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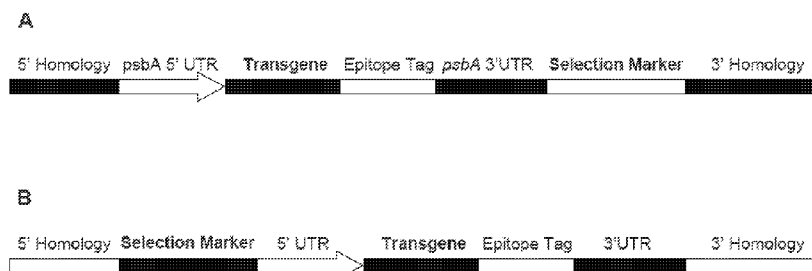
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(54) Title: ANIMAL FEEDSTOCK COMPRISING GENETICALLY MODIFIED ALGAE

Figure 1



(57) Abstract: The present disclosure provides methods for producing a photosynthetic organism, wherein the photosynthetic organism produces one or more enzymes that help degrade or digest a biomass, resulting in a more nutrient composition for use as animal feed. The invention also provides methods for producing a photosynthetic organism, wherein the photosynthetic organism produces one or more fatty acids or lipids that provide additional nutrient value. The fatty acids and lipids produced may also be used for the production of biofuel and the spent photosynthetic organisms can then be used as an animal feed. The invention also provides compositions of animal food comprising modified photosynthetic organisms.

ANIMAL FEEDSTOCK COMPRISING GENETICALLY MODIFIED ALGAE**CROSS REFERENCE TO RELATED APPLICATIONS**

5 [0001] This application claims the benefit of United States Provisional Patent Application serial number 61/197,938 filed October 31, 2008, the contents of which are incorporated by reference in its entirety.

INCORPORATION BY REFERENCE

10 [0002] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application is specifically and individually indicated to be incorporated by reference.

BACKGROUND

15 [0003] Current feed products for commercially important animals, such as cattle, sheep, swine, goats, horses and poultry, often contain ingredients that do not provide nourishment to the animal. Such ingredients in feed products are indigestible by the animal and are therefore incapable of providing nourishment. In some cases ingredients in feed products, for example excess indigestible carbohydrate or fat, can make an animal sick. In some cases ingredients in animal feed can cause disease, either directly or indirectly. Current feed products can also be expensive. The addition of various enzymes to an animal feed can help animals digest certain ingredients found in problematic feed mixtures. However, the addition of such enzymes, under current methods of production, can be expensive and can be economically prohibitive. Improved methods of providing less expensive, safer and more nutritious food to commercial farm animals is highly desired.

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SUMMARY

25 [0004] Disclosed herein are animal feedstocks comprising genetically modified non-vascular photosynthetic organisms (NVPO). The genetically modified non-vascular photosynthetic organisms may produce enzymes which degrade components of animal feedstocks and/or may add to the nutritional value of the feedstocks. Other products, for example, enzymes, isoprenoids, and fatty acids, may be extracted from the NVPOs prior to inclusion in the feedstocks disclosed herein.

30 [0005] In one instance, the present disclosure provides a composition comprising a feedstock and a genetically modified non-vascular photosynthetic organism, or a cellular component thereof, wherein the organism comprises an exogenous nucleic acid encoding a biomass-degrading enzyme or a nucleic acid resulting in increased expression of an endogenous biomass-degrading enzyme compared to the organism without the genetic modification. In some instances, the organism is at least partially depleted of a lipid, fatty acid, isoprenoid, carotenoid, carbohydrate, or selected protein prior to addition to the feedstock. A biomass-degrading enzyme may be, but is not limited to, a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, phytase, or any mixture thereof. In one embodiment, the biomass-degrading enzyme is a carbohydrase, for example, an α -amylase, β -amylase, endo- β -

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glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase. In other instances, the biomass-degrading enzyme is a protease, for example, subtilisin, bromelain, or a fungal acid-stable protease. In still other instances, the biomass-degrading enzyme is a phytase, which may be of bacterial or fungal origin. A biomass-degrading enzyme for use in other embodiments may be an enzyme other than a phytase. In some instances, the non-vascular photosynthetic organism is a non-vascular photosynthetic organism other than *Porphyridium*, for example, *Scenodesmous dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*. In some instances, the genetic modification to the organism impairs photosynthetic capability of the organism. For the purposes of this disclosure, an organism in which that photosynthetic capability has been impaired due to a genetic modification is still considered an NVPO if the organism was photosynthetic prior to the genetic modification. In other instances, the genetically modified non-vascular photosynthetic organism has a higher lipid, fatty acid, or isoprenoid content relative to an unmodified organism of the same species. Useful organisms may be prokaryotic or eukaryotic. In some instances, the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale. A genetically modified organism may further comprise an exogenous nucleic acid encoding enzyme in an isoprenoid biosynthesis pathway or a nucleic acid which results in the increased production of an endogenous isoprenoid at higher levels relative to an unmodified organism of the same species. In some instances, the isoprenoid biosynthesis pathway enzyme is an enzyme in a mevalonate pathway. In other instances, the isoprenoid biosynthesis pathway enzyme is a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase.

[0006] The present disclosure also provides a method for producing an improved feedstock composition, comprising: combining a genetically modified non-vascular photosynthetic organism or a cellular component thereof with a feedstock to generate the improved feedstock composition, wherein the organism comprises at least one exogenous nucleic acid encoding a biomass-degrading enzyme or resulting in increased expression of an endogenous biomass-degrading enzyme compared to said organism without the genetic modification. In some instances, this method additionally comprises removing a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified non-vascular photosynthetic organism prior to combining the organism with the feedstock. A biomass-degrading enzyme for use in a method disclosed herein may be one or more of a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, or phytase. In one embodiment, the biomass-degrading enzyme is a carbohydrase, for example an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase. In other embodiments, the biomass-degrading enzyme is a protease, for example subtilisin, bromelain, or a fungal acid-stable protease. In other instances, the biomass-degrading enzyme is a phytase. An organism for use in the present disclosure may further comprise at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway, for example, an enzyme in a mevalonate pathway, for example a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase. In some instances the genetically modified non-vascular photosynthetic organism is prokaryotic. In other instances, the non-vascular photosynthetic organism is *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*. In some embodiments, methods provided herein may further comprise lysing or drying a genetically modified non-vascular photosynthetic organism prior to

combining with the feedstock. The genetically modified non-vascular photosynthetic organism may be grown under heterotrophic conditions, autotrophic conditions, and/or in darkness. In some embodiments, the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

[0007] The present disclosure further provides a method for increasing the nutrient availability of a feedstock, comprising: combining a genetically modified non-vascular photosynthetic organism or a cellular component thereof with a feedstock to generate the improved feedstock composition, wherein the organism comprises at least one exogenous nucleic acid encoding a biomass-degrading enzyme or resulting in increased expression of an endogenous biomass-degrading enzyme compared to said organism without the genetic modification; and feeding the improved feedstock composition to an animal wherein the improved feedstock composition has an improved nutrient availability as compared to the feedstock prior to combining with the genetically modified non-vascular photosynthetic organism. In some instances, this method additionally comprises removing a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified non-vascular photosynthetic organism prior to combining the organism with the feedstock. A biomass-degrading enzyme for use in a method disclosed herein may be one or more of a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, or phytase. In one embodiment, the biomass-degrading enzyme is a carbohydrase, for example an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase. In other embodiments, the biomass-degrading enzyme is a protease, for example subtilisin, bromelain, or a fungal acid-stable protease. In other instances, the biomass-degrading enzyme is a phytase. An organism for use in the present disclosure may further comprise at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway, for example, an enzyme in a mevalonate pathway, for example a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase. In some instances the genetically modified non-vascular photosynthetic organism is prokaryotic while in other cases it is eukaryotic. In other instances, the non-vascular photosynthetic organism may be *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*. In some embodiments, methods provided herein may further comprise lysing or drying a genetically modified non-vascular photosynthetic organism prior to combining with the feedstock. The genetically modified non-vascular photosynthetic organism may be grown under heterotrophic conditions, autotrophic conditions, and/or in darkness. In some embodiments, the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale. The increased nutrient availability may be one or more of increased digestible protein, increased digestible carbohydrate, or increased mineral availability. The animal to which the improved feedstock composition is fed may be a ruminant or a non-ruminant. Examples of ruminant animals include, but are not limited to cattle, sheep, goats, deer, oxen, buffalo, bison and elk. Examples of non-ruminants include, pigs, horses, donkeys dogs, cats, rabbits, rodents and avian species. Avian species include domestic birds such as chickens, ducks, geese and turkeys as well as game birds such as pheasant, quail, doves, grouse and partridge. The animal may also be a pseudo ruminant,

such as members of the family camelidae. The non-ruminant animal may also be an aquatic species such as a fish or crustacean. Examples of fish, include, but are not limited to trout, salmon, tilapia, catfish, perch, bluebill or carp. Non-limiting examples of crustaceans include lobster, crabs, crayfish and shrimp.

