutyric acid	88.11	11.907	0.1-7.9
meso-butanediol	90.12	11.26	0.1-6.5
glycerol	92.09	16.99	0.8-9

[0201] Samples analyzed for fatty acid butyl esters were run on Agilent 6890 GC with a 7683B injector and a G2614A auto sampler. The column was a HP-DB-FFAP column (15 meters x 0.53 mm ID (Megabore), 1-micron film thickness column (30 m x 0.32 mm ID, 0.25 µm film). The carrier gas was helium at a flow rate of 3.7 mL/min measured at 45ºC with constant head pressure; injector split was 1:50 at 225ºC; oven temperature was 100ºC for 2.0 minutes, 100ºC to 250ºC at 10ºC/min, then 250ºC for 9 minutes for a run time of 26 minutes. Flame ionization detection was used at 300ºC with 40 mL/min helium makeup gas. The following GC standards (Nu-Chek Prep; Elysian, MN) were used to confirm the identity of fatty acid isobutyl ester products: iso-butyl palmitate, iso-butyl stearate, iso-butyl oleate, iso-butyl linoleate, iso-butyl linolenate, iso-butyl arachidate.

[0202] Examples 1-14 describe various fermentation conditions that may be used for the claimed methods. As an example, some fermentations were subjected to Lipase Treatment pre-liquefaction and others were subjected to Lipase Treatment post-liquefaction. In other examples, the fermentation was subjected to Heat inactivation Treatment. Following fermentation, the effective isobutanol titer (Eff Iso Titer) was measured, that is, the total grams of isobutanol produced per liter aqueous volume. Results are shown in Table 3.

Example 1 – (control)

[0203] Experiment identifier 2010Y014 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 2

[0204] Experiment identifier 2010Y015 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Lipase Treatment Post-Liquefaction method, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 3

[0205] Experiment identifier 2010Y016 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Lipase Treatment Post-Liquefaction method, Nutrient Addition Prior to Inoculation method with the exception of the exclusion of ethanol, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 4

[0206] Experiment identifier 2010Y017 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Heat Kill Treatment method Post-Liquefaction, Nutrient Addition Prior to Inoculation method with the exception of the exclusion of ethanol, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1-1.0 hr after inoculation. The butanologen was NGCI-070.

Example 5

[0207] Experiment identifier 2010Y018 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Lipase Treatment Post-Liquefaction method with the exception of only adding 7.2 ppm lipase after liquefaction, Heat Kill Treatment method Post-Liquefaction, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 6 – (control)

[0208] Experiment identifier 2010Y019 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Heat Kill Treatment method Post-Liquefaction, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 7 – (control)

[0209] Experiment identifier 2010Y021 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Lipase Treatment Pre-Liquefaction method, Liquefaction method, Heat Kill Treatment during liquefaction, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1-1.0 hr after inoculation. The butanologen was NGCI-070.

Example 8

[0210] Experiment identifier 2010Y022 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 9

[0211] Experiment identifier 2010Y023 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Lipase Treatment Post-Liquefaction method, no Heat Kill Treatment, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Corn oil fatty acids made from crude corn oil was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 10

[0212] Experiment identifier 2010Y024 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Lipase Treatment Pre-Liquefaction method, Liquefaction method, Heat Kill Treatment during liquefaction, Nutrient Addition Prior to Inoculation method with the exception of there being no addition of ethanol, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Oleyl alcohol was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 11

[0213] Experiment identifier 2010Y029 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Lipase Treatment Pre-

Liquefaction method, Liquefaction method, Heat Kill Treatment during liquefaction, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Corn oil fatty acids made from crude corn oil was added in a single batch between 0.1-1.0 hr after inoculation. The butanologen was NGCI-070.

Example 12

[0214] Experiment identifier 2010Y030 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Lipase Treatment Pre-Liquefaction method, Liquefaction method, Heat Kill Treatment during liquefaction, Nutrient Addition Prior to Inoculation method with the exception of there being no addition of ethanol, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Corn oil fatty acids made from crude corn oil was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 13 – (control)

[0215] Experiment identifier 2010Y031 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Lipase Treatment Post Liquefaction method, no Heat Kill Treatment, Nutrient Addition Prior to Inoculation method with the exception of there being no addition of ethanol, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Corn oil fatty acids made from crude corn oil was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Example 14

[0216] Experiment identifier 2010Y032 included: Seed Flask Growth method, Initial Fermentation Vessel Preparation method, Liquefaction method, Lipase Treatment Post-Liquefaction method, no Heat Kill Treatment, Nutrient Addition Prior to Inoculation method, Fermentation Vessel Inoculation method, Fermentation Vessel Operating Conditions method, and all of the Analytical methods. Corn oil fatty acids made from crude corn oil was added in a single batch between 0.1- 1.0 hr after inoculation. The butanologen was NGCI-070.

Table 3: Fermentation conditions for Examples 1-14.

Example #	Experimental Identifier	Lipase	Max cell Count x 10^7	Ethanol g/L	Solvent	Heat Kill Lipase	Eff Iso Titer g/L*	max Eff Iso rate g/L/h
1	2010Y014	none	27.2	5	Oleyl alcohol	none	56.0	0.79
2	2010Y015	10 ppm	31.5	5	Oleyl alcohol	none	52.4	0.74
3	2010Y016	10 ppm	6.7	0	Oleyl alcohol	none	25.9	0.36
4	2010Y017	none	7.9	0	Oleyl alcohol	post – liquefaction	17.2	0.25
5	2010Y018	7.2 ppm	16.2	5	Oleyl alcohol	post – liquefaction	45.8	0.66
6	2010Y019	none	17.5	5	Oleyl alcohol	post – liquefaction	48.1	0.69
7	2010Y021	10 ppm	21.2	5	Oleyl alcohol	during liquefaction	46.8	0.82
8	2010Y022	none	9	5	Oleyl alcohol	during liquefaction	56.2	0.87
9	2010Y023	10 ppm	12.8	5	Corn Oil Fatty Acids	none	60.3	1.3
10	2010Y024	10 ppm	25.3	0	Oleyl alcohol	during liquefaction	19.8	0.33
11	2010Y029	10 ppm	21.2	5	Corn Oil Fatty Acids	during liquefaction	28.36	0.52
12	2010Y030	10 ppm	9	0	Corn Oil Fatty Acids	during liquefaction	12.71	0.24
13	2010Y031	10 ppm	12.8	0	Corn Oil Fatty Acids	none	18.86	0.35
14	2010Y032	10 ppm	25.3	5	Corn Oil Fatty Acids	none	53.36	0.92

* The “Eff Iso Titer g/L” = total grams of isobutanol produced per liter aqueous volume

Example 15

[0217] The experimental identifier was GLNOR432A. NYLA74 (a butanediol producer - NGCI-047) was grown in 25 mL of medium in a 250 mL flask from a frozen vial to ~ 1 OD. The pre-seed culture was transferred to a 2 L flask and grown to 1.7-1.8 OD. The medium for both flasks was:

3.0 g/L dextrose
3.0 g/L ethanol, anhydrous
6.7 g/L Difco Yeast Nitrogen Base without amino acids (No. 291920)
1.4 g/L Yeast Dropout Mix (Sigma Y2001)
10 mL/L 1% w/v L-Leucine stock solution
2 mL/L 1% w/v L-Tryptophan stock solution

[0218] A 1 L, Applikon fermentation vessel was inoculated with 60 mL of the seed flask. The fermentation vessel contained 700 mL of the following sterile medium:

20.0 g/L dextrose
8.0 mL/L ethanol, anhydrous
6.7 g/L Difco Yeast Nitrogen Base without amino acids (No. 291920)
2.8 g/L Yeast Dropout Mix (Sigma Y2001)
20 mL/L 1% w/v L-Leucine stock solution
4 mL/L 1% w/v L-Tryptophan stock solution
0.5 mL Sigma 204 Antifoam
0.8 mL/L 1% w/v Ergesterol solution in 1:1::Tween 80:Ethanol

[0219] The residual glucose was kept excess with a 50% w/w glucose solution. The dissolved oxygen concentration of the fermentation vessel was controlled at 30% with stir control. The pH was controlled at pH=5.5. The fermentation vessel was sparged with 0.3 slpm of sterile, house air. The temperature was controlled at 30^{o}C.

Example 16

[0220] The experimental identifier was GLNOR434A. This example is the same as example 15 with the exception of the addition of 3 g of oleic acid and the addition of 3 g of palmitic acid prior to inoculation. NYLA74 (a butanediol producer - NGCI-047) was the biocatalyst.

[0221] FIG. 7 shows that there were more grams per liter of glucose consumed in the fermentation vessel that received the fatty acids. The squares represent the fermentation vessel that received oleic acid and palmitic acid. The circles represent the fermentation vessel that did not receive any extra fatty acids.

Example 17

[0222] The experimental identifier was GLNOR435A. This example was the same as example 15 except it was inoculated with NYLA74 (an isobutanol producer - NGCI-049).

Example 18

[0223] The experimental identifier was GLNOR437A. This example was the same as Example 16 except it was inoculated with NYLA74 (an isobutanol producer) (NGCI-049).

[0224] FIG. 8 shows that there were more grams per liter of glucose consumed in the fermentation vessel that received the fatty acids. The squares represent the fermentation vessel that received oleic acid and palmitic acid. The circles represent the fermentation vessel that did not receive any extra fatty acids.

Example 19

[0225] The experimental identifier was 090420_3212. This example was run similarly to Example 15 except it was inoculated with butanologen NYLA84 (an isobutanol producer). This fermentation was run in a 1 L Sartorius fermentation vessel.

Example 20

[0226] The experimental identifier was 2009Y047. This example was run similarly to Example 16 except it was inoculated with butanologen NYLA84 (an isobutanol producer). This fermentation was run in a 1 L Sartorius fermentation.

[0227] FIG. 9 shows that there were more grams per liter of glucose consumed in the fermentation vessel that received the fatty acids. The squares represent the fermentation vessel that received oleic acid and palmitic acid. The circles represent the fermentation vessel that did not receive any extra fatty acids.

[0228] Table 4 shows +/- fatty acid addition, maximum optical density, and g/L glucose consumed.

Table 4

Example #	Experimental Identifier	Strain	Fatty Acids Added	Product	69 hours OD_{600}	69 hours g/L glucose consumed
15	GLNOR432A	NYLA74	-	butanediol	12.8	86.0
16	GLNOR434A	NYLA74	+	butanediol	23.1	95.9
17	GLNOR435A	NYLA74	-	isobutanol	2.4	16.9
18	GLNOR437A	NYLA74	+	isobutanol	4.5	18.3
19	090420_3212	NYLA84	-	isobutanol	9.6	39.3
20	2009Y047	NYLA84	+	isobutanol	20.2	49.1

EXAMPLE 21

Lipase treatment of Liquefied Corn Mash for Simultaneous Saccharification and Fermentation with In-situ Product Removal Using Oleyl Alcohol

[0229] Samples of broth and oleyl alcohol taken from fermentations run as described above in Examples 1, 2, and 3 were analyzed for wt% lipid (derivatized as fatty acid methyl esters, FAME) and for wt% free fatty acid (FFA, derivatized as fatty acid methyl esters, FAME) according to the method described by E. G. Bligh and W. J. Dyer (Canadian Journal of Biochemistry and Physiology, 37:911-17, 1959, *hereafter* Reference 1). The liquefied corn mash that was prepared for each of the three fermentations was also analyzed for wt% lipid and for wt% FFA after treatment with Lipolase® 100 L (Novozymes) (10 ppm of Lipolase® total soluble protein (BCA protein analysis, Sigma Aldrich)) per kg of liquefaction reaction mass containing 30 wt% ground corn kernels). No lipase was added to the liquefied corn mash in Example 1 (control), and the fermentations described in Examples 2 and 3 containing liquefied corn mash treated with lipase (no heat inactivation of lipase) were identical except that no ethanol was added to the fermentation described in Example 3.

[0230] The % FFA in lipase-treated liquefied corn mash prepared for fermentations run as described in Examples 2 and 3 was 88% and 89%, respectively, compared to 31 % without lipase treatment (Example 1). At 70 h (end of run (EOR)), the concentration of FFA in the OA phase of fermentations run as described in Examples 2 and 3 (containing active lipase) was 14% and

20%, respectively, and the corresponding increase in lipids (measured as corn oil fatty acid methyl ester derivatives) was determined by GC/MS to be due to the lipase-catalyzed esterification of COFA by OA, where COFA was first produced by lipase-catalyzed hydrolysis of corn oil in the liquefied corn mash. Results are shown in Table 5.

Table 5: Lipid and free fatty acid content of fermentations containing oleyl alcohol as ISPR solvent and active lipase

fermentation	lipase	time (h), sample	lipids (wt%)	FFA (wt%)	lipids (g)	FFA (g)	lipids + FFA (g)	% FFA
Example 1	none	liq. mash	0.61	0.28	5.3	2.4	7.7	31
Example 1	none	0.8 h, broth	0.49	0.22	5.5	2.5	8.0	31
Example 1	none	31 h, broth	0.19	0.03	2.1	0.3	2.4	13
Example 1	none	31 h, OA	0.36	0.21	3.4	2.0	5.3	37
Example 1	none	70 h, broth	0.15	0.03	1.7	0.3	2.0	15
Example 1	none	70 h, OA	0.57	0.25	5.3	2.3	7.7	31
Example 2	10 ppm	liq. mash	0.13	0.97	1.1	8.5	9.6	88
Example 2	10 ppm	0.8 h, broth	0.15	0.62	1.7	7.0	8.7	81
Example 2	10 ppm	31 h, broth	0.16	0.05	1.8	0.5	2.3	23
Example 2	10 ppm	31 h, OA	0.37	0.23	3.5	2.2	5.7	38
Example 2	10 ppm	70 h, broth	0.17	0.02	1.9	0.3	2.2	13
Example 2	10 ppm	70 h, OA	0.60	0.10	5.7	1.0	6.7	14
Example 3	10 ppm	liq. mash	0.12	0.97	1.0	8.5	9.5	89
Example 3	10 ppm	0.8 h, broth	0.32	0.40	3.6	4.5	8.1	56
Example 3	10 ppm	31 h, broth	0.17	0.05	1.9	0.6	2.5	24
Example 3	10 ppm	31 h, OA	0.38	0.22	3.6	2.1	5.7	37
Example 3	10 ppm	70 h, broth	0.15	0.02	1.7	0.2	1.9	13
Example 3	10 ppm	70 h, OA	0.46	0.12	4.4	1.1	5.6	20

EXAMPLE 22

Heat Inactivation of Lipase in Lipase-treated Liquefied Corn Mash to Limit Production of Oleyl Alcohol Esters of Corn Oil Free Fatty Acids

[0231] Tap water (918.4 g) was added to a jacketed 2-L resin kettle, then 474.6 g wet weight (417.6 g dry weight) of ground whole corn kernels (1.0 mm screen on hammer mill) was added with stirring. The mixture was heated to 55°C with stirring at 300 rpm, and the pH adjusted to 5.8 with 2 N sulfuric acid. To the mixture was added 14.0 g of an aqueous solution containing 0.672 g of Spezyme®-FRED L (Genencor®, Palo Alto, CA), and the temperature of the mixture increased to 85°C with stirring at 600 rpm and pH 5.8. After 120 minutes at 85°C, the mixture was cooled to 50°C and 45.0 mL aliquots of the resulting liquefied corn mash were transferred to 50-mL polypropylene centrifuge tubes and stored frozen at -80°C.

[0232] In a first reaction, 50 g of liquefied corn mash prepared as described above was mixed with 10 ppm Lipolase® 100 L (Novozymes) for 6 h at 55°C and with no inactivation of lipase at 85°C for 1 h, the mixture was cooled to 30°C. In a second reaction, 50 g of liquefied corn mash was mixed with 10 ppm Lipolase® for 6 h at 55°C, then heated to 85°C for 1 h (lipase inactivation), then cooled to 30°C. In a third reaction, 50 g of liquefied corn mash without added lipase was mixed for 6 h at 55°C, and with no heating at 85°C for 1 h, the mixture was cooled to 30°C, 38 g of oleyl alcohol was added, and the resulting mixture stirred for 73 h at 30°C. In a fourth reaction, 50 g of liquefied corn mash without added lipase was mixed for 6 h at 55°C, then heated to 85°C for 1 h, then cooled to 30°C. Each of the four reaction mixtures was sampled at 6 h, then 38 g of oleyl alcohol added, and the resulting mixtures stirred at 30°C and sampled at 25 h and 73 h. Samples (both liquefied mash and oleyl alcohol (OA)) were analyzed for wt% lipid (derivatized as fatty acid methyl esters, FAME) and for wt% free fatty acid (FFA, derivatized as fatty acid methyl esters, FAME) according to the method described by Reference 1.

[0233] The % FFA in the OA phase of the second reaction run with heat inactivation of lipase prior to OA addition was 99% at 25 h and 95% at 73 h, compared to only 40% FFA and 21% FFA at 25 h and 73 h, respectively, when the lipase in lipase-treated liquefied corn mash was not heat inactivated (first reaction). No significant change in % FFA was observed in the two control reactions without added lipase. Results are shown in Table 6.

Table 6: Lipid and free fatty acid content of a mixture of liquefied corn mash and oleyl alcohol in the presence or absence of active or heat-inactivated lipase

reaction conditions	time (h), sample	lipids (wt%)	FFA (wt%)	lipids (mg)	FFA (mg)	lipid+FFA (mg)	% FFA
10 ppm active lipase, no 85 °C heat treatment	6 h, liq. mash	0.08	0.71	41	345	386	89
	25 h, liq. mash	0.22	0.06	105	27	132	20
	25 h, OA	0.58	0.39	212	143	355	40
	73 h, liq. mash	0.25	0.05	121	22	143	18

	73 h, OA	0.91	0.24	333	88	420	21
10 ppm inactive lipase, 85 °C heat treatment	6 h, liq. mash	0.06	0.45	28	224	252	89
	25 h, liq. mash	0.10	0.11	49	54	103	53
	25 h, OA	0.02	0.96	8	366	374	99
	73 h, liq. mash	0.24	0.15	117	72	189	62
	73 h, OA	0.06	1.11	23	424	447	95
no lipase, no 85 °C heat treatment	6 h, liq. mash	0.80	0.40	401	199	599	33
	25 h, liq. mash	0.30	0.05	147	25	173	15
	25 h, OA	0.55	0.36	212	139	351	40
	73 h, liq. mash	0.23	0.05	117	26	143	23
	73 h, OA	0.79	0.42	305	162	467	34
no lipase, 85 °C heat treatment	6 h, liq. mash	0.74	0.36	370	183	553	33
	25 h, liq. mash	0.31	0.05	156	27	183	15
	25 h, OA	0.60	0.35	233	136	369	37
	73 h, liq. mash	0.20	0.05	99	23	121	23
	73 h, OA	0.84	0.41	326	159	486	33

EXAMPLE 23

Heat Inactivation of Lipase in Lipase-treated Liquefied Corn Mash for Simultaneous Saccharification and Fermentation with In-situ Product Removal Using Oleyl Alcohol

[0234] Three fermentations were run as described above in Examples 4, 5, and 6. No lipase was added to the liquefied corn mash in Examples 4 and 6 prior to fermentation, and the Lipase Treatment of the liquefied corn mash in the fermentation described in Example 5 (using 7.2 ppm of Lipolase® total soluble protein) was followed immediately by Heat Inactivation Treatment (to completely inactivate the lipase), and subsequently followed by Nutrient Addition Prior to Inoculation and fermentation. The % FFA in liquefied corn mash prepared without lipase treatment for fermentations run as described in Examples 4 and 6 was 31% and 34%, respectively, compared to 89% with lipase treatment (Example 5). Over the course of the fermentations listed in Table 10, the concentration of FFA in the OA phase did not decrease in any of the three fermentations, including that containing heat-inactivated lipase. The % FFA in the OA phase of the fermentation run according to Example 5 (with heat inactivation of lipase prior to fermentation) was 95% at 70 h (end of run (EOR)), compared to only 33% FFA for the remaining two fermentations (Examples 4 and

6) where liquefied corn mash was not treated with lipase. Results are shown in Table 7.

Table 7: Lipid and free fatty acid content of fermentations containing oleyl alcohol as ISPR solvent and heat-inactivated lipase (after lipase treatment of liquefied mash)

fermentation	lipase	time (h), sample	lipids (wt%)	FFA (wt%)	lipids (g)	FFA (g)	lipid + FFA (g)	% FFA
Example 4	none	liquefied mash	0.65	0.30	7.2	3.3	10.4	31
Example 4	none	0.2 h, broth	0.56	0.28	6.6	3.3	9.9	33
Example 4	none	4.3 h, broth	0.28	0.09	3.3	1.0	4.4	24
Example 4	none	4.3 h, OA	0.45	0.27	4.0	2.4	6.4	37
Example 4	none	30 h, broth	0.17	0.05	2.0	0.6	2.7	24
Example 4	none	30 h, OA	0.63	0.29	5.7	2.6	8.3	32
Example 4	none	53 h, broth	0.13	0.04	1.5	0.5	2.0	23
Example 4	none	53 h, OA	0.67	0.32	6.0	2.9	8.9	32
Example 4	none	70 h, broth	0.13	0.04	1.5	0.4	1.9	23
Example 4	none	70 h, OA	0.64	0.31	5.8	2.8	8.5	33
Example 5	7.2 ppm	liquefied mash	0.11	0.89	1.3	9.9	11.2	89
Example 5	7.2 ppm	0.2 h, broth	0.25	0.83	2.9	9.8	12.8	77
Example 5	7.2 ppm	4.3 h, broth	0.14	0.17	1.6	2.1	3.7	56
Example 5	7.2 ppm	4.3 h, OA	0.02	0.84	0.2	7.9	8.1	97
Example 5	7.2 ppm	30 h, broth	0.08	0.18	1.0	2.1	3.1	68
Example 5	7.2 ppm	30 h, OA	0.04	0.92	0.3	8.6	8.9	96
Example 5	7.2 ppm	53 h, broth	0.07	0.11	0.9	1.3	2.2	61
Example 5	7.2 ppm	53 h, OA	0.08	0.95	0.7	8.9	9.6	93
Example 5	7.2 ppm	70 h, broth	0.08	0.10	0.9	1.2	2.1	55
Example 5	7.2 ppm	70 h, OA	0.05	0.94	0.4	8.8	9.2	95
Example 6	none	liquefied mash	0.66	0.34	7.3	3.8	11.1	34
Example 6	none	0.2 h, broth	0.63	0.34	7.6	4.0	11.6	34
Example 6	none	4.3 h, broth	0.33	0.10	3.9	1.2	5.1	23
Example 6	none	4.3 h, OA	0.45	0.27	4.0	2.4	6.4	38
Example 6	none	30 h, broth	0.17	0.06	2.1	0.8	2.8	26
Example 6	none	30 h, OA	0.69	0.33	6.2	3.0	9.1	32
Example 6	none	53 h, broth	0.14	0.05	1.6	0.5	2.2	25
Example 6	none	53 h, OA	0.72	0.35	6.4	3.1	9.5	33
Example 6	none	70 h, broth	0.15	0.05	1.8	0.6	2.4	25
Example 6	none	70 h, OA	0.70	0.34	6.2	3.0	9.2	33

EXAMPLE 24

Lipase treatment of Ground Whole Corn Kernels prior to Liquefaction

[0235] Tap water (1377.6 g) was added into each of two jacketed 2-L resin kettles, then 711.9 g wet weight (625.8 g dry weight) of ground whole corn kernels (1.0 mm screen on hammer mill) was added to each kettle with stirring.

Each mixture was heated to 55°C with stirring at 300 rpm, and the pH adjusted to 5.8 with 2 N sulfuric acid. To each mixture was added 21.0 g of an aqueous solution containing 1.008 g of Spezyme®-FRED L (Genencor®, Palo Alto, CA). To one mixture was then added 10.5 mL of aqueous solution of Lipolase® 100L Solution (21 mg total soluble protein, 10 ppm lipase final concentration) and to the second mixture was added 1.05 mL of aqueous solution of Lipolase® 100L Solution (2.1 mg total soluble protein, 1.0 ppm lipase final concentration). Samples were withdrawn from each reaction mixture at 1 h, 2 h, 4 h and 6 h at 55°C, then the temperature of the mixture was increased to 85°C with stirring at 600 rpm and pH 5.8, and a sample was taken when the mixture first reached 85°C. After 120 minutes at 85°C, a sample was taken and the mixtures were cooled to 50°C and final samples of the resulting liquefied corn mash were transferred to 50-mL polypropylene centrifuge tubes; all samples were stored frozen at -80°C.

[0236] In two separate reactions, a 50 g sample of the 10 ppm lipase-treated liquefied corn mash or a 55 g sample of the 1.0 ppm lipase-treated liquefied corn mash prepared as described above was mixed with oleyl alcohol (OA) (38 g) at 30°C for 20 h, then the liquefied mash and OA in each reaction mixture were separated by centrifugation and each phase analyzed for wt% lipid (derivatized as fatty acid methyl esters, FAME) and for wt% free fatty acid (FFA, derivatized as fatty acid methyl esters, FAME) according to the method described by Reference 1. The % FFA in the OA phase of the liquefied mash/OA mixture prepared using heat inactivation of 10 ppm lipase during liquefaction was 98% at 20 h, compared to only 62% FFA in the OA phase of the liquefied mash/OA mixture prepared using heat inactivation of 1.0 ppm lipase during liquefaction. Results are shown in Table 8.

Table 8: Lipid and free fatty acid content of a mixture of liquefied corn mash and oleyl alcohol, using lipase treatment of ground corn suspension prior to liquefaction (heat inactivation of lipase during liquefaction)

reaction conditions	time (h), sample	lipids (wt%)	FFA (wt%)	lipids (mg)	FFA (mg)	lipid+FFA (mg)	% FFA
10 ppm lipase at 55 ℃ prior to liquefaction at 85 ℃, mix with OA for 20 h	1 h, pre-liquefaction	0.226	0.627	112	311	424	74
	2 h, pre-liquefaction	0.199	0.650	99	323	422	77
	4 h, pre-liquefaction	0.151	0.673	75	334	410	82
	6 h, pre-liquefaction	0.101	0.700	50	348	398	87
	0 h, 85 ℃, liq. mash	0.129	0.764	64	380	444	86
	2 h, 85 ℃, liq. mash	0.129	0.751	64	373	437	85
	20 h, 30 ℃, liq. mash	0.074	0.068	37	34	71	48
	20 h, 30 ℃, OA	0.015	1.035	5.7	394	400	98
1.0 ppm lipase at 55 ℃ prior to liquefaction at 85 ℃, mix with OA for 20 h	1 h, pre-liquefaction	0.408	0.480	226	266	492	54
	2 h, pre-liquefaction	0.401	0.424	222	235	457	51
	4 h, pre-liquefaction	0.299	0.433	165	240	405	58
	6 h, pre-liquefaction	0.346	0.453	192	251	442	57
	0 h, 85 ℃, liq. mash	0.421	0.407	233	225	458	49
	2 h, 85 ℃, liq. mash	0.424	0.429	235	237	472	50
	20 h, 30 ℃, liq. mash	0.219	0.054	121	30	151	20
	20 h, 30 ℃, OA	0.344	0.573	140	233	373	62

EXAMPLE 25

Lipase Screening for Treatment of Ground Whole Corn Kernels prior to Liquefaction

[0237] Seven reaction mixtures containing tap water (67.9 g) and ground whole corn kernels (35.1 g wet wt., ground with 1.0 mm screen using a hammer mill) at pH 5.8 were stirred at 55℃ in stoppered flasks. A 3-mL sample (t = 0 h) was removed from each flask and the sample immediately frozen on dry ice, then ca. 0.5 mL of 10 mM sodium phosphate buffer (pH 7.0) containing 1 mg total soluble protein (10 ppm final concentration in reaction mixture) of one of the following lipases (Novozymes) were added to one of each flask: Lipolase® 100 L, Lipex® 100L, Lipoclean® 2000T, Lipozyme® CALB L, Novozyme® CALA L, and Palatase 20000L; no lipase was added to the seventh flask. The resulting mixtures were stirred at 55℃ in stoppered flasks, and 3-mL samples were withdrawn from each reaction mixture at 1 h, 2 h, 4 h and 6 h and immediately frozen in dry ice until analyzed for wt% lipid (derivatized as fatty acid methyl esters, FAME) and for wt% free fatty acid (FFA, derivatized as fatty acid methyl esters, FAME) according to the method described by Reference 1, and the percent free fatty acid content was calculated relative to the total combined concentrations of lipid and free fatty acid was determined for each sample. Results are shown in Table 9.