[0008] Also provided is a method for decreasing the amount of phytic acid in animal manure comprising combining a genetically modified non-vascular photosynthetic organism or a cellular component thereof with a feedstock to generate an improved feedstock composition, wherein the genetically modified non-vascular photosynthetic organism comprises at least one exogenous nucleic acid encoding a phytase. The improved feedstock composition is fed to an animal which can result in the manure of the animal ingesting the improved feedstock composition having a lower phytic acid composition as compared to manure of the same animal or an animal of the same species not fed the improved feedstock composition. The genetically modified non-vascular photosynthetic organism can be prokaryotic or eukaryotic. In one embodiment, the genetically modified non-vascular photosynthetic organism is a cyanobacterium (blue green algae). In other embodiments the genetically modified non-vascular photosynthetic organism is a green algae. In particular embodiments, the non-vascular photosynthetic organism can be *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*. In some embodiments, methods provided herein may further comprise lysing or drying a genetically modified non-vascular photosynthetic organism prior to combining with the feedstock. The non-vascular photosynthetic organism may further comprise at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway, for example, an enzyme in a mevalonate pathway, for example a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase. In some instances, this method additionally comprises removing a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified non-vascular photosynthetic organism prior to combining the organism with the feedstock. The genetically modified non-vascular photosynthetic organism may be grown under heterotrophic conditions, autotrophic conditions, and/or in darkness. In some embodiments, the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale. The animal to which the improved feedstock composition is fed may be a ruminant or a non-ruminant. Examples of ruminant animals include, but are not limited to cattle, sheep, goats, deer, oxen, buffalo, bison and elk. Examples of non-ruminants include, pigs, horses, donkeys, dogs, cats, rabbits, rodents and avian species. Avian species include domestic birds such as chickens, ducks, geese and turkeys as well as game birds such as pheasant, quail, doves, grouse and partridge. The animal may also be a pseudo ruminant, such as members of the family camelidae. The non-ruminant animal may also be an aquatic species such as a fish or crustacean. Examples of fish, include, but are not limited to trout, salmon, tilapia, catfish, perch, bluebill or carp. Non-limiting examples of crustaceans include lobster, crabs, crayfish and shrimp.

[0009] In certain embodiments any of the improved feedstocks described herein are suitable for consumption by an animal. In particular embodiments, the improved feedstocks described herein are suitable for consumption by an animal to be used as food by humans or producing a product to be used as food by humans.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following

detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0011] Figure 1 illustrates two constructs for insertion of a gene into a chloroplast genome.

[0012] Figure 2 illustrates primer pairs for PCR screening of transformants and expected band profiles for wild-type, heteroplasmic and homoplasmic strains.

[0013] Figure 3 illustrates results from PCR screening and Western blot analysis of endo- β -glucanase transformed *C. reinhardtii* clones.

[0014] Figure 4 is a graphic representation of additional nucleic acid constructs.

[0015] Figure 5 shows PCR and Western analysis of *C. reinhardtii* transformed with FPP synthase and bisabolene synthase.

[0016] Figure 6 shows gas chromatography - mass spectrometry analysis of *C. reinhardtii* transformed with FPP synthase and bisabolene synthase.

[0017] Figure 7 shows Western blot analysis of *S. dimorphus* transformed with a phytase (FD6, SEQ ID NO 28) alone or in combination with an endoxylanase (BD11, SEQ ID NO. 31). SE70 designates wild type. +C is a positive control.

[0018] Figure 8 shows Western blot analysis of *S. dimorphus* transformed with a phytase (FD7, SEQ ID NO 29). Three positive clones are in the center of the blot designated by the arrow. Wild type is designated wt.

[0019] Figure 9 shows phytase production by Western blot analysis in *C. reinhardtii*. Arrows show the presence of proteins encoded by SEQ ID NO. 28 (FD6) and SEQ ID NO. 30 (FD11).

DETAILED DESCRIPTION

[0020] The present disclosure relates to compositions of an animal feedstock and methods of modifying or making an animal feedstock. The feedstocks disclosed herein can comprise non-vascular photosynthetic organisms (NVPOs) wherein the NVPOs can be genetically modified.

[0021] The host organisms or cells disclosed herein (e.g. NVPOs) can be genetically modified or modified (e.g. by methods disclosed herein) for use as a feedstock. The compositions of genetically modified NVPOs disclosed here can be used directly as a feedstock or can be added to a feedstock to generate a modified or improved feedstock. For example a composition can comprise a feedstock and a genetically modified NVPO. Genetic modification of an NVPO can comprise engineering an NVPO to express one or more enzymes. In some aspects the enzyme can be a biomass degrading enzyme and in some aspects the enzyme can be a biosynthetic enzyme. Genetically modified NVPOs can also express both types of enzymes (e.g. a biomass degrading enzyme and a biosynthetic enzyme). The enzyme expressed can be one that is naturally expressed in the NVPO or not naturally expressed in the NVPO. In some aspects the enzyme produced is not naturally expressed in the NVPO. For example an enzyme (e.g. a biomass degrading enzyme) can be an exogenous enzyme. In some aspects the exogenous enzyme is a biodegrading enzyme other than a phytase. In another example a composition can comprise a feedstock and a genetically modified NVPO wherein the NVPO is modified to increase the expression of a naturally occurring enzyme (e.g. a biomass-degrading enzyme). In some aspects an enzyme can be secreted from a genetically modified NVPO or added to the feedstock as an independent ingredient.

[0022] Biomass degrading enzymes can improve the nutrient value of an existing feedstock by breaking down complex components of the feedstock (e.g. indigestible components) into components that can be absorbed and used by the animal. A biomass-degrading enzyme can be expressed and retained in the NVPO or secreted or expelled (i.e. produced

ex vivo) from the NVPO. Genetically modified NVPOs that provide the biomass degrading enzymes can also be utilized by the animal for the inherent nutrient value of the NVPO. For example, a composition can comprise a feedstock, a genetically modified non-vascular photosynthetic organism, and a biomass-degrading enzyme that is ex vivo to the genetically modified non-vascular photosynthetic organism. In another example, a genetically modified non-vascular photosynthetic organism is modified to increase expression of a naturally occurring biomass-degrading enzyme.

[0023] The expression of certain exogenous biosynthetic enzymes in an NVPO can allow the biosynthesis of nutrient rich lipids, fatty acids and carbohydrates. Genetically modified NVPOs that express such nutrient rich components can be added to an existing feedstock to supplement the nutritional value of the feedstock. In some aspects such genetically modified NVPOs can comprise as much as 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% of the improved feedstock. NVPOs can be genetically modified to produce or increase production of one or more fatty acids, lipids or hydrocarbons. In one example, a genetically modified NVPO comprises at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway. In some aspects a genetically modified NVPO can have a higher content of fatty acids, lipids or hydrocarbons (e.g. isoprenoids) than an unmodified NVPO of the same species. Therefore in one aspect a composition can comprise a feedstock and a genetically modified NVPO wherein the NVPO has a higher lipid, fatty acid, or isoprenoid content relative to an unmodified NVPO of the same species. The biosynthetic enzymes can also be one found in a mevalonate pathway. For example, the enzyme can be farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase.

[0024] An improved feedstock can be comprised entirely or partially of a genetically modified NVPO. In some aspects a genetically modified NVPO can be added to a composition to generate an improved feedstock. The composition may not be considered an improved feedstock until after the addition of a genetically modified NVPO. In some aspects a genetically modified NVPO can be added to an existing feedstock to generate an improved feedstock. In some aspects a genetically modified NVPO can be added to an existing feedstock at a ratio of at least 1:20 (weight of NVPO/wt of feedstock). In some aspects, an improved feedstock can comprises up to 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, or 100 percent of a genetically modified NVPO. In some aspects a viable genetically modified NVPO can be added to a feedstock (e.g. as a seed culture) at a concentration of less than 5% (w/w) of the feedstock wherein the genetically modified NVPO multiplies to become up to 10, 20, 30, 40, 50, 60, 70, 80, 90 or 95% percent of the feedstock (w/w). A feedstock or improved feedstock can also comprise additional nutrients, ingredients or supplements (e.g. vitamins). An improved feedstock comprising a genetically modified NVPO can also comprise any normal ingredient of an animal feed including but not limited any vegetable, fruit, seed, root, flower, leaf, stem, stalk or plant product of any plant. An improved feedstock comprising a genetically modified NVPO can also comprise any animal parts or products (e.g. meat, bone, milk, excrement, skin). An improved feedstock comprising a genetically modified NVPO can also comprise any product or bi-product of a manufacturing process (e.g. sawdust or brewers waste). Additional non limiting examples of ingredients of a feedstock or an improved feedstock as disclosed herein include alfalfa, barley, blood meal, grass, legumes, silage, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale. Therefore, in one aspect a composition can comprise a feedstock and a genetically modified NVPO wherein the

feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

5 **[0025]** In some aspects, a genetically modified NVPO can be used for a purpose (e.g. in producing a recombinant product or biofuel) and the remaining portion thereof can be used for an improved feedstock. Therefore an improved feedstock can comprise a portion of a genetically modified NVPO. For example a composition of an animal feed ingredient can comprise whole and/or defatted algae (e.g. after removal of fatty acids, lipids or hydrocarbons, e.g. after hexane extraction) or a mixture of whole and defatted algae, which provides both the feed enzyme and the inherent
10 nutritive value of the algae. In another example a genetically modified NVPO can be washed, dehydrated, centrifuged, filtered, defatted, lysed, dried, processed (e.g. extracted), or milled. The remaining portion thereof can be used as a feedstock, as an improved feedstock or as a supplement to improve a feedstock. For example, a composition can comprise a feedstock and a portion of a genetically modified NVPO wherein the genetically modified NVPO is at least partially depleted of a lipid, fatty acid, isoprenoid, carotenoid, carbohydrate, or selected protein. The genetically
15 modified NVPO can also be genetically modified to produce a biomass-degrading enzyme as disclosed herein.