Table 9: Percent free fatty acid content (% FFA) of a mixture of ground whole corn kernels using lipase treatment at 55°C prior to liquefaction

	% FFA				
time	0 h	1 h	2 h	4 h	6 h
Lipolase® 100L	33	56	74	76	79
Lipex® 100L	34	66	81	83	83
Lipoclean® 2000T	38	55	73	69	65
Lipozyme® CALB L	39	38	37	43	41
Novozyme® CALA L	37	40	44	44	45
Palatase® 20000L	37	49	59	62	66
no enzyme	38	33	37	41	42

EXAMPLE 26

Lipase treatment of Ground Whole Corn Kernels prior to Simultaneous Saccharification and Fermentation with In-situ Product Removal Using Oleyl Alcohol

[0238] Three fermentations were run as described above in Examples 7, 8, and 10. For fermentations run as described in Examples 7 and 10, lipase (10 ppm of Lipolase® total soluble protein) was added to the suspension of ground corn and heated at 55°C for 6 h prior to Liquefaction to produce a liquefied corn mash containing heat-inactivated lipase. No lipase was added to the suspension of ground corn used to prepare liquefied corn mash for the fermentation described in Example 8, but the suspension was subjected to the same heating step at 55°C prior to liquefaction. The % FFA in lipase-treated liquefied corn mash prepared for fermentations run as described in Examples 7 and 10 was 83% and 86%, respectively, compare to 41% without lipase treatment (Example 8). Over the course of the fermentations, the concentration of FFA did not decrease in any of the fermentations, including that containing heat-inactivated lipase. The % FFA in the OA phase of the fermentation run according to Examples 7 and 10 (with heat inactivation of lipase prior to fermentation) was 97% at 70 h (end of run

(EOR)), compared to only 49% FFA for the fermentation run according to Example 8 where ground whole corn kernels had not been treated with lipase prior to liquefaction. Results are shown in Table 10.

Table 10: Lipid and free fatty acid content of fermentations containing oleyl alcohol as ISPR solvent and heat-inactivated lipase (lipase treatment of ground corn suspension prior to liquefaction)

fermentation	lipase	time (h), sample	lipids (wt%)	FFA (wt%)	lipids (g)	FFA (g)	lipid + FFA (g)	% FFA
Example 7	10 ppm	pre-lipase/pre-liq.	0.65	0.22	7.1	2.4	9.4	25
Example 7	10 ppm	post-lipase/pre-liq.	0.22	0.65	2.4	7.0	9.5	74
Example 7	10 ppm	liquefied mash	0.17	0.79	1.8	8.5	10.3	83
Example 7	10 ppm	0.3 h, broth	0.16	0.79	1.8	8.9	10.7	83
Example 7	10 ppm	4.8 h, broth	0.14	0.31	1.6	3.5	5.1	69
Example 7	10 ppm	4.8 h, OA	0.04	0.68	0.3	5.4	5.6	95
Example 7	10 ppm	29 h, broth	0.10	0.12	1.2	1.3	2.5	53
Example 7	10 ppm	29 h, OA	0.03	1.05	0.2	8.2	8.4	98
Example 7	10 ppm	53 h, broth						
Example 7	10 ppm	53 h, OA	0.07	1.14	0.5	9.0	9.5	95
Example 7	10 ppm	70 h, broth	0.11	0.07	1.2	0.8	2.0	39
Example 7	10 ppm	70 h, OA	0.03	1.10	0.2	8.7	8.9	97
Example 8	none	pre-lipase/pre-liq.	0.62	0.23	6.7	2.5	9.2	27
Example 8	none	post-lipase/pre-liq.	0.57	0.26	6.2	2.8	9.0	31
Example 8	none	liquefied mash	0.52	0.36	5.6	4.0	9.6	41
Example 8	none	0.3 h, broth	0.50	0.33	5.7	3.8	9.4	40
Example 8	none	4.8 h, broth	0.47	0.14	5.3	1.6	6.9	24
Example 8	none	4.8 h, OA	0.12	0.32	1.0	2.9	3.9	73
Example 8	none	29 h, broth	0.30	0.05	3.4	0.6	4.0	16
Example 8	none	29 h, OA	0.31	0.46	2.7	4.1	6.9	60
Example 8	none	53 h, broth						
Example 8	none	53 h, OA	0.47	0.50	4.2	4.4	8.6	51
Example 8	none	70 h, broth	0.22	0.04	2.5	0.5	3.0	17
Example 8	none	70 h, OA	0.40	0.39	3.6	3.5	7.0	49
Example 10	10 ppm	pre-lipase/pre-liq.	0.67	0.23	7.4	2.5	9.9	25
Example 10	10 ppm	post-lipase/pre-liq.	0.19	0.69	2.1	7.6	9.7	78
Example 10	10 ppm	liquefied mash	0.14	0.85	1.6	9.4	11.0	86
Example 10	10 ppm	0.3 h, broth	0.13	0.82	1.5	9.4	10.9	86
Example 10	10 ppm	4.8 h, broth	0.11	0.29	1.3	3.3	4.6	72
Example 10	10 ppm	4.8 h, OA	0.04	0.60	0.3	5.2	5.6	94
Example 10	10 ppm	29 h, broth	0.09	0.14	1.0	1.6	2.6	61
Example 10	10 ppm	29 h, OA	0.01	0.96	0.1	8.4	8.5	99
Example 10	10 ppm	53 h, broth						
Example 10	10 ppm	53 h, OA	0.02	0.95	0.2	8.3	8.4	98
Example 10	10 ppm	70 h, broth	0.09	0.08	1.1	0.9	1.9	45
Example 10	10 ppm	70 h, OA	0.03	0.99	0.3	8.7	9.0	97

EXAMPLE 27

Lipase treatment of Ground Whole Corn Kernels or Liquefied Corn Mash for Simultaneous Saccharification and Fermentation with In-situ Product Removal Using Corn Oil Fatty Acids (COFA)

[0239] Five fermentations were run as described above in Examples 9, 11, 12, 13, and 14. For the fermentations run as described in Examples 9, 13, and 14, lipase (10 ppm of Lipolase® total soluble protein) was added after Liquefaction and there was no heat-inactivation of lipase. Fermentations run as described in Examples 9 and 14 had 5 g/L of ethanol added prior to inoculation, whereas the fermentation run as described in Example 13 had no added ethanol. The fermentations run as described in Examples 11 and 12 employed the addition of 10 ppm Lipolase® total soluble protein to the suspension of ground corn prior to liquefaction, resulting in heat inactivation of lipase during liquefaction. The fermentation run as described in Example 11 had 5 g/L of ethanol added prior to inoculation, whereas the fermentation run as described in Example 12 had no added ethanol. The final total grams of isobutanol (*i*-BuOH) present in the COFA phase of the fermentations containing active lipase was significantly greater than the final total grams of *i*-BuOH present in the COFA phase of the fermentations containing inactive lipase. The final total grams of isobutanol (*i*-BuOH) present in the fermentation broths containing active lipase were only slightly less than the final total grams of *i*-BuOH present in the fermentation broths containing inactive lipase, such that the overall production of *i*-BuOH (as a combination of free *i*-BuOH and isobutyl esters of COFA (FABE)) was significantly greater in the presence of active lipase when compared to that obtained in the presence of heat-inactivated lipase. Results are shown in Tables 11 and 12.

Table 11: Dependence of the production of free isobutanol (*i*-BuOH) and isobutyl esters of COFA (FABE) in fermentations containing corn oil fatty acids (COFA) as ISPR solvent on presence (Examples 9, 13, and 14) or absence (Examples 11 and 12) of active lipase (COFA phase analysis)

fermentation	fermentation time (h)	g *i*-BuOH/ kg COFA	g FABE/ kg COFA	g *i*-BuOH from FABE/ kg COFA	total g *i*-BuOH/ kg COFA
Example 9	4.5	2.4	0.0	0	2.4
Example 9	28.8	5.4	70.9	16.5	22.0
Example 9	52.4	8.9	199.0	46.4	55.3
Example 9	69.3	4.9	230.9	53.9	69.3
Example 11	6.6	2.3	0.0	0.0	2.3
Example 11	53.5	25.1	2.9	0.6	25.7
Example 11	71.1	24.4	6.3	1.4	25.8
Example 12	6.6	2.3	0.0	0.0	2.3
Example 12	53.5	12.8	1.6	0.4	13.2
Example 12	71.1	12.8	3.0	0.7	13.5
Example 13	6.6	2.3	0.0	0.0	2.3
Example 13	53.5	4.9	72.1	16.0	20.9
Example 13	71.1	4.6	91.4	20.3	24.9
Example 14	6.6	2.1	0.0	0.0	2.1
Example 14	53.5	9.8	197.2	43.8	53.6
Example 14	71.1	4.9	244.5	54.3	59.2

Table 12: Dependence of the production of free isobutanol (*i*-BuOH) and isobutyl esters of COFA (FABE) in fermentations containing corn oil fatty acids (COFA) as ISPR solvent on presence (Examples 9, 13, and 14) or absence (Examples 11 and 12) of active lipase (fermentation broth analysis)

sample	fermentation time (h)	g *i*-BuOH/ kg broth	g FABE/ kg broth	g *i*-BuOH from FABE/ kg broth	total g *i*-BuOH/ kg broth
Example 9	4.5	0.0	0.0	0	0
Example 9	28.8	0.0	12.6	2.9	2.9
Example 9	52.4	0.0	30.3	7.1	7.1
Example 9	69.3	0.0	24.7	5.8	5.8
Example 11	6.6	0.0	0.0	0	0.0
Example 11	53.5	9.8	0.0	0	9.8
Example 11	71.1	9.5	0.0	0	9.5
Example 12	6.6	0.0	0.0	0	0
Example 12	53.5	3.8	0.0	0.0	3.8
Example 12	71.1	5.1	0.0	0.0	5.1
Example 13	6.6	0.0	0.0	0	0
Example 13	53.5	2.1	3.0	0.7	2.8
Example 13	71.1	2.1	7.4	1.6	3.7
Example 14	6.6	0.0	0.0	0	0.0
Example 14	53.5	2.9	22.4	5.0	7.9
Example 14	71.1	3.3	19.3	4.3	7.6

EXAMPLE 28

Dependence of isobutyl-COFA ester concentration on aqueous/COFA ratio in lipase-catalyzed reactions

[0240] Reaction mixtures containing aqueous 2-(*N*-morpholino)ethanesulfonic acid buffer (0.20 M, pH 5.2), isobutanol (2-methyl-1-propanol), lipase (Lipolase® 100 L; Novozymes) and corn oil fatty acids prepared from corn oil (Table 13) were stirred at 30 °C, and samples were withdrawn from each reaction mixture at predetermined times, immediately centrifuged, and the aqueous and organic layers separated and analyzed for isobutanol (*i*-BuOH) and isobutyl esters of corn oil fatty acids (*i*-BuO-COFA) (Table 14).

Table 13: Reaction conditions for conversion of isobutanol (*i*-BuOH) to isobutyl esters of corn oil fatty acids (*i*-BuO-COFA)

reaction	MES (0.2 M) (g)	*i*-BuOH (g)	COFA (g)	lipase (ppm)
1	45.96	3.6	43.4	10
2	45.96	3.6	21.7	10
3	45.96	3.6	10.85	10
4	45.96	3.6	43.4	4
5	45.96	3.6	43.4	0

Table 14: Weights of isobutanol (*i*-BuOH) and isobutyl esters of corn oil fatty acids (*i*-BuO-COFA) present in the aqueous fraction (AQ) and organic fraction (ORG) for reactions described in Table 13

reaction	time (h)	total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	free *i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-COFA (g) (ORG)	*i*-BuO-COFA (g) (ORG)
1	0.1	0.77	2.83	2.77	0.05	0.24
1	1	0.76	2.84	2.58	0.25	1.13
1	2	0.74	2.86	2.41	0.44	2.00
1	4	0.66	2.94	2.05	0.89	4.03
1	6	0.63	2.97	1.43	1.54	6.93
1	21.5	0.28	3.32	0.34	2.98	13.4
1	25.5	0.23	3.37	0.29	3.08	13.8
2	0.1	1.17	2.43	2.36	0.07	0.30
2	1	1.09	2.51	2.26	0.24	1.10
2	2	1.07	2.53	2.19	0.34	1.52
2	4	1.03	2.57	1.99	0.59	2.64
2	6	1.00	2.60	1.70	0.90	4.04
2	21.5	0.75	2.85	0.58	2.27	10.2
2	25.5	0.59	3.01	0.49	2.52	11.4
3	0.1	1.56	2.04	1.98	0.06	0.27
3	1	1.55	2.05	1.77	0.28	1.24
3	2	1.49	2.11	1.65	0.46	2.08
3	4	1.45	2.15	1.28	0.87	3.92
3	6	1.33	2.27	0.96	1.31	5.92
3	21.5	1.12	2.48	0.26	2.22	10.0
3	25.5	0.88	2.72	0.26	2.46	11.1
4	0.1	0.84	2.76	2.75	0.02	0.07
4	1	0.78	2.82	2.73	0.09	0.40
4	2	0.83	2.77	2.59	0.17	0.79
4	4	0.78	2.82	2.44	0.38	1.71

4	6	0.78	2.82	2.10	0.72	3.25
4	21.5	0.58	3.02	1.12	1.90	8.57
4	25.5	0.51	3.09	0.97	2.11	9.51
5	0.1	0.90	2.70	2.70	0.00	0.00
5	1	0.90	2.70	2.70	0.00	0.00
5	2	0.92	2.68	2.68	0.00	0.00
5	4	0.89	2.71	2.70	0.00	0.02
5	6	0.92	2.68	2.62	0.06	0.29
5	21.5	0.90	2.70	2.62	0.08	0.37
5	25.5	0.89	2.71	2.62	0.09	0.41

EXAMPLE 29

Dependence of isobutyl-COFA ester concentration on aqueous/COFA ratio in lipase-catalyzed reactions

[0241] Reaction mixtures containing aqueous 2-(*N*-morpholino)ethanesulfonic acid buffer (0.20 M, pH 5.2), isobutanol (2-methyl-1-propanol) or n-butanol, lipase (Lipolase® 100 L; Novozymes) and corn oil fatty acids prepared from corn oil (Table 15) were stirred at 30°C, and samples were withdrawn from each reaction mixture at predetermined times, immediately centrifuged, and the aqueous and organic layers separated and analyzed for isobutanol (*i*-BuOH) or *n*-butanol (*n*-BuOH) and isobutyl- or butyl esters of corn oil fatty acids (BuO-COFA) (Table 16).

Table 15: Reaction conditions for conversion of isobutanol (*i*-BuOH) or *n*-butanol (*n*-BuOH) to butyl esters of corn oil fatty acids (BuO-COFA)

reaction	butanol	MES(0.2 M) (g)	butanol (g)	COFA (g)	lipase (ppm)
6	iso-butanol	45.96	3.6	13.5	10
7	n-butanol	45.96	3.6	13.5	10
8	iso-butanol	45.96	3.6	13.5	0
9	isobutanol	45.96	3.6	13.5	4

<u>Table 16:</u> Weights of isobutanol (*i*-BuOH) or *n*-butanol (*n*-BuOH) and butyl esters of corn oil fatty acids (BuO-COFA) present in the aqueous fraction (AQ) and organic fraction (ORG) for reactions described in Table 15

reaction	time (h)	total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	total *i*-BuOH (g) (ORG)	*i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-COFA (g) (ORG)
6	0	1.46	2.14	2.11	0.04	0.16
6	2	1.41	2.19	1.63	0.56	2.51
6	4	1.27	2.33	1.31	1.02	4.58
6	21	0.66	2.94	0.29	2.65	12.0
6	25	0.60	3.00	0.26	2.73	12.3
6	46	0.54	3.06	0.22	2.83	12.8
		total *n*-BuOH (g) (AQ)	total *n*-BuOH (g) (ORG)	*n*-BuOH (g) (ORG)	*n*-BuOH from *n*-BuO-COFA (g) (ORG)	*n*-BuO-COFA (g) (ORG)
7	0	1.31	2.29	2.26	0.03	0.11
7	2	1.26	2.34	1.89	0.45	2.03
7	4	1.20	2.40	1.66	0.74	3.35
7	21	0.81	2.79	0.50	2.29	10.3
7	25	0.77	2.83	0.40	2.43	11.0
7	46	0.50	3.10	0.23	2.87	12.9
		total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	*i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-COFA (g) (ORG)	*i*-BuO-COFA (g) (ORG)
8	0	1.62	1.98	1.98	0.00	0.01
8	2	1.56	2.04	2.04	0.00	0.00
8	4	1.59	2.01	2.01	0.00	0.00
8	21	1.59	2.01	2.00	0.01	0.04
8	25	1.55	2.05	2.04	0.01	0.04
8	46	1.45	2.15	2.12	0.02	0.11
		total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	*i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-COFA (g) (ORG)	*i*-BuO-COFA (g) (ORG)
9	0	1.57	2.03	2.02	0.01	0.04
9	2	1.54	2.06	1.86	0.19	0.86
9	4	1.44	2.16	1.79	0.36	1.64
9	21	1.14	2.46	0.95	1.51	6.82
9	25	1.10	2.50	0.83	1.67	7.50
9	46	0.78	2.82	0.44	2.37	10.7

EXAMPLE 30

Production of iso-butyl oleate by lipase-catalyzed reaction of iso-butanol and oleic acid

[0242] Reaction mixtures containing aqueous 2-(*N*-morpholino)ethanesulfonic acid buffer (0.20 M, pH 5.2), isobutanol (2-methyl-1-propanol), lipase (0 ppm or

10 ppm Lipolase® 100 L; Novozymes) and oleic acid (Alfa Aesar) (Table 17) were stirred at 30℃, and samples were withdrawn from each reaction mixture at predetermined times, immediately centrifuged, and the aqueous and organic layers separated and analyzed for isobutanol (*i*-BuOH) and iso-butyl oleate (*i*-BuO-oleate) (Table 18).

Table 17: Reaction conditions for conversion of isobutanol (*i*-BuOH) to iso-butyl oleate (*i*-BuO-oleate)

reaction	MES (0.2 M) (g)	*i*-BuOH (g)	oleic acid (g)	lipase (ppm)
10	46.11	3.64	14.62	10
11	46.10	3.59	14.40	0

Table 18: Weights of isobutanol (*i*-BuOH) and iso-butyl oleate (*i*-BuO -COFA) present in the aqueous fraction (AQ) and organic fraction (ORG) for reactions described in Table 17.

reaction	time (h)	total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	*i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-oleate (g) (ORG)	*i*-BuO-oleate (g) (ORG)
10	0	1.37	2.28	2.24	0.04	0.18
10	2	1.30	2.34	1.95	0.40	1.81
10	4	1.28	2.37	1.82	0.55	2.53
10	6	1.22	2.42	1.71	0.72	3.27
10	23	0.92	2.72	0.71	2.01	9.20
10	27	0.89	2.75	0.65	2.11	9.62
10	47	0.81	2.84	0.55	2.29	10.5
10	51	0.82	2.83	0.54	2.29	10.5
11	0	1.44	2.16	2.16	0.00	0.00
11	2	1.45	2.15	2.15	0.00	0.00
11	4	1.44	2.16	2.16	0.00	0.00
11	6	1.43	2.16	2.16	0.00	0.00
11	23	1.49	2.10	2.10	0.01	0.02
11	27	1.46	2.14	2.13	0.01	0.04
11	47	1.48	2.12	2.09	0.02	0.10
11	51	1.52	2.07	2.05	0.02	0.11

EXAMPLE 31

Production of iso-butyl oleate by lipase-catalyzed reaction of iso-butanol and oleic acid

[0243] Reaction mixtures containing aqueous 2-(*N*-morpholino)ethanesulfonic acid buffer (MES, 0.20 M, pH 5.2), isobutanol (2-methyl-1-propanol), oleic acid (Alfa Aesar), and lipase (10 ppm) from Lipolase® 100L, Lipex® 100L, Lipozyme® CALB L, Novozyme® CALA L, Palatase® from Novozymes, or lipase (10 ppm) from *Pseudomonas fluorescens, Pseudomonas cepacia, Mucor miehei, hog pancreas, Candida cylindracea, Rhizopus niveus, Candida antarctica, Rhizopus arrhizus or Aspergillus* from SigmaAldrich (Table 19), were stirred at 30℃, and samples were withdrawn from each reaction mixture at predetermined times, immediately centrifuged, and the aqueous and organic layers separated and analyzed for isobutanol (*i*-BuOH) and iso-butyl oleate (*i*-BuO-oleate) (Table 20).

<u>Table 19:</u> Reaction conditions for conversion of isobutanol (*i*-BuOH) to iso-butyl oleate (*i*-BuO-oleate)

MES (0.2 M) (g)	*i*-BuOH (g)	oleic acid (g)	lipase (ppm)
46.105	3.601	13.72	10

<u>Table 20:</u> Weights of isobutanol (*i*-BuOH) and iso-butyl oleate (*i*-BuO-oleate) present in the aqueous fraction (AQ) and organic fraction (ORG) for reactions described in Table 19

lipase	time (h)	total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	*i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-oleate (g) (ORG)	*i*-BuO-oleate (g) (ORG)
Lipolase® 100L	23	1.55	2.05	1.47	0.59	2.68
Lipex® 100L	23	0.65	2.95	0.30	2.65	12.09
Lipozyme® CALB L	23	1.01	2.59	0.82	1.77	8.08
Novozyme® CALA L	23	1.39	2.22	2.16	0.06	0.27
Palatase®	23	1.27	2.33	1.43	0.91	4.14
Pseudomonas fluorescens	23	1.38	2.22	1.97	0.25	1.14
Pseudomonas cepacia	23	1.39	2.21	1.95	0.26	1.20
Mucor miehei	23	1.29	2.31	1.57	0.75	3.42
hog pancreas	23	1.40	2.20	2.19	0.01	0.04
Candida cylindracea	23	1.15	2.45	1.08	1.37	6.25
Rhizopus niveus	23	1.39	2.21	2.19	0.02	0.11
Candida antarctica	23	1.37	2.24	2.08	0.15	0.69
Rhizopus arrhizus	23	1.01	2.59	0.81	1.78	8.12
Aspergillus	23	1.36	2.24	2.06	0.18	0.82

Example 32

Production of iso-butyl COFA esters by phospholipase-catalyzed reaction of iso-butanol and corn oil fatty acids (COFA)

[0244] Reaction mixtures containing aqueous 2-(*N*-morpholino)ethanesulfonic acid buffer (0.20 M, pH 5.3), isobutanol (2-methyl-1-propanol), phospholipase (Phospholipase A; SigmaAldrich, L3295-250) and corn oil fatty acids prepared from corn oil were stirred at 30°C (Table 21), and samples were withdrawn from each reaction mixture at predetermined times, immediately centrifuged, and the aqueous and organic layers separated and analyzed for isobutanol (*i*-BuOH) and isobutyl esters of corn oil fatty acids (*i*-BuO-COFA) (Table 22).

Table 21. Reaction conditions for conversion of isobutanol (*i*-BuOH) to isobutyl esters of corn oil fatty acids (*i*-BuO-COFA)

reaction #	MES buffer (0.2 M) (g)	*i*-BuOH (g)	COFA (g)	lipase (ppm)
1	46.1	3.6	14.7	10
2	46.1	3.6	14.7	3
3	46.1	3.6	14.7	0

Table 22. Weights of isobutanol (*i*-BuOH) and isobutyl esters of corn oil fatty acids (*i*-BuO-COFA) present in the aqueous fraction (AQ) and organic fraction (ORG) for reactions described in Table 21

reaction	time (h)	total *i*-BuOH (g) (AQ)	total *i*-BuOH (g) (ORG)	free *i*-BuOH (g) (ORG)	*i*-BuOH from *i*-BuO-COFA (g) (ORG)	*i*-BuO-COFA (g) (ORG)
1	0.1	1.29	2.39	2.39	0.00	0.00
1	2	1.24	2.44	2.38	0.06	0.26
1	20	1.25	2.43	2.22	0.21	0.96
1	24	1.26	2.42	2.19	0.23	1.03
1	44	1.27	2.41	2.13	0.28	1.28
1	48	1.22	2.46	2.15	0.31	1.41
2	0.1	1.27	2.34	2.34	0.00	0.00
2	2	1.25	2.35	2.33	0.02	0.08
2	20	1.24	2.37	2.30	0.07	0.30
2	24	1.22	2.38	2.31	0.07	0.32
2	44	1.33	2.28	2.18	0.10	0.44

2	48	1.23	2.38	2.27	0.11	0.48
3	0.1	1.27	2.33	2.33	0.00	0.00
3	2	1.26	2.34	2.34	0.00	0.00
3	20	1.22	2.38	2.37	0.01	0.07
3	24	1.25	2.35	2.33	0.02	0.08
3	44	1.24	2.36	2.32	0.04	0.18
3	48	1.24	2.36	2.32	0.04	0.18

EXAMPLE 33

Comparison of partition coefficients for isobutanol between water and extractant

[0245] Aqueous solutions of isobutanol (30 g/L) were mixed with corn oil fatty acids (COFA), oleic acid, or corn oil triglycerides, and their measured partition coefficients reported in the table relative to the measured partition coefficient for oleyl alcohol. Results are shown in Table 23.

Table 23: Relative partition coefficients for isobutanol (30 g/L) between water and extractant

extractant	isobutanol partition coefficient, relative to oleyl alcohol
oleyl alcohol	100 %
corn oil fatty acids	91 %
corn oil fatty acid isobutyl esters	43 %
corn oil triglycerides	10 %

Example 34

Hydroxylated Triglycerides from Corn Oil

[0246] To a three-neck 500mL flask equipped with a mechanical stirrer and addition funnel was added corn oil (50.0 g), toluene (25.0 mL), Amberlyte IR-120 resin (12.5 g), and glacial acetic acid (7.5 g). The resulting mixture was heated to 60°C, and then hydrogen peroxide (41.8 g of 30% H_2O_2 in water) was added dropwise over one hour. The mixture was stirred at 60°C for two hours, upon which time the reaction mixture was worked up: resin was removed by filtration, and the filtrate partitioned between ethyl acetate (75 mL) and water (50 mL). After the layers were separated, the organic layer was washed with sat. aq. $NaHCO_3$ solution (50 mL), and brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 48.9g of yellow oil. The ^1H NMR analysis of the crude reaction product showed that 63% of double bonds were epoxidized.

A. Corn oil hydroxylation (63% hydroxylation)

[0247] To a three-neck 500mL flask equipped with a mechanical stirrer and addition funnel was added corn oil (50.0 g), toluene (25.0 mL), Amberlyte IR-120 resin (12.5 g), and glacial acetic acid (7.5 g). The resulting mixture was heated to 60°C, and then hydrogen peroxide (41.8 g of 30% H_2O_2 in water) was added dropwise over one hour. The mixture was stirred at 60°C for two hours, upon which time the reaction mixture was worked up: resin was removed by filtration, and the filtrate partitioned between ethyl acetate (75 mL) and water (50 mL). After the layers were separated, the organic layer was washed with sat. aq. $NaHCO_3$ solution (50 mL), and brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 48.9g of yellow oil. The ^1H NMR analysis of the crude reaction product showed that 63% of double bonds were epoxidized.

[0248] To a 500 mL round bottom flask was added epoxidized corn oil (20.0 g), tetrahydrofuran (THF) (100.0 mL), and sulfuric acid (50 mL of 1.7 M aqueous solution). The cloudy mixture was stirred for two hours at 50°C, and then worked up by partitioning between water (100 mL) and ethyl acetate (200 mL). The organic layer was washed with water (3x50 mL) and then brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 19.9 g of dark yellow oil (63% hydroxylation corn oil).

B. Corn oil hydroxylation (47% hydroxylation)

[0249] To a three-neck 500 mL flask, equipped with a mechanical stirrer and addition funnel was added corn oil (50.0 g), toluene (25.0 mL), Amberlyte IR-120 resin (12.5 g), and glacial acetic acid (7.5 g). The resulting mixture was heated to

60°C, and then hydrogen peroxide (41.8 g of 30% H_2O_2 in water) was added dropwise over one hour. The mixture was stirred at 60_oC for one hour, upon which time the reaction mixture was worked up: the resin was removed by filtration, and the filtrate partitioned between ethyl acetate (75 mL) and water (50 mL). After the layers were separated, the organic layer was washed with sat. aq. $NaHCO_3$ solution (50 mL), and brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 49.8g of yellow oil. The ^{1}H NMR analysis of the crude reaction product showed that 47% of double bonds were epoxidized.

[0250] To a 500 mL round bottom flask was added epoxidized corn oil (20.0 g), THF (100.0 mL), and sulfuric acid (50 mL of 1.7M aqueous solution). The cloudy mixture was stirred for two hours at 50°C, and then worked up by partitioning between water (100 mL) and ethyl acetate (200 mL). The organic layer was washed with water (3x50 mL) and then brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 19.2 g of dark yellow oil (47% hydroxylation corn oil).

C. Corn oil hydroxylation (28% hydroxylation)

[0251] To a three-neck 500 mL flask, equipped with a mechanical stirrer and addition funnel was added corn oil (50.0 g), toluene (25.0 mL), Amberlyte IR-120 resin (12.5 g), and glacial acetic acid (7.5g). The resulting mixture was heated to 60°C, and then hydrogen peroxide (41.8 g of 30% H_2O_2 in water) was added dropwise over one hour. The mixture was stirred at 60°C for two hours, upon which time the reaction mixture was worked up: the resin was removed by filtration, and the filtrate partitioned between ethyl acetate (75 mL) and water (50 mL). After the layers were separated, the organic layer was washed with sat. aq. $NaHCO_3$ solution (50 mL), and brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 47.2 g of yellow oil. The ^{1}H NMR analysis of the crude reaction product showed that 28% of double bonds were epoxidized.