[0026] Methods of generating, modifying, supplementing or improving a feedstock composition are also disclosed herein. The methods can comprise combining a genetically modified NVPO or a portion thereof with a feedstock to generate the improved feedstock. In one example the method comprises removing a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified NVPO. The remaining genetically modified NVPO, or a portion thereof, can
20 be combined with a feedstock to generate the improved feedstock composition. In one example, the modified NVPO does not express an exogenous phytase. The genetically modified NVPO or a portion thereof can comprise at least one nucleic acid (e.g. an exogenous nucleic acid). The nucleic acid can be a vector. In one example, the nucleic acid encodes a biomass degrading enzyme. The biomass-degrading enzyme can be a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, or phytase. The biomass-degrading enzyme can be a
25 carbohydrase, for example, an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase. The biomass-degrading enzyme can be a protease, for example, a subtilisin, bromelain, or fungal acid-stable protease. The biomass-degrading enzyme can be a phytase. In another example the genetically modified NVPO further comprises at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway. The enzyme in the isoprenoid biosynthesis pathway can be a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase. The enzyme in the isoprenoid biosynthesis
30 pathway can be in a mevalonate pathway. In yet another example, the method can further comprise removing a lipid, fatty acid, or isoprenoid, from the genetically modified NVPO prior to combining with a feedstock to generate the improved feedstock.

[0027] The method can further comprise refining the removed lipid, fatty acid, isoprenoid, or carbohydrate into a
35 biofuel. In some aspects, the method further comprises lysing the genetically modified NVPO prior to combining with the feedstock. In some embodiments, the method further comprises drying the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock. The genetically modified NVPO can be prokaryotic or eukaryotic. In some embodiments the NVPO can be *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*.

The genetically modified NVPO can be grown under heterotrophic conditions or autotrophic conditions. The genetically modified NVPO can be grown in darkness. The feedstock or improved feedstock to which the NVPO is added can comprise one or more of the following of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

[0028] Compositions disclosed herein can comprise a host organism transformed with one or more of the nucleic acids described herein. The host organism can be photosynthetic. In some cases, the host organism can be photosynthetic and non-vascular. In other cases, the host organism can be photosynthetic and vascular. The host organism can be eukaryotic or prokaryotic. Therefore in one aspect a composition can comprise a feedstock and a genetically modified NVPO wherein the NVPO is prokaryotic. In another example the NVPO is eukaryotic. The host organism can be unicellular or multicellular. In one embodiment, the organism is a unicellular algae or microalgae. The host organism can be an autotroph or a heterotroph. A photosynthetic organism is one that naturally photosynthesizes (has a plastid) or that is genetically engineered or otherwise modified to be photosynthetic. In some instances, a photosynthetic organism can be transformed with a construct which renders all or part of the photosynthetic apparatus inoperable (e.g. genetically modified to have impaired photosynthesis). Examples of some prokaryotic organisms that may be used include, but are not limited to, cyanobacteria (e.g., *Synechococcus*, *Synechocystis*, *Athrospira*). In some aspects the host organism can be eukaryotic (e.g. green algae). Some non-limiting examples of eukaryotic NVPOs used herein include *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, *D. terciolecta*, and *H. pluvalis*. Additional non-limiting examples of NVPOs contemplated for use herein include cyanophyta, prochlorophyta, rhodophyta, chlorophyta, heterokontophyta, tribophyta, glaucophyta, chlorarachniophytes, euglenophyta, euglenoids, haptophyta, chrysophyta, cryptophyta, cryptomonads, dinophyta, dinoflagellata, pyrmnesiophyta, bacillariophyta, xanthophyta, eustigmatophyta, raphidophyta, phaeophyta, porphyridium and phytoplankton. Therefore in one example a composition can comprise a feedstock and a genetically modified NVPO. The NVPO (e.g. genetically modified NVPO) can be *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina* or *H. pluvalis*. In some aspects the NVPO is a NVPO other than Porphyridium.

[0029] An exemplary group of useful organisms are the green algae. One non-limiting example is of the genus *Chlamydomonas*, a genus of unicellular green algae (phylum Chlorophyta).

[0030] As disclosed herein host organisms (e.g. NVPOs) can be transfected or transformed (i.e. genetically modified) with at least one nucleic acid encoding one or more proteins (e.g. enzymes). A single genetically modified host organism can comprise exogenous nucleic acids encoding one, two, three or more proteins or subunits thereof (e.g. *C. reinhardtii* can be genetically modified to produce both an endoxylanase and an endo- β -glucanase). A host organism can be genetically modified to contain multiple copies of a nucleic acid that encodes the same protein. A host organism can be engineered to contain one or more nucleic acids with one or more mutations. The engineered nucleic acids can comprise a plastid promoter or a nuclear promoter to direct expression in the nucleus, in the chloroplast or plastid of the host organism. The nucleic acid (e.g. vector) may also encode a fusion protein or agent that selectively targets the expressed protein of interest to the nucleus, the chloroplast or plastid. Exogenous nucleic acids described herein can be introduced into the host organisms by any suitable method.

[0031] Disclosed herein is a genetically modified, non-vascular photosynthetic organism comprising at least one exogenous nucleic acid. The nucleic acid can encode at least one enzyme. The encoded enzyme can be a biomass degrading enzyme or a biosynthetic enzyme. The biosynthetic enzyme can be in an isoprenoid biosynthesis pathway. In some aspects two enzymes are encoded by one or more exogenous nucleic acids. In one example, a genetically modified non-vascular photosynthetic organism comprises at least one nucleic acid encoding a first and a second enzyme, wherein the first enzyme is an enzyme in an isoprenoid biosynthesis pathway, and the second enzyme is a biomass-degrading enzyme. In one example, the first and second enzymes are encoded on a vector. In some aspects, the first and second enzymes are encoded on a single vector. A genetically modified non-vascular photosynthetic organism can comprise a first vector and a second vector, wherein the first vector encodes an enzyme in an isoprenoid biosynthesis pathway, and the second vector encodes a biomass-degrading enzyme. The enzyme in the isoprenoid biosynthesis pathway can be farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase. In another example, the enzyme in the isoprenoid biosynthesis pathway can be an enzyme in a mevalonate pathway. The biomass-degrading enzyme can be a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, or phytase. In one example the biomass-degrading enzyme is a carbohydrase. In another example the biomass-degrading enzyme is a protease. In yet another example the biomass-degrading enzyme is a phytase.

[0032] The term "nucleic acid" refers to deoxyribonucleotides, deoxyribonucleosides, ribonucleosides, or ribonucleotides and polymers thereof in either single- or double-stranded form. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (including but not limited to, degenerate codon substitutions) and complementary sequences as well as the sequence explicitly indicated.

[0033] Nucleic acids can comprise promoters, enhancers, restriction enzyme sites, selection markers, open reading frames, and regulatory sequences, etc. Nucleic acids can encode polypeptides and can comprise mutations. Nucleic acids can be in the form of vectors. Vectors can be used as a vehicle for introducing into and expressing one or more desired genes in a host organism. Non limiting examples of vectors include plasmids, phages, phagemids, viruses and retroviruses. A vector can be linear (e.g. a PCR product) or circular.