[0252] To a 500 mL round bottom flask was added epoxidized corn oil (20.0 g), THF (100.0 mL), and sulfuric acid (50 mL of 1.7M aqueous solution). The cloudy mixture was stirred for two hours at 50°C, and then worked up by partitioning between water (100 mL) and ethyl acetate (200 mL). The organic layer was washed with water (3x50 mL) and then brine (50 mL). The organic layer was dried over anh. Na_2SO_4 and concentrated *in vacuo* to obtain 20.3 g of dark yellow oil (28% hydroxylation corn oil).

Partition coefficient measurement

[0253] To a 5 mL vial was added 0.910 g of the 67% hydroxylated corn oil, and 0.910 mL of 3wt% *i*-BuOH water solution. The biphasic mixture was vigorously stirred using Vortex Genie® for 10 minutes. Upon mixing, the separation of layers was aided by centrifuging the mixture using Fisher Scientific Centrific 228 centrifuge (3300 rpm) for 10 minutes. 0.100 g of both layers were taken. The organic, upper layer was diluted to 1.00 mL with toluene solution of ethylene glycol diethylether (10.1 mg/mL), and the water layer was diluted to 1.00 mL with methanol solution of ethylene glycol diethylether (10.2 mg/mL). The concentrations of *i*-BuOH in both phases were measured using a calibrated gas chromatograph (GC). The same procedure was repeated for 47% and 28% hydroxylated corn oil. The partition coefficient thus measured was 3.2 for the 67% hydroxylated corn oil, 2.3 for the 47% hydroxylated corn oil, and 2.1 for the 28% hydroxylated corn oil.

[0254] The above outlined procedure was repeated with 6% *i*-BuOH water solution. The partition coefficients for 67% -, 47% -, and 28% -hydroxylated corn oils were 2.9, 2.9, and 2.0, respectively.

Example 34

Fatty Amides Plus Fatty Acids, and Pure Fatty Amides from Corn Oil

[0255] Corn oil was reacted with aqueous ammonium hydroxide in a manner similar to that described by Roe, et al., J. Am. Oil Chem. Soc. 29:18-22, 1952. Mazola® corn oil (0.818 L, 755 g) was placed in a 1 gallon stainless steel reactor to which was added 1.71 L (1540 g) of aqueous ammonium hydroxide (28% as NH_3). The reactor was heated with stirring to 160ºC and was maintained at that temperature with stirring for 7 h during which time the pressure reached 400 psi. The reactor was cooled and the product, a creamy white solid, was removed and the reactor rinsed with ethyl acetate. The product was dissolved in 5 L ethyl acetate and washed 5 times with 500 mL each of water which was neutralized with H_2SO_4. The ethyl acetate was then dried over anhydrous Na_2SO_4 and the solvent removed on a rotary evaporator leaving a light brown soft solid.

[0256] ^{13}C NMR in $CDCl_3$ indicated that the product contained an approximate 2:1 ratio of fatty amide to fatty acid and that the conversion of the corn oil to product was quantitative. The product had a melting point of 57-58ºC, but dropped about 11ºC when saturated with water.

[0257] Pure corn oil fatty amide was synthesized from corn oil according to Kohlhase, et al., J. Am. Oil Chem. Soc. 48:265-270, 1971 using anhydrous ammonia with ammonium acetate as a catalyst.

[0258] Three grams of ammonium acetate were placed in a 400 mL stainless steel shaker tube to which was added 51.8 g of corn oil. Anhydrous ammonia (89.7 g) was then added and the reactor sealed and heated for 7 h at 125ºC during which time the pressure reached 1300 psi. The reactor was cooled, the light colored solid removed and the reactor rinsed with ethyl acetate. The product dissolved in ethyl acetate was then worked up as in the case of the fatty amide/fatty acid mixture above.

[0259] Fatty acids were synthesized from corn oil by base hydrolysis using NaOH. Round bottom flask (5L) was equipped with a mechanical stirrer, thermocouple, heating mantle, condenser, and nitrogen tee. Charged with 500 g of food grade corn oil, 1 L of water and 75 g of sodium hydroxide. Mixture was heated to 90°C and held for three hours, during which time it became a single thick, emulsion-like single phase. At the end of this time, TLC shows no remaining corn oil in the mixture. The mixture was then cooled to 72ºC and 500 mL of 25% sulfuric acid was added to acidify the mixture. It was then cooled to room temperature and 2 L of diethyl ether was added. The ether layer was washed 3x1 L with 1% sulfuric acid, 1x1 L with saturated brine, dried over $MgSO_4$, and filtered. The ether was removed by rotovap and then the oil was purged with nitrogen overnight, obtaining 470 g of a yellow oil that partially crystallized overnight. Titration for free fatty acids via AOCS method Ca 5a-40 shows a fatty acid content of 95% expressed as oleic acid. A sample was silanized by reacting 104 mg with 100 uL of N-methyl-N-(trimethylsilyl)trifluoroacetamide in 1 mL of dry pyridine. Gas chromatography-mass spectrometry (GCMS) analysis of the silanized product shows the presence of the TMS derivatives of the 16:0, 18:2, 18:1, 18:0, and 20:0 acids

[0260] Three preparations: (1) the 2:1 mixture of corn oil fatty amide and corn oil fatty acid from aqueous ammonia, (2) a 2:1 mixture of pure corn oil fatty amide:pure corn oil fatty acid, and (3) a 1:2 mixture of pure corn oil fatty amide:corn oil fatty acid, were all tested for their ability to extract isobutanol from a 3% solution in water. Seven hundred milligrams of each was added to 2.1 mL of water containing 3% isobutanol in a 20 mL scintillation vial and placed on a rotary shaker overnight at 30ºC. In all three cases, the organic phase became liquid at this temperature, indicating a further lowering of the melting point with the uptake of isobutanol. Fifty microliters of the upper phase were diluted with either 200 µL of toluene containing ethylene glycol diethylether (10.068 mg/mL) as a GC standard or 200 µL of isopropanol containing the same concentration of ethylene glycol diethylether. Fifty microliters of the lower phase was diluted with 150 µL of methanol and 50 µL of isopropanol containing the same concentration of ethylene glycol diethylether. The concentrations of isobutanol in both phases were determined using a calibrated GC. The partition coefficients measured were as follows: 3.81 for (1), 4.31 for (2), and 3.58 for (3).

[0261] Fatty amide/fatty acid aqueous ammonia preparation (1), and a preparation (1a) constituted by preparation (1) mixed 1:1 with pure corn oil fatty acid (equivalent to 1:2 fatty amide:fatty acid) were incubated in shake flasks with fermentation broth containing the *Saccharomyces* butanologen NGCI-070 at a ratio of 3 parts broth to 1 part amide/acid mixture. Preparation (1) was a soft solid, while preparation (1a) was a liquid at 30ºC. Starting at a glucose concentration of 8.35 g/L, the shake flasks were then incubated for 25 h on an incubator shaker and the consumption of glucose followed as a function of time. Table 24 indicates that the fatty amide/fatty acid mixtures at both ratios were not toxic to the butanologen and even showed higher rates of glucose uptake than with oleyl alcohol.

Table 24

Flask	Glucose conc. (g/L)		
	Time = 0	18hrs	25hrs
Oleyl Alcohol	8.35	4.26	0
Oleyl Alcohol	8.35	4.46	0
2:1 Synthesized Fatty Amide:Fatty Acid Mix (Preparation (1))	8.35	3.06	0
2:1 Synthesized Fatty Amide:Fatty Acid Mix (Preparation (1))	8.35	3.22	0
1:1 Synthesized Fatty Amide Fatty Acid Mix:Pure Fatty Acids (Preparation (1a))	8.35	2.73	0
1:1 Synthesized Fatty Amide Fatty Acid Mix:Pure Fatty Acids (Preparation (1a))	8.35	2.73	0

Example 35

Fatty Alcohols from Corn Oil

[0262] With reference to the reaction of Equation IV above for producing fatty alcohols from corn oil, a 22L, round-bottom flask equipped with a mechanical stirrer, reflux condenser with N_2 source, addition funnel, internal thermocouple, and rubber septum was flame-dried under nitrogen. The flask was charged with 132 g (3.30 moles) of 95% lithium aluminum hydride powder that is weighed out in a dry box and loaded into a solids addition funnel. The 22L flask was cooled with an ice bath, and 9.0 liters of anhydrous THF were added into the reactor via a cannula. The resulting slurry was cooled to 0-5°C and a solution of 956 g (1.10 moles) of Wesson® corn oil in 1.00 liter of anhydrous THF was added dropwise over 2-3 hours while holding the reaction temperature at 5-20°C. After adding the corn oil, the slurry was stirred overnight at ambient temperature. When the reaction was done, as verified by TLC chromatography, it was quenched by the dropwise addition of a solution of 130 g of water dissolved in 370 mL of THF. Then 130 g of 15% aqueous NaOH solution was added followed by the addition of 400 g of water. The mixture was vigorously stirred while warming to room temperature and produced a white granular solid. The solids were filtered off using a fritted-glass filter funnel and washed with additional THF. The THF was removed on a rotary evaporator and the residue was taken up in 3.00 liters of ethyl acetate. The product solution was washed with 2x 1.00 L of water, 1 x 1.00 L of brine, dried over Na_2SO_4, filtered, and concentrated *in vacuo* to give 836 g (97%) of fatty alcohols as yellow oil. The crude fatty alcohol mixture was then distilled (140°C/1mmHg), and used in the following partition coefficients experiments.

Partition coefficient experiments

[0263] To each of the five 5-mL vials were added 1 mL of fatty alcohol mixture, and 1 mL of 3 wt% *i*-BuOH water solution. The biphasic mixture was vigorously stirred using Vortex Genie® for 10, 20, 30, 40, and 60 minutes, respectively. Upon mixing, the separation of layers was aided by centrifuging the mixture using Fisher Scientific Centrific 228 centrifuge (3300 rpm) for 10 minutes. 0.100 mL of both layers were taken. The organic, upper layer was diluted to 1.00 mL with toluene solution of ethylene glycol diethylether, and the water layer was diluted to 1.00 mL with methanol solution of ethylene glycol diethylether. The concentrations of *i*-BuOH in both phases were measured using a calibrated GC. The partition coefficient thus measured was 2.70.

[0264] The same partition coefficient measurement, as described above was run for 6 wt% *i*-BuOH concentration. The partition coefficient thus measured was 3.06.

[0265] While various embodiments of the present invention have been described above, it should be understood that they have been presented by way of example only, and not limitation. It will be apparent to persons skilled in the relevant art that various changes in form and detail can be made therein without departing from the spirit and scope of the invention. Thus, the breadth and scope of the present invention should not be limited by any of the above-described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents.

[0266] All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

[0267] Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component or group thereof.

[0268] Further, any prior art reference or statement provided in the specification is not to be taken as an admission that such art constitutes, or is to be understood as constituting, part of the common general knowledge.

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The Claims defining the invention are as follows:

1. A method comprising:
contacting biomass comprising water, fermentable carbon source, and oil with one or more catalyst whereby at least a portion of the oil is hydrolyzed by one or more catalyst to form an extractant, wherein the fermentable carbon source and the oil are both derived from the biomass.

2. The method of claim 1, further comprising:
contacting the biomass with a fermentation broth in a fermentation vessel;
fermenting the carbon source of the biomass to produce a product alcohol; and removing *in situ* the product alcohol from the fermentation broth by contacting the broth with the extractant.

3. A method for producing an alcohol comprising:
(a) providing biomass comprising water, fermentable carbon source, and oil;
(b) liquefying the biomass to produce a liquefied biomass;
(c) contacting the liquefied biomass with one or more catalysts whereby at least a portion of the oil is hydrolyzed to form an extractant;
(d) contacting the liquefied biomass with a saccharification enzyme capable of converting oligosaccharides into fermentable sugar;
(e) contacting the liquefied biomass with a fermentation broth in a fermentation vessel;
(f) fermenting the carbon source of the liquefied biomass to produce a product alcohol;
(g) removing *in situ* the product alcohol from the fermentation broth by contacting the broth with the extractant;
and optionally steps (c) and (d) occur concurrently

4. A composition comprising:
(a) a recombinant microorganism capable of producing an alcohol;
(b) fermentable carbon source;

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(c) one or more catalysts capable of hydrolyzing glycerides into fatty acids;
(d) oil comprising glycerides; and
(e) fatty acids.

5. The composition of claim 4, wherein the one or more catalysts is selected from esterase, lipase, phospholipase, and lysophospholipase.

6. The composition of claim 4 or claim 5, wherein the oil is corn, tallow, canola, capric/caprylic triglycerides, castor, coconut, cottonseed, fish, jojoba, lard, linseed, neetsfoot, oiticica, palm, peanut, rapeseed, rice, safflower, soya, sunflower, tung, jatropha and vegetable oil blends.

7. The composition of claim 4, wherein the fermentable carbon source and the oil are derived from biomass.

8. The composition of claim 7, wherein the biomass comprises corn grain, corn cobs, crop residues, corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, sugar cane, soy, grains, cellulosic material, lignocellulosic material, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

9. The composition of any one of Claims 4 to 8, further comprising a saccharification enzyme.

10. The composition of any one of Claims 4 to 9, further comprising undissolved solids.

11. The composition of any one of Claims 4 to 10, further comprising at least one or more of monoglycerides, diglycerides, triglycerides, glycerol, monosaccharides, oligosaccharides, or alcohol.

12. The composition of any one of Claims 4 to 11, wherein the alcohol is butanol.

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13. The method of claim 1 or 3, wherein the biomass comprises corn grain, corn cobs, crop residues, corn husks, corn stover, grasses, wheat, rye, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, sugar cane, soy, grains, cellulosic material, lignocellulosic material, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers, animal manure, and mixtures thereof.

14. The method of claim 1 or 3, wherein the oil comprises glycerides and wherein the one or more catalysts hydrolyze the glycerides to form fatty acids.

15. The method of claim 1 or 3, wherein the extractant comprises fatty acids, fatty amides, fatty alcohols, fatty esters, triglycerides, or mixtures thereof.