[0034] The vectors disclosed herein can be used for stable introduction and integration of a nucleic acid into a host organism, herein referred to as stable transformation. Vectors can be engineered to direct the expression of heterologous (i.e. from a different species) and/or native protein(s) of interest (e.g., carbohydrate degrading enzymes, proteases, phytases, etc.) in a host organism. Such vectors can be used to modify the natural phenotype of the host organism (e.g., increasing levels of carotenoids, tocopherols, isoprenoids, etc.). Vectors can comprise one or more coding sequences and direct the expression of one or more proteins. A vector can comprise a nucleic acid sequence encoding a first and a second polypeptide (e.g. a first and a second enzyme). The first and/or second polypeptides can be enzymes of a biosynthesis pathway (e.g. an isoprenoid biosynthesis pathway). The first and/or second polypeptides can be biomass-degrading enzymes (e.g. a carbohydrase). Therefore an exemplary composition comprises a nucleic acid sequence encoding a first enzyme and a second enzyme wherein the first enzyme is an enzyme in an isoprenoid biosynthesis pathway, and the second enzyme is a biomass-degrading enzyme. The nucleic acid can be a vector. The nucleic acid sequence can comprise a promoter for directing expression in a plastid, chloroplast or nucleus of a non-vascular photosynthetic organism. Such nucleic acids or vectors can be constructed using standard techniques known in the art.

[0035] A vector can be constructed wherein any gene of interest is inserted into the vector for subsequent transformation and expression of the gene in a host organism. A vector can be constructed with a promoter or regulatory element positioned on the 5' or upstream side of a multiple cloning sequence. The multiple cloning sequence allows splicing of any desired coding sequence into the vector wherein the upstream promoter or regulatory element drives expression of the coding sequence in the host organism. The vector can comprise one or more promoter or regulatory elements positioned on the 5' or upstream side of a desired coding region. Additional regulatory elements can be located downstream or 3' of a coding region. Regulator elements can come from any source (e.g., viral, bacterial, fungal, protist, animal). The regulator elements contemplated herein can be specific to photosynthetic organisms, non-vascular photosynthetic organisms (NVPO), and vascular photosynthetic organisms (e.g., flowering plants). As used herein, the term "NVPO," refers to any macroscopic or microscopic organism which does not have a vascular system (e.g. xylem and phloem) such as that found in higher plants. A coding sequence may be flanked by sequences which direct expression of an inserted coding sequence into a targeted cellular region or compartment (e.g., a nucleus or plastid). A vector encoding a protein (e.g. an enzyme) can be inserted into a nuclear genome of a host organism (e.g. nuclear or plastid genome), such that protein expression is controlled by a site-specific regulatory element native to that particular organelle (e.g. a plastid specific promoter). Examples of regulatory elements include, but are not limited to, constitutive promoters, light-inducible promoters, quorum-sensing promoters, temperature-sensitive promoters, or nitrogen-starvation responsive promoters. An example of light-inducible promoter is described in U.S. Patent No. 6858429. Some non limiting examples of promoters contemplated for insertion of a nucleic acid into a chloroplast include those disclosed in US Application No. 2004/0014174. A promoter can be a constitutive promoter or an inducible promoter. Any regulatory element situated 5' or 3' of the coding region of interest and which helps direct efficient expression of one or more desired proteins in a NVPO can be used.

[0036] A regulatory element, as the term is used herein, broadly refers to a nucleotide sequence that regulates the transcription or translation of a nucleic acid or the localization of a polypeptide to which it is operatively linked. A regulatory element may be native or foreign to the nucleotide sequence encoding the polypeptide. Such elements include, but are not limited to, an RBS, a leader, a polyadenylation sequence, a pro-peptide sequence, a promoter, a signal peptide sequence, a transcription terminator, an enhancer, an initiation (start) codon, a splicing signal for intron excision and maintenance of a correct reading frame, a STOP codon, an amber or ochre codon, or an IRES. In one non-limiting example, the typical vector may comprise regulatory elements such as a promoter or enhancer, a transcriptional and translational start signal and a transcriptional and translational stop signal. A vector may comprise various linkers for the purpose of introducing one or more restriction sites that facilitate ligation of regulatory elements and coding regions into the vector. A vector can comprise sequences and regulatory elements indigenous to NVPO such as viral sequences and bacterial sequences which are naturally associated with the NVPO.

[0037] A vector can be engineered to express one or more proteins wherein the expressed protein can be an individual protein, a pro-protein or a fusion protein. A vector can be engineered to express proteins comprising additional elements that allow the expressed proteins to be targeted to an organelle, targeted to the cell surface, anchored on the cell surface, or secreted to the environment. A vector can be engineered to express proteins comprising elements that allow compartmentalization (i.e., targets a polypeptide to the cytosol, nucleus, chloroplast membrane or cell membrane). Such signals are well known in the art and have been widely reported (see, e.g., U.S. Pat. No. 5,776,689).

[0038] Any suitable plasmid can be used as the backbone of a vector described herein. Non-limiting examples of such plasmids include pUC or its derivatives, pBR322, pBluescript, or pGEM. A particular plasmid can be chosen based on the nature of the markers, availability of convenient restriction sites, copy number and the like. The nucleic acid encoding the polypeptide of interest may be synthetic, naturally derived, or a combination thereof. Codons can be altered or customized to increase expression efficiency of a desired protein in a specific NVPO. The codon bias of *C. reinhardtii* has been reported. See U.S. Application 2004/0014174. Codon bias can be variously skewed in different plants, including, for example, in algae as compared to tobacco. Generally, the codon bias selected reflects codon usage of the plant (or organelle therein) which is being transformed with the nucleic acids of the present invention. For example, where *C. reinhardtii* is the host, the chloroplast codon usage may be biased to reflect nuclear or chloroplast codon usage (e.g., about 74.6% AT bias in the third codon position for sequences targeting the chloroplast).

[0039] Vectors disclosed herein can contain an origin of replication isolated from various sources. For example a vector can comprise a prokaryote origin of replication (ori), for example, an *E. coli* ori or a cosmid ori, thus allowing passage of the vector in a prokaryote host organism, as well as in a plant chloroplast, as desired. Such features, combined with appropriate selectable markers, allows for the vector to be "shuttled" between the target host cell (e.g. a bacteria or yeast cell).

[0040] Vectors can be engineered to express a selectable marker or reporter for efficient selection of a host organism (e.g. green algae) transformed by the vector. A selectable marker or reporter can be operably linked downstream or upstream of one or more regulatory elements. The term "reporter" or "selectable marker" refers to an encoded polypeptide that confers a detectable phenotype. A reporter generally encodes a detectable polypeptide, for example, a green fluorescent protein or an enzyme such as luciferase, which, when contacted with an appropriate agent (a particular wavelength of light or luciferin, respectively) generates a signal that can be detected by eye or using appropriate instrumentation (Giacomin, *Plant Sci.* 116:59-72, 1996; Srikantha, *J. Bacteriol.* 178:121, 1996; Gerdes, *FEBS Lett.* 389:44-47, 1996; see, also, Jefferson, *EMBO J.* 6:3901-3907, 1987, fl-glucuronidase). A reporter or selectable marker can be a cell surface marker. A selectable marker generally is a molecule that, when present or expressed in a cell, provides a selective advantage (or disadvantage) to the cell containing the marker, for example, the ability to grow in the presence of an agent that otherwise would kill the cell. A selectable marker can provide a means to isolate a genetically modified host organism that expresses the marker (see, for example, Bock, R. (2001) *J.Mol.Biol.* 312:425-438). Non limiting examples of selectable markers include those that confer antimetabolite resistance, for example, dihydrofolate reductase, which confers resistance to methotrexate (Reiss, *Plant Physiol. (Life Sci. Adv.)* 13:143-149, 1994); neomycin phosphotransferase, which confers resistance to the aminoglycosides neomycin, kanamycin and paromycin (Herrera-Estrella, *EMBO J.* 2:987-995, 1983), hygromycin, which confers resistance to hygromycin (Marsh, *Gene* 32:481-485, 1984), *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells to utilize histidinol in place of histidine (Hartman, *Proc. Natl. Acad. Sci., USA* 85:8047, 1988); mannose-6-phosphate isomerase which allows cells to utilize mannose (WO 94/20627); and deaminase from *Aspergillus terreus*, which confers resistance to Blasticidin S (Tamura, *Biosci. Biotechnol. Biochem.* 59:2336-2338, 1995). Additional non-limiting examples of selection markers may include kanamycin, phleomycin, bleomycin and zeocin. Additional selectable markers include those that confer herbicide resistance, for example, phosphinothricin acetyltransferase gene, which confers resistance to phosphinothricin (White et al., *Nucl. Acids Res.* 18:1062, 1990; Spencer et al., *Theor. Appl. Genet.* 79:625-631, 1990), a mutant EPSPV-

synthase, which confers glyphosate resistance (Hinchee et al., *BioTechnology* 91:915-922, 1998), a mutant acetolactate synthase, which confers imidazolinone or sulfonylurea resistance (Lee et al., *EMBO J.* 7:1241-1248, 1988), a mutant psbA, which confers resistance to atrazine (Smeda et al., *Plant Physiol.* 103:911-917, 1993), or a mutant protoporphyrinogen oxidase (see U.S. Pat. No. 5,767,373), or other markers conferring resistance to an herbicide such as glufosinate. Selectable markers include polynucleotides that confer dihydrofolate reductase (DHFR) or neomycin resistance for eukaryotic cells and tetracycline; ampicillin resistance for prokaryotes such as *E. coli*; and bleomycin, gentamycin, glyphosate, hygromycin, kanamycin, methotrexate, phleomycin, phosphinotricin, spectinomycin, streptomycin, sulfonamide and sulfonylurea resistance in plants (see, for example, Maliga et al., *Methods in Plant Molecular Biology*, Cold Spring Harbor Laboratory Press, 1995, page 39). An additional selectable marker can be produced by complementing a wild type gene (e.g. nitrate reductase) into an auxotrophic mutant strain of algae (e.g. *C. reinhardtii* 137c, which lacks nitrate reductase and therefore cannot grow on nitrate as the sole nitrogen source).