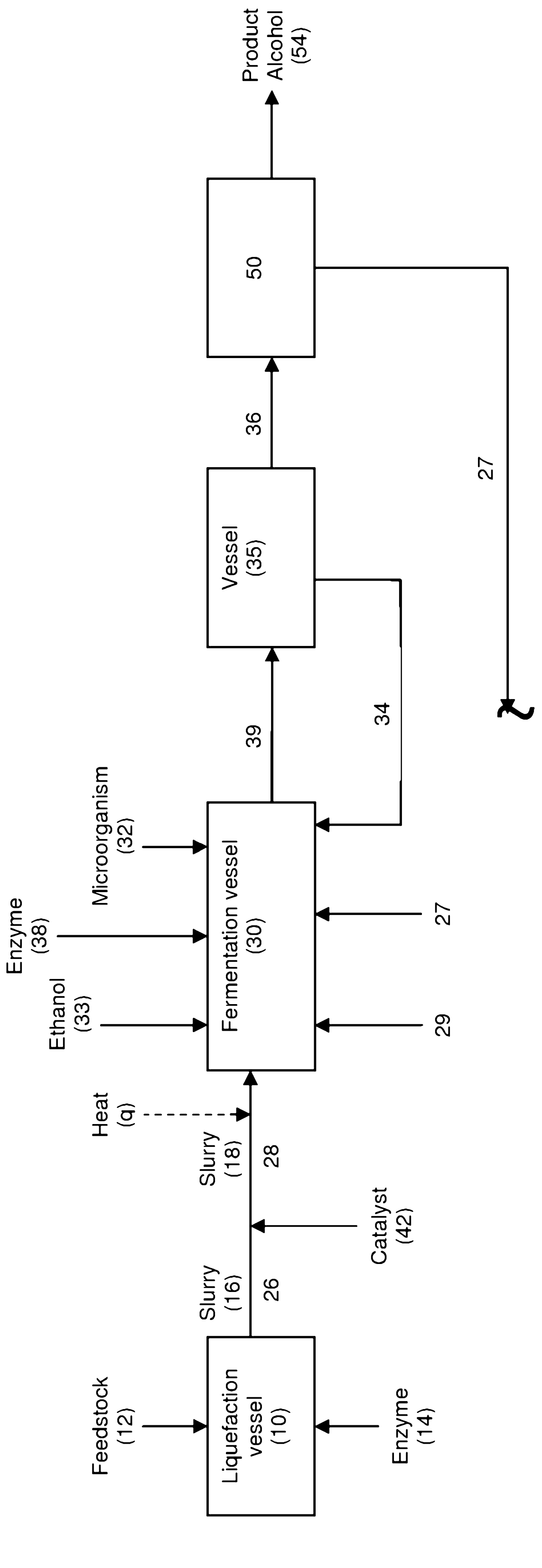
16. The method of claim 1 or 3, wherein the one or more catalysts is selected from esterase, lipase, phospholipase, and lysophospholipase.

17. The method of claim 1 or 3, wherein a partition coefficient of the extractant for the product alcohol is greater than a partition coefficient of the oil of the biomass for the product alcohol.

18. The method of claim 1 or 3, further comprising:
inactivating the catalyst after at least a portion of the oil is hydrolyzed.

19. The method of claim 1 or 3, further comprising:
separating the oil from the biomass prior to hydrolysis by one or more catalyst.

20. The method of claim 2 or 3, wherein the product alcohol is butanol.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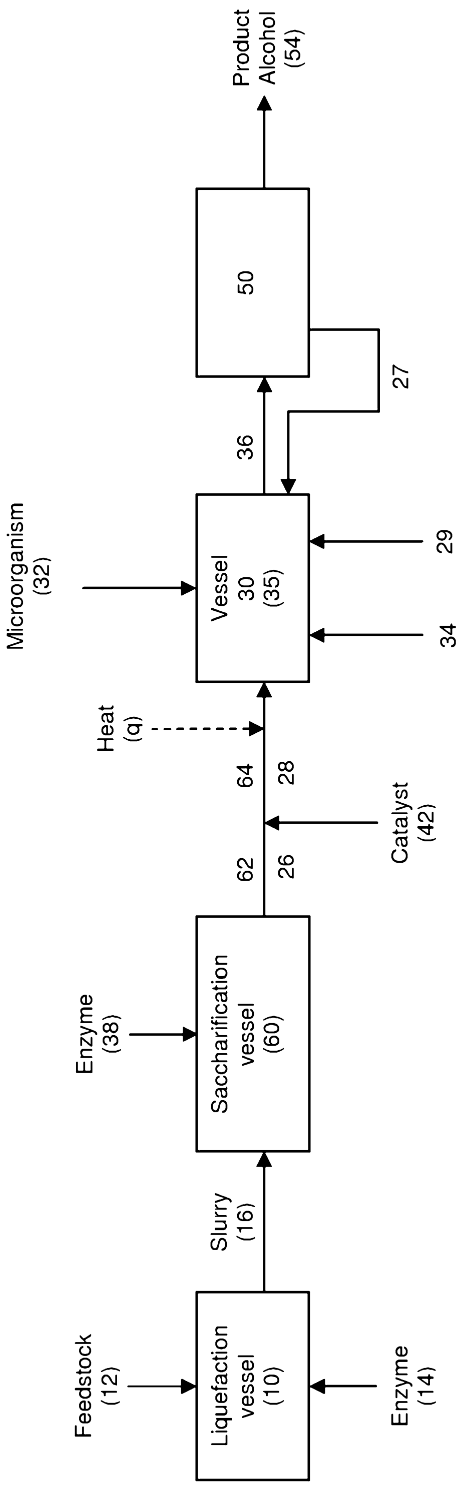
Product Alcohol (54)

FIG. 1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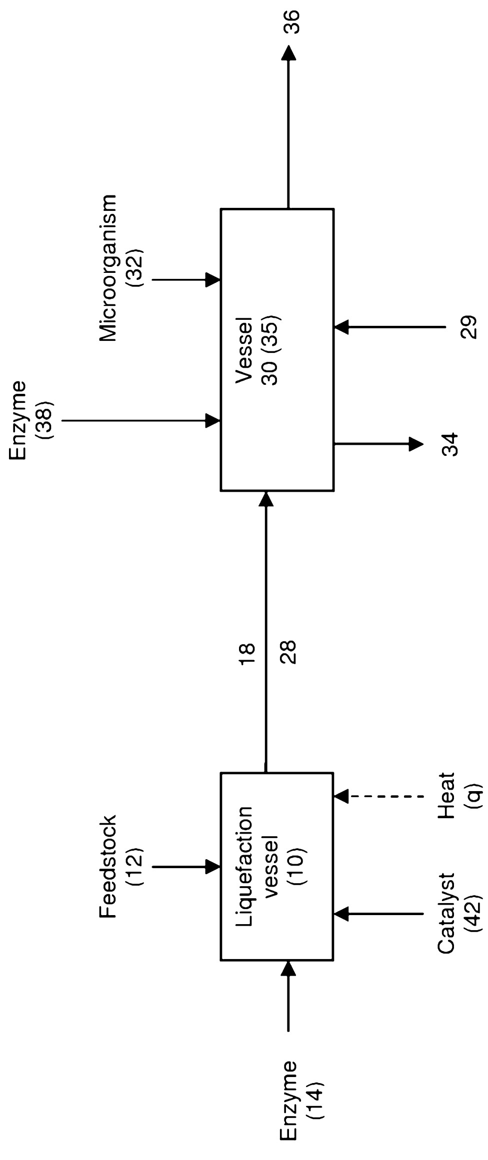
Product Alcohol (54)

FIG. 2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34
36

FIG. 3

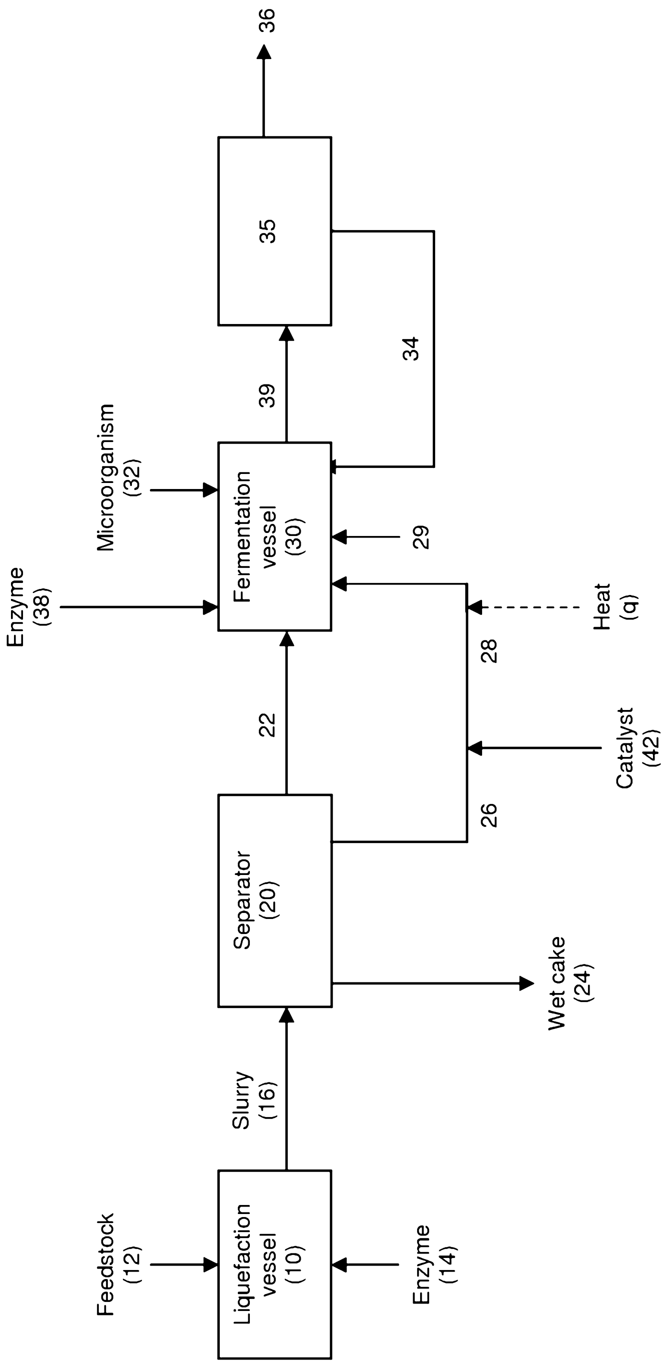
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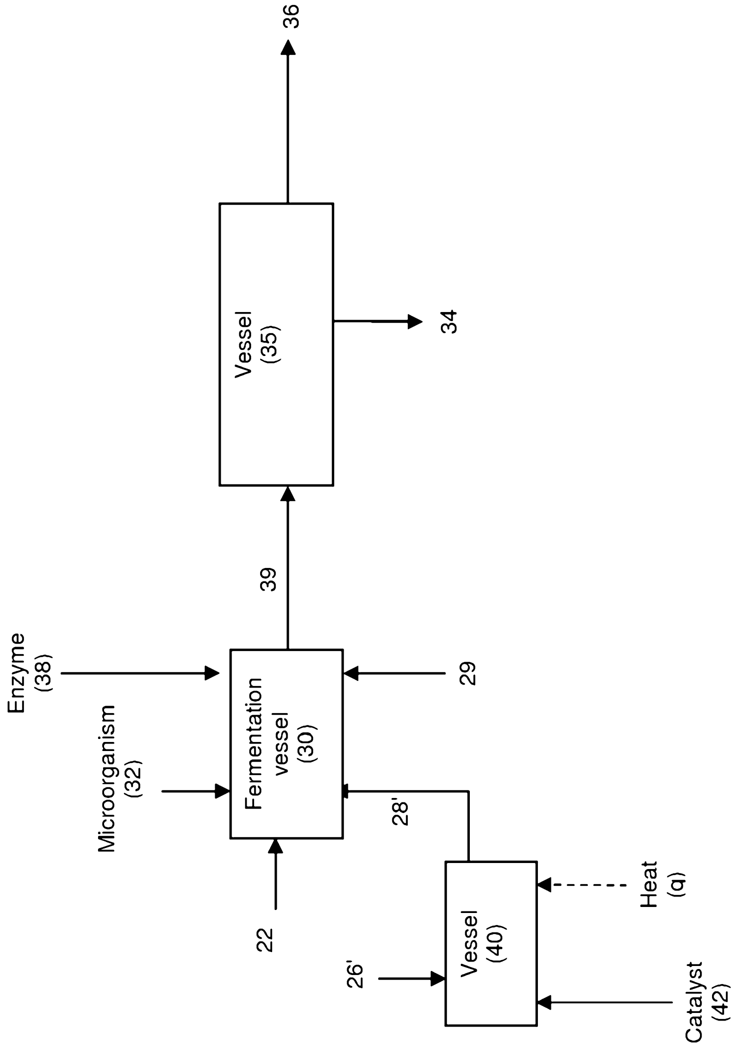
FIG. 4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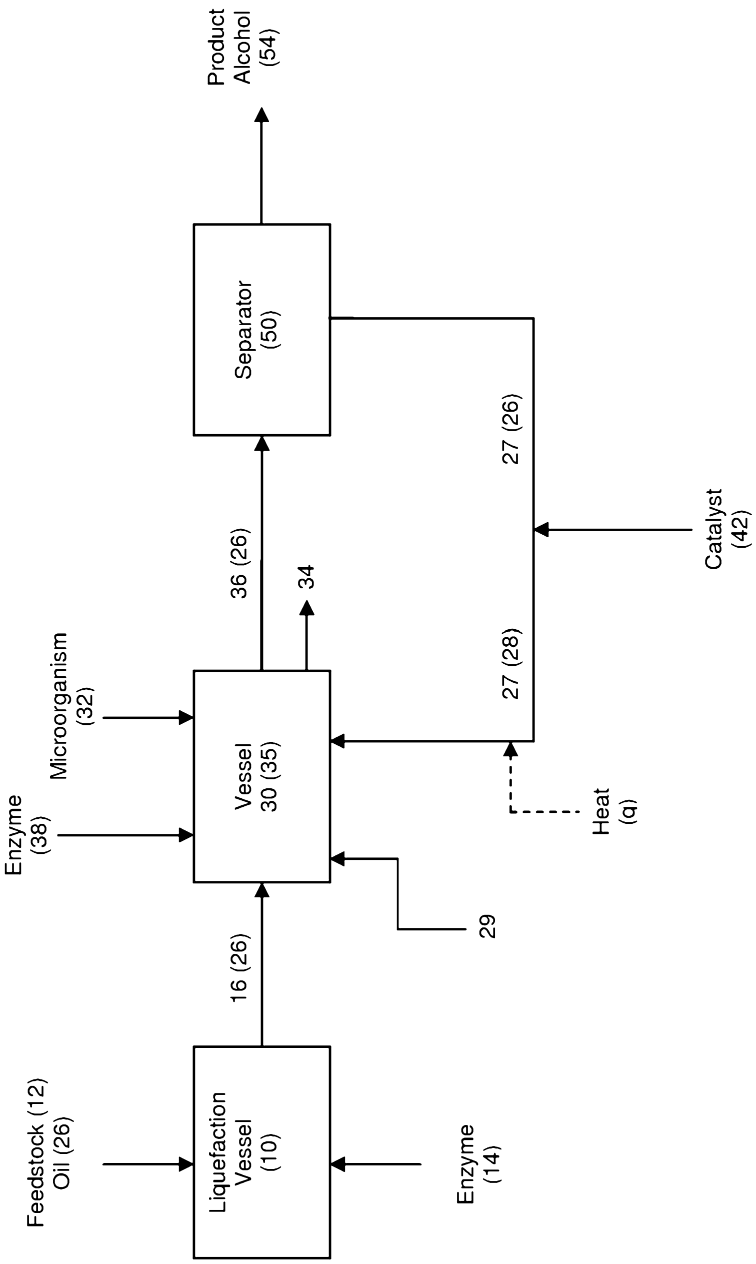
Catalyst (42)

FIG. 5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Vessel
30 (35)
29
Heat
(q)
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36 (26)
Catalyst
(42)
27 (26)
Separator
(50)
Product
Alcohol
(54)

FIG. 6

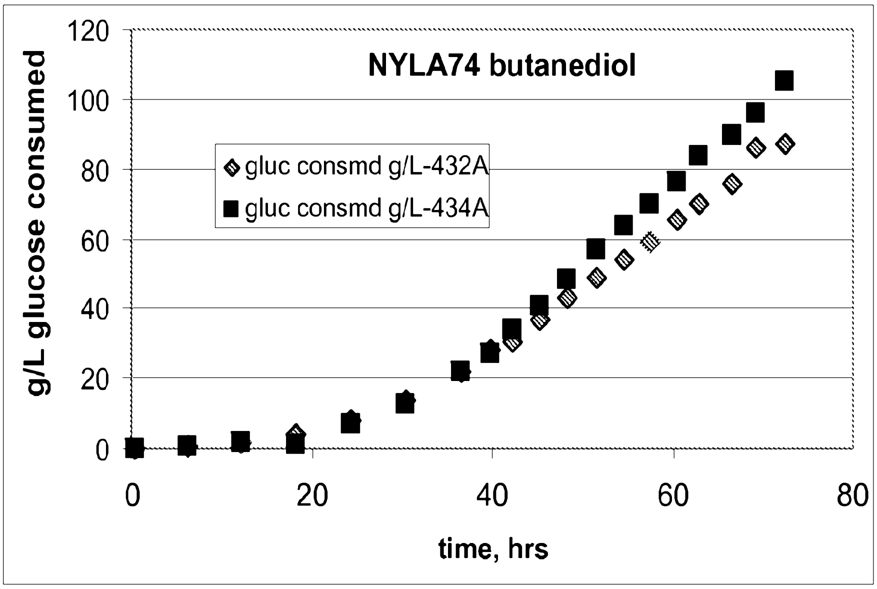
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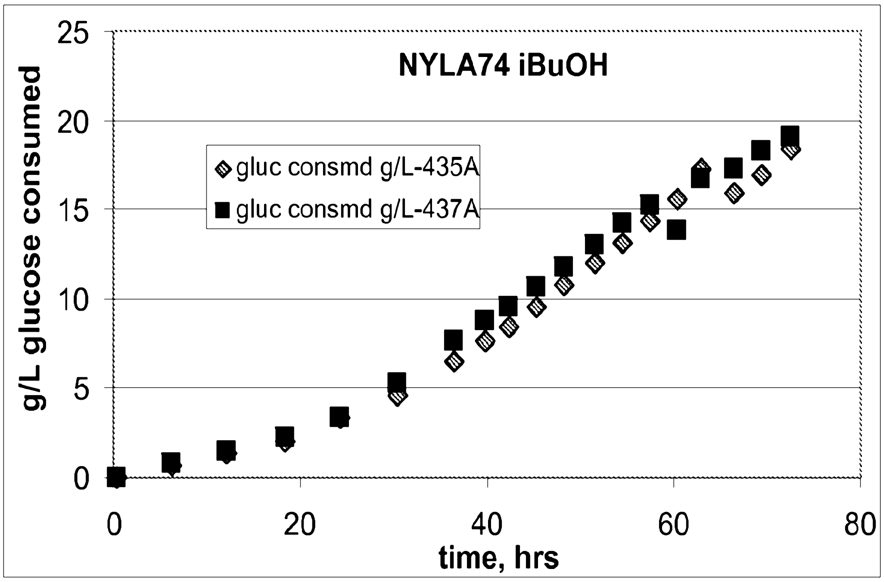
FIG. 7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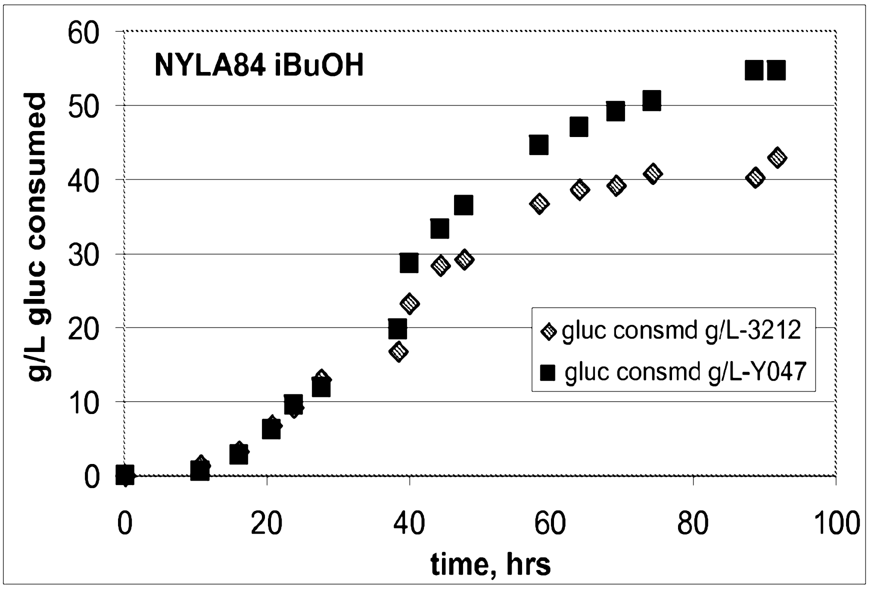
20
40
60
80

FIG. 8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gluc consmd g/L-Y047

FIG. 9

SEQUENCE LISTING

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<151> 2010-07-28

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<212> DNA
<213> Artificial sequence

<220>
<223> Template

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tcgtataatg tatgctatac gaacggtacc agtgatgata caacgagtta gccaaggtg 1559

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<212> DNA
<213> Achromobacter xylosoxidans

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gcggtgggca taccggcgac ttgggacatc tgccaggaga tcgtcaagcc cggcgcgcac   780

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gtcgcctcga ccgacaagct tccgttgaag aagatgatta cccatcgctt cgagctggcc   960

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atcctctcga acgcaggcgc tgcctga   1047

<210> 70
<211> 348
<212> PRT
<213> Achromobacter xylosoxidans

<400> 70

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        35   40   45

Asn Pro Glu Val Ala Asp Gly Arg Ile Leu Gly His Glu Gly Val Gly
    50   55   60

Val Ile Glu Glu Val Gly Glu Ser Val Thr Gln Phe Lys Lys Gly Asp
65   70   75   80