[0041] After a vector is constructed to direct expression of at least one polypeptide in a NVPO, the vector can be introduced into the host organism using any of several methods, some of which might be particular to the host organism. One or more vectors can be introduced into a host. Variations on these methods are amply described in the general literature.

[0042] Transformed cells can be produced by introducing a nucleic acid (e.g. vector) into a population of target cells and selecting the cells which have taken up the nucleic acid. The basic techniques used for transformation and expression in photosynthetic organisms are similar to those commonly used for *E. coli*, *Saccharomyces cerevisiae* and other species. Transformation methods customized for a NVPO, e.g., the chloroplast of a strain of algae, are known in the art. One or more vectors can be introduced into a host NVPO using any suitable method. These methods have been described in a number of texts for standard molecular biological manipulation (see Packer & Glaser, 1988, "Cyanobacteria", *Meth. Enzymol.*, Vol. 167; Weissbach & Weissbach, 1988, "Methods for plant molecular biology," Academic Press, New York, Sambrook, Fritsch, Maniatis, *Molecular Cloning, A Laboratory Manual*, 2nd edition, vol. 1, 2 & 3 Cold Spring Harbor Press, 1989, New York; and Clark M S, 1997, *Plant Molecular Biology*, Springer, N.Y.). Some non-limiting examples include biolistic devices ((see, e.g.; Klein et al., *Nature* 327:70-73, 1987; Christou, *Trends in Plant Science* 1:423-431, 1996; Sanford, *Trends In Biotech.* (1988) 6: 299-302; U.S. Pat. No. 4,945,050; Duan et al., *Nature Biotech.* 14:494-498, 1996; Shimamoto, *Curr. Opin. Biotech.* 5:158-162, 1994); electroporation (Fromm et al., *Proc. Nat'l. Acad. Sci. (USA)* (1985) 82: 5824-5828); use of a laser beam, protoplast transformation, electroporation of partially permeabilized cells, introduction of DNA using glass fibers, the glass bead agitation method and microinjection. Any suitable method for introducing a nucleic acid into a host organism (e.g., an NVPO) can be used.

[0043] Plastid transformation is an established method for introducing a polynucleotide into a plant cell chloroplast (see U.S. Pat. Nos. 5,451,513, 5,545,817, and 5,545,818; WO 95/16783; McBride et al., *Proc. Natl. Acad. Sci., USA* 91:7301-7305, 1994). In some embodiments, chloroplast transformation involves introducing regions of chloroplast DNA flanking a desired nucleotide sequence and allowing for homologous recombination of the exogenous DNA into the target chloroplast genome. In some instances one to 1.5 kb flanking nucleotide sequences of chloroplast genomic DNA can be used. Using this method, point mutations in the chloroplast 16S rRNA and rps12 genes, which confer resistance to spectinomycin and streptomycin, can be utilized as selectable markers for transformation (Svab et al., *Proc. Natl. Acad. Sci., USA* 87:8526-8530, 1990), and can result in stable homoplasmic transformants, at a frequency of

approximately one per 100 bombardments of target leaves. Transformation frequency can be increased by replacement of recessive rRNA or r-protein antibiotic resistance genes with a dominant selectable marker, including, but not limited to the bacterial *aadA* gene (Svab and Maliga, *Proc. Natl. Acad. Sci.*, USA 90:913-917, 1993).

[0044] Candidate genes for directing the expression of proteins (e.g. enzymes) in genetically modified NVPOs for use in animal feeds can be obtained from a variety of organisms including eukaryotes, prokaryotes, or viruses. In some instances, an expressed enzyme is one member of a metabolic pathway (e.g. an isoprenoid biosynthesis pathway). Several enzymes may be introduced into the NVPO to produce increased levels of desired metabolites, or several enzymes may be introduced to produce a NVPO containing multiple useful feed enzyme activities (e.g. simultaneous production of xylanase, endo- β -glucanase, and phytase activities).

[0045] Feed enzymes can be expressed in host organisms (e.g. genetically modified host organisms) and purified to a useful level. The purified enzymes can be added to animal feed in a manner similar to current practice. Feed enzymes can also be expressed in host organisms (e.g. a genetically modified NVPO (e.g. algae)), and the resulting host organisms can be added as a feed ingredient, adding both nutritive value and desired enzyme activity to the animal feed product. In this application, the genetically modified host organisms (e.g. NVPO) can be added to a feedstock alive, whole and non-viable or as a lysate wherein the NVPO organisms are lysed by any suitable means (e.g. physical, chemical or thermal).

[0046] Disclosed herein are various non-limiting exemplary enzymes that can be expressed in a genetically modified host organism (e.g. a NVPO), and various non-limiting examples of metabolites produced as a result of expression of such enzymes in the host organism. These examples are meant to provide illustration of the types of molecules which can be utilized and are not intended to be limiting.

[0047] Any suitable biomass-degrading enzyme is contemplated for use herein. Non limiting examples of biomass-degrading enzymes included those selected from the group consisting of amylases (e.g. β -amylase, glucoamylase), phosphatases, phytases, cellulases, β -glucanases, hemicellulases (e.g. xylanases and galactanases), proteases, peptidases (e.g. lysozyme), galactosidases, pectinases, esterases, lipases, phospholipases, glucose oxidases, other digestive related oxidases, reductases and transglutaminases. Non limiting examples of biomass-degrading enzymes also include those disclosed in WO/2008/017661. In one example a composition can comprise a feedstock and a genetically modified NVPO. The genetically modified NVPO can be genetically modified to produce a biomass-degrading enzyme such as a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, phytase, or mixture thereof.

[0048] Many animal feeds can contain plant seeds, including soybeans, maize, wheat, and barley among others. Plant seeds can contain high levels of myo-inositol polyphosphate (phytic acid). This phytic acid is indigestible to non-ruminant animals, and so feeds with high levels of phytic acid may have low levels of bioavailable phosphorous. The phytic acid can also chelate many important nutritive minerals, such as calcium and magnesium. Incorporation of a phytase into the feed, which can act in the animal's upper gut, can release both the chelated mineral nutrients and significant levels of bioavailable phosphorous. The net result is that less free phosphorous needs to be added to the animal feed product. In addition, phosphorous levels in the excreta (manure) can be reduced, which can reduce downstream phosphorous pollution.

[0049] Genetically modified NVPOs that express phytases or similar enzymes can be added to a feedstock to improve the nutrient or digestible properties of the feed. Phytases contemplated for use herein can be from any organism (e.g.

bacterial or fungal derived). Non limiting examples of types of phytases contemplated for use herein include 3-phytase (alternative name 1- phytase; a myo-inositol hexaphosphate 3-phosphohydrolase, EC 3.1.3.8), 4- phytase (alternative name 6-phytase, name based on 1 L-numbering system and not 1 D- numbering, EC 3.1.3.26), and 5-phytase (EC 3.1.3.72). Additional non limiting examples of phytases include microbial phytases, such as fungal, yeast or bacterial

5 phytases such as disclosed in EP 684313, US 6139902, EP 420358, WO 97/35017, WO 98/28408, WO 98/28409, JP 1 1000164, WO98/13480, AU 724094, WO 97/33976, US 6110719, WO 2006/038062, WO 2006/038128, WO 2004/085638, WO 2006/037328, WO 2006/037327, WO 2006/043178, US 5830732 and under UniProt designations P34753, P34752, P34755, 000093, 031097, P42094, 066037 and P34754 (UniProt, (2008) <http://www.uniprot.org/>).

Polypeptides having an amino acid sequence of at least 75% identity to an amino acid sequence (comprising the active site) of any one of the phytases disclosed above are also contemplated for use herein. In one example a composition can comprise a feedstock and a genetically modified NVPO. The genetically modified NVPO can be genetically modified to produce a biomass-degrading enzyme such as a phytase. In one aspect the phytase is a phytase of bacterial or fungal origin. In one aspect the biomass-degrading enzyme is an enzyme other than a phytase.