Lys Val Leu Ile Ser Cys Val Thr Ser Cys Gly Ser Cys Asp Tyr Cys
                85   90   95

```
Lys Lys Gln Leu Tyr Ser His Cys Arg Asp Gly Gly Trp Ile Leu Gly
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Tyr Met Ile Asp Gly Val Gln Ala Glu Tyr Val Arg Ile Pro His Ala
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Asp Asn Ser Leu Tyr Lys Ile Pro Gln Thr Ile Asp Asp Glu Ile Ala
    130                 135                 140

Val Leu Leu Ser Asp Ile Leu Pro Thr Gly His Glu Ile Gly Val Gln
145                 150                 155                 160

Tyr Gly Asn Val Gln Pro Gly Asp Ala Val Ala Ile Val Gly Ala Gly
                165                 170                 175

Pro Val Gly Met Ser Val Leu Leu Thr Ala Gln Phe Tyr Ser Pro Ser
            180                 185                 190

Thr Ile Ile Val Ile Asp Met Asp Glu Asn Arg Leu Gln Leu Ala Lys
        195                 200                 205

Glu Leu Gly Ala Thr His Thr Ile Asn Ser Gly Thr Glu Asn Val Val
    210                 215                 220

Glu Ala Val His Arg Ile Ala Ala Glu Gly Val Asp Val Ala Ile Glu
225                 230                 235                 240

Ala Val Gly Ile Pro Ala Thr Trp Asp Ile Cys Gln Glu Ile Val Lys
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Pro Gly Ala His Ile Ala Asn Val Gly Val His Gly Val Lys Val Asp
            260                 265                 270

Phe Glu Ile Gln Lys Leu Trp Ile Lys Asn Leu Thr Ile Thr Thr Gly
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Leu Val Asn Thr Asn Thr Thr Pro Met Leu Met Lys Val Ala Ser Thr
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Asp Lys Leu Pro Leu Lys Lys Met Ile Thr His Arg Phe Glu Leu Ala
305                 310                 315                 320

Glu Ile Glu His Ala Tyr Gln Val Phe Leu Asn Gly Ala Lys Glu Lys
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<213> artificial sequence

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atgaaatggg tgggaaatgc taatgagtta aatgcctcct atatggccga cgggtacgca   180

agaacgaaaa aggctgcggc attcttgact acatttggtg ttggcgaatt atccgcagtt   240

aatggcttag cgggctccta tgctgagaac ctgcctgttg ttgagatcgt gggatctcct   300

acctcgaaag tgcagaacga aggtaagttt gttcaccata cgttggctga tggtgatttc   360

aagcacttta tgaagatgca cgaaccggtt actgctgcca ggactttatt gacagccgag   420

aatgcaactg ttgaaattga tagagtgttg tctgccttac taaaggaaag aaagccggtt   480

tacatcaatt tacctgtaga tgtagctgcc gctaaggctg aaaaaccatc cttgcctctt   540

aagaaggaaa attccacgtc gaatacatct gatcaagaga ttctgaacaa aatacaggaa   600

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acagaaaatg aattcgtatc cgtgatgaaa gaagctcaag cagatccaaa taggatgtat   1560

tggatagaac ttattctagc aaaggagggt gcacctaaag ttttgaaaaa gatgggtaag   1620

ttatttgcag aacaaaacaa gagc   1644

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<211> 753
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<213> Saccharomyces cerevisiae

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aactctttcc tgtaggtcag gttgctttct caggtatagc atgaggtcgc tcttattgac   180

cacacctcta ccggcatgcc gagcaaatgc ctgcaaatcg ctccccattt cacccaattg   240

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ggacaacacc tgtggt   316

<210> 74
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<212> DNA
<213> artificial sequence

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<223> primer

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<210> 75
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gcgtccaggg cgtcaaagat caggcagc   28

<210> 76
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<220>
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<220>
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<210> 94
<211> 26
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<220>
<223> primer

<400> 94
ccaggccaat tcaacagact gtcggc   26

<210> 95
<211> 2347
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<220>
<223> constructed URA3 marker with flanking homologous repeat sequences
       for HIS gene replacement and marker excision

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gatgcacgca ccgaccgaac gtctggatga agtcatccag tgatgataca acgagttagc 2340

caaggtg 2347

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<220>
<223> primer

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ttacgtattc taatgttcag 80

<210> 97
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<212> DNA
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<220>
<223> primer

<400> 97
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aactcgttgt atcatcactg g 81

<210> 98
<211> 26
<212> DNA
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<220>
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<400> 98
gacttgaata atgcagcggc gcttgc 26

<210> 99
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<220>
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<400> 99
ccaccctctt caattagcta agatcatagc 30

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<220>
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<400> 100
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<210> 101
<211> 20
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<400> 101
ctgcagcgag gagccgtaat 20

<210> 102
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gcattgcgga ttacgtattc taatgttcag 90

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<210> 105
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ccaattccgt gatgtctctt tgttgc   26

<210> 106
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<220>
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<400> 107
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<210> 108
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<220>
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<400> 108

ggatccgcat gcttgcattt agtcgtgc 28

<210> 109
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<220>
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<400> 109
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<210> 110
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<212> DNA
<213> Saccharomyces cerevisiae

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tctccacccct tgcacatgtc ttcagattcg tccaagatca agaggaagcg taacagaatt 180

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tcaaagtatg actgacaaaa aaactcttaa agacttaag 99

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<220>
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aatatatttc tccatac 77

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<211> 571
<212> PRT
<213> Streptococcus mutans

<400> 116

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Gly Met Gln Asp Glu Asp Phe Glu Lys Pro Ile Val Gly Val Ile Ser
35 40 45

Thr Trp Ala Glu Asn Thr Pro Cys Asn Ile His Leu His Asp Phe Gly
50 55 60

Lys Leu Ala Lys Val Gly Val Lys Glu Ala Gly Ala Trp Pro Val Gln
65 70 75 80

Phe Gly Thr Ile Thr Val Ser Asp Gly Ile Ala Met Gly Thr Gln Gly
85 90 95

Met Arg Phe Ser Leu Thr Ser Arg Asp Ile Ile Ala Asp Ser Ile Glu
100 105 110

Ala Ala Met Gly Gly His Asn Ala Asp Ala Phe Val Ala Ile Gly Gly
115 120 125

Cys Asp Lys Asn Met Pro Gly Ser Val Ile Ala Met Ala Asn Met Asp
130 135 140

Ile Pro Ala Ile Phe Ala Tyr Gly Gly Thr Ile Ala Pro Gly Asn Leu
145 150 155 160

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Asp Gly Lys Asp Ile Asp Leu Val Ser Val Phe Glu Gly Val Gly His
                165                 170                 175

Trp Asn His Gly Asp Met Thr Lys Glu Glu Val Lys Ala Leu Glu Cys
            180                 185                 190

Asn Ala Cys Pro Gly Pro Gly Gly Cys Gly Gly Met Tyr Thr Ala Asn
        195                 200                 205

Thr Met Ala Thr Ala Ile Glu Val Leu Gly Leu Ser Leu Pro Gly Ser
    210                 215                 220

Ser Ser His Pro Ala Glu Ser Ala Glu Lys Lys Ala Asp Ile Glu Glu
225                 230                 235                 240

Ala Gly Arg Ala Val Val Lys Met Leu Glu Met Gly Leu Lys Pro Ser
                245                 250                 255

Asp Ile Leu Thr Arg Glu Ala Phe Glu Asp Ala Ile Thr Val Thr Met
            260                 265                 270

Ala Leu Gly Gly Ser Thr Asn Ser Thr Leu His Leu Leu Ala Ile Ala
        275                 280                 285

His Ala Ala Asn Val Glu Leu Thr Leu Asp Asp Phe Asn Thr Phe Gln
    290                 295                 300

Glu Lys Val Pro His Leu Ala Asp Leu Lys Pro Ser Gly Gln Tyr Val
305                 310                 315                 320

Phe Gln Asp Leu Tyr Lys Val Gly Gly Val Pro Ala Val Met Lys Tyr
                325                 330                 335

Leu Leu Lys Asn Gly Phe Leu His Gly Asp Arg Ile Thr Cys Thr Gly
            340                 345                 350

Lys Thr Val Ala Glu Asn Leu Lys Ala Phe Asp Asp Leu Thr Pro Gly
        355                 360                 365

Gln Lys Val Ile Met Pro Leu Glu Asn Pro Lys Arg Glu Asp Gly Pro
    370                 375                 380

Leu Ile Ile Leu His Gly Asn Leu Ala Pro Asp Gly Ala Val Ala Lys
385                 390                 395                 400

Val Ser Gly Val Lys Val Arg Arg His Val Gly Pro Ala Lys Val Phe
                405                 410                 415

Asn Ser Glu Glu Glu Ala Ile Glu Ala Val Leu Asn Asp Asp Ile Val
            420                 425                 430
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Asp Gly Asp Val Val Val Val Arg Phe Val Gly Pro Lys Gly Gly Pro
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Gly Met Pro Glu Met Leu Ser Leu Ser Ser Met Ile Val Gly Lys Gly
    450   455   460

Gln Gly Glu Lys Val Ala Leu Leu Thr Asp Gly Arg Phe Ser Gly Gly
465   470   475   480

Thr Tyr Gly Leu Val Val Gly His Ile Ala Pro Glu Ala Gln Asp Gly
                485   490   495

Gly Pro Ile Ala Tyr Leu Gln Thr Gly Asp Ile Val Thr Ile Asp Gln
            500   505   510

Asp Thr Lys Glu Leu His Phe Asp Ile Ser Asp Glu Glu Leu Lys His
        515   520   525

Arg Gln Glu Thr Ile Glu Leu Pro Pro Leu Tyr Ser Arg Gly Ile Leu
    530   535   540

Gly Lys Tyr Ala His Ile Val Ser Ser Ala Ser Arg Gly Ala Val Thr
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Asp Phe Trp Lys Pro Glu Glu Thr Gly Lys Lys
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<210> 117
<211> 548
<212> PRT
<213> Lactococcus lactis

<400> 117

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Asp Gln Ile Ile Ser His Lys Asp Met Lys Trp Val Gly Asn Ala Asn
        35   40   45

Glu Leu Asn Ala Ser Tyr Met Ala Asp Gly Tyr Ala Arg Thr Lys Lys
    50   55   60

Ala Ala Ala Phe Leu Thr Thr Phe Gly Val Gly Glu Leu Ser Ala Val
65   70   75   80

Asn Gly Leu Ala Gly Ser Tyr Ala Glu Asn Leu Pro Val Val Glu Ile
                85   90   95

Val Gly Ser Pro Thr Ser Lys Val Gln Asn Glu Gly Lys Phe Val His

100 105 110

His Thr Leu Ala Asp Gly Asp Phe Lys His Phe Met Lys Met His Glu
115 120 125

Pro Val Thr Ala Ala Arg Thr Leu Leu Thr Ala Glu Asn Ala Thr Val
130 135 140

Glu Ile Asp Arg Val Leu Ser Ala Leu Leu Lys Glu Arg Lys Pro Val
145 150 155 160

Tyr Ile Asn Leu Pro Val Asp Val Ala Ala Ala Lys Ala Glu Lys Pro
165 170 175

Ser Leu Pro Leu Lys Lys Glu Asn Ser Thr Ser Asn Thr Ser Asp Gln
180 185 190

Glu Ile Leu Asn Lys Ile Gln Glu Ser Leu Lys Asn Ala Lys Lys Pro
195 200 205

Ile Val Ile Thr Gly His Glu Ile Ile Ser Phe Gly Leu Glu Lys Thr
210 215 220

Val Thr Gln Phe Ile Ser Lys Thr Lys Leu Pro Ile Thr Thr Leu Asn
225 230 235 240

Phe Gly Lys Ser Ser Val Asp Glu Ala Leu Pro Ser Phe Leu Gly Ile
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Tyr Asn Gly Thr Leu Ser Glu Pro Asn Leu Lys Glu Phe Val Glu Ser
260 265 270

Ala Asp Phe Ile Leu Met Leu Gly Val Lys Leu Thr Asp Ser Ser Thr
275 280 285

Gly Ala Phe Thr His His Leu Asn Glu Asn Lys Met Ile Ser Leu Asn
290 295 300

Ile Asp Glu Gly Lys Ile Phe Asn Glu Arg Ile Gln Asn Phe Asp Phe
305 310 315 320

Glu Ser Leu Ile Ser Ser Leu Leu Asp Leu Ser Glu Ile Glu Tyr Lys
325 330 335

Gly Lys Tyr Ile Asp Lys Lys Gln Glu Asp Phe Val Pro Ser Asn Ala
340 345 350

Leu Leu Ser Gln Asp Arg Leu Trp Gln Ala Val Glu Asn Leu Thr Gln
355 360 365

Ser Asn Glu Thr Ile Val Ala Glu Gln Gly Thr Ser Phe Phe Gly Ala

370 375 380

Ser Ser Ile Phe Leu Lys Ser Lys Ser His Phe Ile Gly Gln Pro Leu
385 390 395 400

Trp Gly Ser Ile Gly Tyr Thr Phe Pro Ala Ala Leu Gly Ser Gln Ile
405 410 415

Ala Asp Lys Glu Ser Arg His Leu Leu Phe Ile Gly Asp Gly Ser Leu
420 425 430

Gln Leu Thr Val Gln Glu Leu Gly Leu Ala Ile Arg Glu Lys Ile Asn
435 440 445

Pro Ile Cys Phe Ile Ile Asn Asn Asp Gly Tyr Thr Val Glu Arg Glu
450 455 460

Ile His Gly Pro Asn Gln Ser Tyr Asn Asp Ile Pro Met Trp Asn Tyr
465 470 475 480

Ser Lys Leu Pro Glu Ser Phe Gly Ala Thr Glu Asp Arg Val Val Ser
485 490 495

Lys Ile Val Arg Thr Glu Asn Glu Phe Val Ser Val Met Lys Glu Ala
500 505 510

Gln Ala Asp Pro Asn Arg Met Tyr Trp Ile Glu Leu Ile Leu Ala Lys
515 520 525

Glu Gly Ala Pro Lys Val Leu Lys Lys Met Gly Lys Leu Phe Ala Glu
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Gln Asn Lys Ser
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<211> 1125
<212> DNA
<213> artificial sequence

<220>
<223> horse ADH coding region codon optimized for S. cerevisiae expression

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atggttgcca ccggaatctg tagatccgac gaccatgtgg tgagtggcac tctagttact 180

cctttgccag taatcgcggg acacgaggct gccggaatcg ttgaatccat aggtgaaggt 240

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atcgatgcag cttcaccact ggaaaaagtt tgcttgatag ggtgcggatt ttccaccggt 540

tacggttccg cagttaaagt tgcaaaggtt acacagggtt cgacttgtgc agtattcggt 600

ttaggaggag taggactaag cgttattatg ggtgtaaag ctgcaggcgc agcgaggatt 660

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tgtgttaacc ctcaagatta taagaaacca atacaagaag tccttactga aatgtcaaac 780

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aatttgtcga tgaatcctat gctgttgcta agcggtcgta catggaaggg agctatattt 960

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<211> 375
<212> PRT
<213> Equus caballus

<400> 119

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Lys Ala His Glu Val Arg Ile Lys Met Val Ala Thr Gly Ile Cys Arg
35 40 45

Ser Asp Asp His Val Val Ser Gly Thr Leu Val Thr Pro Leu Pro Val
50 55 60

Ile Ala Gly His Glu Ala Ala Gly Ile Val Glu Ser Ile Gly Glu Gly
65 70 75 80

Val Thr Thr Val Arg Pro Gly Asp Lys Val Ile Pro Leu Phe Thr Pro
85 90 95

Gln Cys Gly Lys Cys Arg Val Cys Lys His Pro Glu Gly Asn Phe Cys
100 105 110

Leu Lys Asn Asp Leu Ser Met Pro Arg Gly Thr Met Gln Asp Gly Thr
115 120 125

Ser Arg Phe Thr Cys Arg Gly Lys Pro Ile His His Phe Leu Gly Thr

130   135   140

Ser Thr Phe Ser Gln Tyr Thr Val Val Asp Glu Ile Ser Val Ala Lys
145   150   155   160

Ile Asp Ala Ala Ser Pro Leu Glu Lys Val Cys Leu Ile Gly Cys Gly
                165   170   175

Phe Ser Thr Gly Tyr Gly Ser Ala Val Lys Val Ala Lys Val Thr Gln
            180   185   190

Gly Ser Thr Cys Ala Val Phe Gly Leu Gly Gly Val Gly Leu Ser Val
        195   200   205

Ile Met Gly Cys Lys Ala Ala Gly Ala Ala Arg Ile Ile Gly Val Asp
    210   215   220

Ile Asn Lys Asp Lys Phe Ala Lys Ala Lys Glu Val Gly Ala Thr Glu
225   230   235   240

Cys Val Asn Pro Gln Asp Tyr Lys Lys Pro Ile Gln Glu Val Leu Thr
                245   250   255

Glu Met Ser Asn Gly Gly Val Asp Phe Ser Phe Glu Val Ile Gly Arg
            260   265   270

Leu Asp Thr Met Val Thr Ala Leu Ser Cys Cys Gln Glu Ala Tyr Gly
        275   280   285

Val Ser Val Ile Val Gly Val Pro Pro Asp Ser Gln Asn Leu Ser Met
    290   295   300

Asn Pro Met Leu Leu Leu Ser Gly Arg Thr Trp Lys Gly Ala Ile Phe
305   310   315   320

Gly Gly Phe Lys Ser Lys Asp Ser Val Pro Lys Leu Val Ala Asp Phe
                325   330   335

Met Ala Lys Lys Phe Ala Leu Asp Pro Leu Ile Thr His Val Leu Pro
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Phe Glu Lys Ile Asn Glu Gly Phe Asp Leu Leu Arg Ser Gly Glu Ser
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Ile Arg Thr Ile Leu Thr Phe
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aaaatctcaa aaatgtgtgg gtcattacgt aaataatgat aggaatggga ttcttctatt   180

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aatggaaaag catgagctta gcgttgctcc aaaaaagtat tggatggtta ataccatttg   360

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accataccac agcttttcaa ttcaattcat cattttttt ttattctttt ttttgatttc   240

ggtttctttg aaattttttt gattcggtaa tctccgaaca gaaggaagaa cgaaggaagg   300

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caacgaatcg tatatactaa catgcgcgcg cttcctatat acacatatac atatatatat 540

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<213> Saccharomyces cerevisiae

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<213> Bacillus subtilis

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<211> 571
<212> PRT
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<400> 138

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35 40 45

Gln Asp Lys Gly Pro Glu Ile Ile Val Ala Arg His Glu Gln Asn Ala
50 55 60

Ala Phe Met Ala Gln Ala Val Gly Arg Leu Thr Gly Lys Pro Gly Val
65 70 75 80

Val Leu Val Thr Ser Gly Pro Gly Ala Ser Asn Leu Ala Thr Gly Leu
85 90 95

Leu Thr Ala Asn Thr Glu Gly Asp Pro Val Val Ala Leu Ala Gly Asn
100 105 110

Val Ile Arg Ala Asp Arg Leu Lys Arg Thr His Gln Ser Leu Asp Asn
115 120 125

Ala Ala Leu Phe Gln Pro Ile Thr Lys Tyr Ser Val Glu Val Gln Asp
130 135 140

Val Lys Asn Ile Pro Glu Ala Val Thr Asn Ala Phe Arg Ile Ala Ser
145 150 155 160

Ala Gly Gln Ala Gly Ala Ala Phe Val Ser Phe Pro Gln Asp Val Val
165 170 175

Asn Glu Val Thr Asn Thr Lys Asn Val Arg Ala Val Ala Ala Pro Lys
180 185 190

Leu Gly Pro Ala Ala Asp Asp Ala Ile Ser Ala Ala Ile Ala Lys Ile
195 200 205

Gln Thr Ala Lys Leu Pro Val Val Leu Val Gly Met Lys Gly Gly Arg

210 215 220

Pro Glu Ala Ile Lys Ala Val Arg Lys Leu Leu Lys Lys Val Gln Leu
225 230 235 240

Pro Phe Val Glu Thr Tyr Gln Ala Ala Gly Thr Leu Ser Arg Asp Leu
245 250 255

Glu Asp Gln Tyr Phe Gly Arg Ile Gly Leu Phe Arg Asn Gln Pro Gly
260 265 270

Asp Leu Leu Leu Glu Gln Ala Asp Val Val Leu Thr Ile Gly Tyr Asp
275 280 285

Pro Ile Glu Tyr Asp Pro Lys Phe Trp Asn Ile Asn Gly Asp Arg Thr
290 295 300

Ile Ile His Leu Asp Glu Ile Ile Ala Asp Ile Asp His Ala Tyr Gln
305 310 315 320

Pro Asp Leu Glu Leu Ile Gly Asp Ile Pro Ser Thr Ile Asn His Ile
325 330 335

Glu His Asp Ala Val Lys Val Glu Phe Ala Glu Arg Glu Gln Lys Ile
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Leu Ser Asp Leu Lys Gln Tyr Met His Glu Gly Glu Gln Val Pro Ala
355 360 365

Asp Trp Lys Ser Asp Arg Ala His Pro Leu Glu Ile Val Lys Glu Leu
370 375 380

Arg Asn Ala Val Asp Asp His Val Thr Val Thr Cys Asp Ile Gly Ser
385 390 395 400

His Ala Ile Trp Met Ser Arg Tyr Phe Arg Ser Tyr Glu Pro Leu Thr
405 410 415

Leu Met Ile Ser Asn Gly Met Gln Thr Leu Gly Val Ala Leu Pro Trp
420 425 430

Ala Ile Gly Ala Ser Leu Val Lys Pro Gly Glu Lys Val Val Ser Val
435 440 445

Ser Gly Asp Gly Gly Phe Leu Phe Ser Ala Met Glu Leu Glu Thr Ala
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Val Arg Leu Lys Ala Pro Ile Val His Ile Val Trp Asn Asp Ser Thr
465 470 475 480

Tyr Asp Met Val Ala Phe Gln Gln Leu Lys Lys Tyr Asn Arg Thr Ser

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Leu Arg Gln Gly Met Asn Ala Glu Gly Pro Val Ile Ile Asp Val Pro
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