[0050] Many plant parts (e.g. seeds, fruits, stems, roots, leaves and flowers) from plants such as, for example, soybeans, wheat, and barley contain polysaccharides that are indigestible by some animals (e.g. non-ruminant animals). Non limiting examples of such carbohydrates include xylans, raffinose, stachyose, and glucans. The presence of indigestible carbohydrates in animal feed can reduce nutrient availability. Indigestible carbohydrates in poultry feed can result in sticky feces, which can increase disease levels. The presence of one or more carbohydrate degrading enzymes (e.g. α -amylase) in the animal feed can help break down polysaccharides, increase nutrient availability, increase the bio-

15 available energy content of the animal feed, and reduce health risks. Non limiting examples of carbohydrate degrading enzymes contemplated for use herein include amylases (e.g. α -amylase and β -amylase), β -mannanase, maltase, lactase, β -glucanase, endo- β -glucanase, glucose isomerase, endoxylanase, α -galactosidase, glucose oxidase, pullulanase, invertase and any carbohydrate digesting enzyme of bacterial, fungal, plant or animal origin. In one example a composition can comprise a feedstock and a genetically modified NVPO. The genetically modified NVPO can be

20 genetically modified to produce a biomass-degrading enzyme such as a carbohydrase. In one aspect the carbohydrase can be an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase.

[0051] Many feedstocks contain plant parts (e.g. seeds) with anti-nutritive proteins (e.g. protease inhibitors, amylase inhibitors and others) that reduce the availability of nutrients in an animal feed. Addition of a broad spectrum protease (e.g. bromelain, subtilisin, or a fungal acid-stable protease) can break down these anti-nutritive proteins and increase the

25 availability of nutrients in the animal's feed. Non limiting examples of proteases contemplated for use herein include endopeptidases and exopeptidases. Non limiting examples of proteases contemplated for use herein include serine proteases (e.g. subtilisin, chymotrypsins, glutamyl peptidases, dipeptidyl-peptidases, carboxypeptidases, dipeptidases, and aminopeptidases), cyteine proteases (e.g. papain, calpain-2, and papain-like peptidases and bromelain), aspartic peptidases (e.g. pepsins and pepsin A), glutamic proteases, threonine proteases, fungal acid proteases and acid stable

30 proteases such as those disclosed in (US 6855548). In one example, a composition can comprise a feedstock and a genetically modified NVPO. The genetically modified NVPO can be genetically modified to produce a biomass-degrading enzyme such as a protease. In one aspect the protease can be a subtilisin, bromelain or fungal acid-stable protease.

[0052] Non limiting examples of lipases contemplated for use herein include pancreatic lipase, lysosomal lipase, lysosomal acid lipase, acid cholesteryl ester hydrolase, hepatic lipase, lipoprotein lipase, gastric lipase, endothelial lipase, pancreatic lipase related protein 2, pancreatic lipase related protein 1, lingual lipase and phospholipases (e.g. phospholipase A1 (EC 3.1.1.32), phospholipase A2, phospholipase B (lysophospholipase), phospholipase C and phospholipase D).

[0053] An improved feedstock can be generated by combining a feedstock with a NVPO that is genetically altered to produce an enzyme (e.g. a carbohydrase, protease or lipase). In some aspects the enzyme is produced ex vivo to the organism. In some aspects the enzyme is secreted. Enzymes produced ex vivo to the organisms can break down components of a feedstock prior to ingestion by an animal. Therefore an improved feedstock can be generated by combining a feedstock with a NVPO that is genetically altered to produce an enzyme (e.g. a carbohydrase, protease or lipase) and subjecting the mixture to a holding period. A holding period can allow the genetically altered NVPO to multiply and to secrete more enzyme into the feedstock. A holding period can be from several hours up to several days. In some aspects a holding period is for up to 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 days. In some aspects a holding period is for up to several days to several weeks. In some aspects a holding period is indefinite. An indefinite holding period allows intermittent removal and use of the improved feedstock and intermittent addition of the base feedstock.

[0054] In some instances, enzymes of the present invention may be modified to exhibit characteristics which are useful in feed production and/or supplementation. For example, as some feeds are heated during processing, enzymes may be engineered to withstand high temperatures for extended periods of time. An enzyme of the present invention may be engineered to withstand elevated temperatures of 40-100°C for 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 or more minutes. In some instances, thermostable biomass-degrading enzymes are also contemplated for use herein such as those disclosed in WO/2003/062409. Other modifications may also be made, such as resistance to high pH (8-10), resistance to low pH (3-5), function in the presence of high ion (sodium, calcium, magnesium, etc.) concentrations, function in the presence of low water concentration, etc.

[0055] Enzymes that modify the isoprenoid and carotenoid pathways (e.g. increase isoprenoid or carotenoid levels) and enzymes that increase levels of nutritive lipids, isoprenoids and tocopherols (e.g. β -carotene, lutein, lycopene, vitamin E, arachadonic acid, and docosahexanoic acid) from a host organism (e.g. algae) can dramatically increase the value of a NVPO (e.g. as an animal feed) and are contemplated for use herein. For example, high levels of carotenoids in poultry diets help increase the yellow color found in poultry skin and egg yolks. Upregulating the levels of these pigments and lipids in the algae can increase the commercial value of the algae as animal feed (e.g. as poultry feed). Dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP) serve as intermediates that feed isoprenoid biosynthesis. These two molecules can be generated by two pathways, the mevalonate pathway (i.e. the HMG-CoA reductase pathway) or the methylerythritol phosphate (MEP) pathway. Isoprenoid biosynthesis (i.e. the isoprenoid biosynthesis pathway) comprises both the mevalonate and MEP pathways. The enzymes that make up these pathways and the genes that encode such enzymes are all contemplated for use herein. Non limiting examples of enzymes that make up the mevalonate pathway include thiolase, HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, mevalonate-5-pyrophosphate decarboxylase, and isopentenyl pyrophosphate isomerase. Non limiting examples of enzymes that make up the MEP pathway include 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR). Additional non limiting examples of isoprenoid biosynthetic enzymes

contemplated for use herein include geranyl geranyl pyrophosphate (GGPP) synthase, geranyl pyrophosphate synthase, phytoene dehydrogenase, phytoene synthase/lycopene cyclase, farnesyl pyrophosphate (FPP) synthase, HMB-PP reductase, malic enzyme, malate dehydrogenase, AMP deaminase, glucose 6 phosphate dehydrogenase, malate dehydrogenase homolog 2, GND1-6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, IDH2-isocitrate dehydrogenase polypeptide, fructose 1,6 bisphosphatase polypeptide, fatty acyl-CoA desaturase, acetoacetyl CoA thiolase, ATP citrate lyase subunit 2, ATP citrate lyase subunit 1, squalene synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, squalene epoxidase, 2,3-oxidosqualene-lanosterol cyclase, cytochrome P450 lanosterol 14 α -demethylase, C-14 sterol reductase, C-4 sterol methyl oxidase, SAM:C-24 sterol methyltransferase, C-8 sterol isomerase, C-5 sterol desaturase, C-22 sterol desaturase, and C-24 sterol reductase, ketolase, phytoene dehydrogenase (or desaturase), lycopene cyclase, carotenoid ketolase, carotenoid hydroxylase, astaxanthin synthase, carotenoid epsilon hydroxylase, lycopene cyclase (beta and epsilon subunits), carotenoid glucosyltransferase, acyl CoA:diacylglycerol acyltransferase, hydroxylase and thioesterase.

[0056] Therefore a composition can comprise a feedstock and a genetically modified NVPO wherein the genetically modified NVPO comprises at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway. For example the enzyme in the isoprenoid biosynthesis pathway can be farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase. The enzyme in the isoprenoid biosynthesis pathway can also be an enzyme found in mevalonate pathway. The enzyme in the isoprenoid biosynthesis pathway can also be an enzyme found in an MEP pathway.

[0057] In some aspects, a genetically modified NVPO is defatted (e.g. depleted or partially depleted of lipid, fatty acids or hydrocarbons, including but not limited to isoprenoids and carotenoids) prior to use as an animal feed. In this aspect the genetically modified NVPO can first be used as a source of producing a biofuel or other product (e.g. a pharmaceutical product) and the remaining defatted NVPO can then be used for animal feed. Therefore a genetically modified NVPO can be lysed (for example by mechanical, chemical, enzymatic or other means) prior to use as an animal feed. In some aspects a genetically modified NVPO can be dried prior to or after lysis and used as an animal feed. In some aspects a genetically modified NVPO is first depleted or partially depleted of a selected protein or carbohydrate. For example, a genetically modified NVPO can be used to produce a commercially valuable recombinant protein. After removal (e.g. extraction) of the protein from the NVPO or from the media used to cultivate the NVPO, the remaining organisms can be used as an animal feed. In another example, lysis of the NVPO can be desired to release a biomass-degrading enzyme into the feedstock or to make the biomass-degrading enzyme more available to the animal. Therefore a composition can comprise a feedstock and a genetically modified NVPO wherein the genetically modified NVPO is lysed. In another example the NVPO is dried prior to or after being lysed.

[0058] The improved feedstocks disclosed herein can be feed to any animal. In some instances the animal is a non-human animal. In certain embodiments the animal is a ruminant animal. Non-limiting examples of ruminant animals include cattle, sheep, goats, deer, elk, antelope, oxen, yaks, bison or buffalo. In other embodiments, the animal is a non-ruminant or monogastric animal. Non-limiting examples of non-ruminants include horses, donkeys, pigs, dogs, cats, rabbits, rodents, avian species and aquatic species. Examples of avian species include, but are not limited to, chickens, geese, turkeys, pheasants, partridges, grouse, doves and quail. Examples of aquatic species include trout, salmon, tilapia,

catfish, perch bluegill, carp and crustaceans. Non-limiting examples of crustaceans include shrimp, lobster, crabs and crayfish.

[0059] An NVPO host organism can be grown under any suitable condition. A host organism can be grown under conditions which permit photosynthesis, however, this is not a requirement (e.g., a host organism can be grown in the absence of light). Therefore in one aspect a composition can comprise a feedstock and a genetically modified NVPO wherein the NVPO is grown under heterotrophic conditions. In another example, the NVPO can be grown under autotrophic conditions. In some instances, the host organism can be genetically modified in such a way that the photosynthetic capability is altered, diminished or destroyed. Therefore in one aspect a composition can comprise a feedstock and a genetically modified NVPO wherein the NVPO is genetically modified to have impaired photosynthesis. In growth conditions where a host organism is not capable of photosynthesis (e.g., because of the absence of light and/or genetic modification), the organism can be provided with the necessary supplemental nutrients to sustain growth or viability. One of skill in the art will recognize that not all organisms will be able to sufficiently metabolize a particular nutrient and that nutrient mixtures may need to be modified from one organism to another in order to provide the appropriate nutrient mix.

[0060] A host organism can be grown on land or in water using any suitable method. Host organism can also be grown in a bioreactor (e.g. a photobioreactor). A pond covered with a greenhouse can be considered a photobioreactor.

[0061] It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. 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 the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing, specific examples of appropriate materials and methods are described herein.

[0062] Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, molecular biology, nucleic acid chemistry, and protein chemistry described below are those well known and commonly employed by those of ordinary skill in the art. In accordance with the present invention, common recombinant DNA techniques, molecular biology techniques, molecular genetics, and microbiology techniques may be used by one of skill in the art. For example, techniques such as those described in Sambrook J et al. (2000) Molecular Cloning: A Laboratory Manual (Third Edition), Goeddel, ed. (1990) Methods in Enzymology 185, Current Protocols In Molecular Biology, F. M. Ausubel et al., eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc. (1994, supplemented through 1999), DNA Cloning: A Practical Approach, Vols. I-II (Glover ed. 1985); Animal Cell Culture: A Practical Approach (Freshney ed. 1986) may be used for recombinant methods, nucleic acid synthesis, cloning methods, cell culture methods, transfection and transformation, and transgene incorporation, e.g., electroporation, injection, gene gun, impressing through the skin, and lipofection. Generally, oligonucleotide synthesis and purification steps are performed according to specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references that are provided throughout this document. The procedures therein are believed to be well known to those of ordinary skill in the art and are provided for the convenience of the reader.

[0063] While certain embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and

substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments described herein may be employed. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

EXAMPLES

Example 1. Transformation and Expression of endo- β -glucanase into *C.reinhardtii*

[0064] A nucleic acid encoding (a biomass degrading enzyme) was introduced into *C. reinhardtii*. Transforming DNA (SEQ ID NO. 16) is shown graphically in FIG. 1A. In this instance the segment labeled "Transgene" is the endo- β -glucanase gene (SEQ ID NO. 16) encoding the endo- β -glucanase protein (SEQ ID NO. 15), the segment labeled "psbA 5' UTR" is the 5' UTR and promoter sequence for the *psbA* gene from *C. reinhardtii*, the segment labeled "psbA 3' UTR" contains the 3' UTR for the *psbA* gene from *C. reinhardtii*, and the segment labeled "Selection Marker" is a kanamycin resistance encoding gene from bacteria, which is regulated by the 5' UTR and promoter sequence for the *atpA* gene from *C. reinhardtii* and the 3' UTR sequence for the *rbcL* gene from *C. reinhardtii*. The transgene cassette is targeted to the *psbA* loci of *C. reinhardtii* via the segments labeled "5' Homology" and "3' Homology," which are identical to sequences of DNA flanking the *psbA* locus on the 5' and 3' sides, respectively. All DNA manipulations carried out in the construction of this transforming DNA were essentially as described by Sambrook, Fritsch, Maniatis, Molecular Cloning, A Laboratory Manual, 2nd edition, vol. 1, 2 & 3 Cold Spring Harbor Press, 1989, New York and Cohen et al., *Meth. Enzymol.* 297, 192-208, 1998.

[0065] For these experiments, all transformations were carried out on *C. reinhardtii* strain 137c (mt+). Cells were grown to late log phase (approximately 7 days) in the presence of 0.5 mM 5-fluorodeoxyuridine in TAP medium (Gorman and Levine, *Proc. Natl. Acad. Sci., USA* 54:1665-1669, 1965, which is incorporated herein by reference) at 23°C under constant illumination of 450 Lux on a rotary shaker set at 100 rpm. Fifty ml of cells were harvested by centrifugation at 4,000xg at 23°C for 5 min. The supernatant was decanted and cells resuspended in 4 ml TAP medium for subsequent chloroplast transformation by particle bombardment (Cohen et al., *supra*, 1998). All transformations were carried out under kanamycin selection (150 μ g/ml) in which resistance was conferred by the gene encoded by the segment in Figure 1 labeled "Selection Marker".

[0066] PCR was used to identify transformed strains. For PCR analysis, 10⁶ algae cells (from agar plate or liquid culture) were suspended in 10 mM EDTA and heated to 95°C for 10 minutes, then cooled to near 23°C. A PCR cocktail consisting of reaction buffer, MgCl₂, dNTPs, PCR primer pair(s) (Table 1 and shown graphically in FIG. 2A), DNA polymerase, and water was prepared. Algae lysate in EDTA was added to provide template for reaction. Magnesium concentration is varied to compensate for amount and concentration of algae lysate in EDTA added. Annealing temperature gradients were employed to determine optimal annealing temperature for specific primer pairs.

[0067] To identify strains that contain the endo- β -glucanase gene, a primer pair was used in which one primer anneals to a site within the *psbA* 5'UTR (SEQ ID NO. 1) and the other primer anneals within the endo- β -glucanase coding segment (SEQ ID NO. 3). Desired clones are those that yield a PCR product of expected size. To determine the degree to which the endogenous gene locus is displaced (heteroplasmic vs. homoplasmic), a PCR reaction consisting of two sets of primer pairs (in the same reaction) was employed. The first pair of primers amplifies the endogenous locus targeted by the expression vector and consists of a primer that anneals within the *psbA* 5'UTR (SEQ ID NO. 8) and one that

anneals within the *psbA* coding region (SEQ ID NO. 9). The second pair of primers (SEQ ID NOs. 6 and 7) amplifies a constant, or control region that is not targeted by the expression vector, so should produce a product of expected size in all cases. This reaction confirms that the absence of a PCR product from the endogenous locus did not result from cellular and/or other contaminants that inhibited the PCR reaction. Concentrations of the primer pairs are varied so that both reactions work in the same tube; however, the pair for the endogenous locus is 5X the concentration of the constant pair. The number of cycles used was >30 to increase sensitivity. The most desired clones are those that yield a product for the constant region but not for the endogenous gene locus. Desired clones are also those that give weak-intensity endogenous locus products relative to the control reaction.

[0068] Results from this PCR on 96 clones were determined and the results are shown in FIG. 3. Figure 3A shows PCR results using the transgene-specific primer pair. As can be seen, multiple transformed clones are positive for insertion of the *exo-β-glucanase* gene (e.g. numbers 1-14). Figure 3B shows the PCR results using the primer pairs to differentiate homoplasmic from heteroplasmic clones. As can be seen, multiple transformed clones are either homoplasmic or heteroplasmic to a degree in favor of incorporation of the transgene (e.g. numbers 1-14). Unnumbered clones demonstrate the presence of wild-type *psbA* and, thus, were not selected for further analysis.

Table 1. PCR primers.

SEQ ID NO.	Use	Sequence
1.	<i>psbA</i> 5' UTR forward primer	GTGCTAGGTAACCTAAGTTTATTTT
2.	<i>Exo-β-glucanase</i> reverse primer	AACCTTCCACGTTAGCTTGA
3.	<i>Endo-β-glucanase</i> reverse primer	GCATTAGTTGGACCACCTTG
4.	<i>β-glucosidase</i> reverse primer	ATCACCTGAAGCAGGTTTGA
5.	<i>Endoxylanase</i> reverse primer	GCACTACCTGATGAAAAATAACC
6.	Control forward primer	CCGAAGTGAAGTTGGGTTTA
7.	Control reverse primer	GGGGGAGCGAATAGGATTAG
8.	<i>psbA</i> 5' UTR forward primer (wild-type)	GGAAGGGGACGTAGGTACATAAA
9.	<i>psbA</i> 3' reverse primer (wild-type)	TTAGAACGTGTTTTGTTCCCAAT
10.	<i>psbC</i> 5' UTR forward primer	TGGTACAAGAGGATTTTTGTTGTT
11.	<i>psbD</i> 5' UTR forward primer	AAATTTAACGTAACGATGAGTTG
12.	<i>atpA</i> 5' UTR forward primer	CCCCTTACGGGCAAGTAAAC
13.	3HB forward primer (wild-type)	CTCGCCTATCGGCTAACAAG
14.	3HB forward primer (wild-type)	CACAAGAAGCAACCCCTTGA

[0069] To ensure that the presence of the *endo-β-glucanase*-encoding gene led to expression of the *endo-β-glucanase* protein, a Western blot was performed. Approximately 1×10^8 algae cells were collected from TAP agar medium and suspended in 0.5 ml of lysis buffer (750 mM Tris, pH=8.0, 15% sucrose, 100 mM beta-mercaptoethanol). Cells were lysed by sonication (5x30sec at 15% power). Lysate was mixed 1:1 with loading buffer (5% SDS, 5% beta-mercaptoethanol, 30% sucrose, bromophenol blue) and proteins were separated by SDS-PAGE, followed by transfer to PVDF membrane. The membrane was blocked with TBST + 5% dried, nonfat milk at 23°C for 30 min, incubated with anti-FLAG antibody (diluted 1:1,000 in TBST + 5% dried, nonfat milk) at 4°C for 10 hours, washed three times with

TBST, incubated with horseradish-linked anti-mouse antibody (diluted 1:10,000 in TBST + 5% dried, nonfat milk) at 23°C for 1 hour, and washed three times with TBST. Proteins were visualized with chemiluminescent detection. Results from multiple clones (FIG. 4C) show that expression of the endo- β -glucanase gene in *C. reinhardtii* cells resulted in production of the protein.

[0070] Cultivation of *C. reinhardtii* transformants for expression of endo- β -glucanase was carried out in liquid TAP medium at 23°C under constant illumination of 5,000 Lux on a rotary shaker set at 100 rpm, unless stated otherwise. Cultures were maintained at a density of 1×10^7 cells per ml for at least 48 hr prior to harvest.

[0071] To determine if the endo- β -glucanase produced by transformed alga cells was functional, endo- β -glucanase activity was tested using a filter paper assay (Xiao et al., *Biotech. Bioengineer.* 88, 832-37, 2004). Briefly, 500 ml of algae cell culture was harvested by centrifugation at 4000xg at 4°C for 15 min. The supernatant was decanted and the cells resuspended in 10 ml of lysis buffer (100 mM Tris-HCl, pH=8.0, 300 mM NaCl, 2% Tween-20). Cells were lysed by sonication (10x30sec at 35% power). Lysate was clarified by centrifugation at 14,000xg at 4°C for 1 hour. The supernatant was removed and incubated with anti-FLAG antibody-conjugated agarose resin at 4°C for 10 hours. Resin was separated from the lysate by gravity filtration and washed 3x with wash buffer ((100 mM Tris-HCl, pH=8.0, 300 mM NaCl, 2% Tween-20). Endo- β -glucanase was eluted by incubation of the resin with elution buffer (TBS, 250 ug/ml FLAG peptide). Results from Western blot analysis of samples collect after each step (FIG. 4D) show that the endo- β -glucanase protein was isolated. A 20 μ l aliquot of diluted enzyme was added into wells containing 40 μ l of 50 mM NaAc buffer and a filter paper disk. After 60 minutes incubation at 50°C, 120 μ l of DNS was added to each reaction and incubated at 95°C for 5 minutes. Finally, a 36 μ l aliquot of each sample was transferred to the wells of a flat-bottom plate containing 160 μ l water. The absorbance at 540 nm was measured. The results for two transformed strains indicated that the isolated enzyme was functional (absorbance of 0.33 and 0.28).

Example 2. Production of FPP synthases and sesquiterpene synthases in *C. reinhardtii*

[0072] In this example nucleic acids encoding FPP synthase from *G. gallus* and bisabolene synthase from *P. abies* were introduced into *C. reinhardtii*. Transforming DNA is shown graphically in FIG. 4. In this instance the gene encoding FPP synthase (SEQ ID NO. 17) is the segment labeled "transgene" in FIG. 4 and is regulated by the 5' UTR and promoter sequence for the *psbA* gene from *C. reinhardtii* and the 3' UTR for the *psbA* gene from *C. reinhardtii*, and the segment labeled "Resistance Marker" is a kanamycin resistance encoding gene from bacteria, which is regulated by the 5' UTR and promoter sequence for the *atpA* gene from *C. reinhardtii* and the 3' UTR sequence for the *rbcl* gene from *C. reinhardtii*. The bisabolene synthase gene (SEQ ID NO. 18 or SEQ ID NO. 20) is the segment labeled "transgene" in FIG. 4 and is regulated by the 5' UTR and promoter sequence for the *psbA* gene from *C. reinhardtii* and the 3' UTR for the *psbA* gene from *C. reinhardtii*, and the segment labeled "Resistance Marker" is the streptomycin resistance encoding gene from bacteria, which is regulated by the 5' UTR and promoter sequence for the *atpA* gene from *C. reinhardtii* and the 3' UTR sequence for the *rbcl* gene from *C. reinhardtii*. The FPP synthase transgene cassette is targeted to the *psbA* loci of *C. reinhardtii* via the segments labeled "Homology A" and "Homology B," which are identical to sequences of DNA flanking the *psbA* loci on the 5' and 3' sides, respectively. The bisabolene synthase transgene cassette is targeted to the 3HB locus of *C. reinhardtii* via the segments labeled "Homology C" and "Homology D," which are identical to sequences of DNA flanking the 3HB locus on the 5' and 3' sides, respectively. All DNA

manipulations carried out in the construction of this transforming DNA were essentially as described by Sambrook, Fritsch, Maniatis, Molecular Cloning, A Laboratory Manual, 2nd edition, vol. 1, 2 & 3 Cold Spring Harbor Press, 1989, New York and Cohen et al., *Meth. Enzymol.* 297, 192-208, 1998.

[0073] For these experiments, all transformations were carried out on *C. reinhardtii* strain 137c (mt+). Cells were grown to late log phase (approximately 7 days) in the presence of 0.5 mM 5-fluorodeoxyuridine in TAP medium (Gorman and Levine, *Proc. Natl. Acad. Sci., USA* 54:1665-1669, 1965, which is incorporated herein by reference) at 23°C under constant illumination of 450 Lux on a rotary shaker set at 100 rpm. Fifty ml of cells were harvested by centrifugation at 4,000xg at 23°C for 5 min. The supernatant was decanted and cells resuspended in 4 ml TAP medium for subsequent chloroplast transformation by particle bombardment (Cohen et al., *supra*, 1998). All transformations were carried out under kanamycin selection (100 µg/ml) in which resistance was conferred by the gene encoded by the segment in FIG. 4 labeled "Resistance Marker".

[0074] PCR was used to identify transformed strains. For PCR analysis, 10⁶ algae cells (from agar plate or liquid culture) were suspended in 10 mM EDTA and heated to 95°C for 10 minutes, then cooled to near 23°C. A PCR cocktail consisting of reaction buffer, MgCl₂, dNTPs, PCR primer pair(s) (Table 2), DNA polymerase, and water was prepared. Algae lysate in EDTA was added to provide template for reaction. Magnesium concentration is varied to compensate for amount and concentration of algae lysate in EDTA added. Annealing temperature gradients were employed to determine optimal annealing temperature for specific primer pairs.

[0075] To identify strains that contain the FPP synthase gene, a primer pair was used in which one primer anneals to a site within the *psbA* 5'UTR (SEQ ID NO. 21) and the other primer (SEQ ID NO. 26) anneals within the FPP synthase coding segment. Desired clones are those that yield a PCR product of expected size. To identify strains that contain the bisabolene synthase gene, a primer pair was used in which one primer anneals to a site within the *psbA* 5'UTR (SEQ ID NO. 21) and the other primer anneals within the bisabolene synthase coding segment (SEQ ID NO. 27). Desired clones are those that yield a PCR product of expected size in both reactions.

[0076] To determine the degree to which the endogenous *psbA* gene locus is displaced (heteroplasmic vs. homoplasmic), a PCR reaction consisting of two sets of primer pairs (in the same reaction) was employed. The first pair of primers amplifies the endogenous locus targeted by the expression vector and consists of a primer that anneals within the *psbA* 5'UTR (SEQ ID NO. 22) and one that anneals within the *psbA* coding region (SEQ ID NO. 23). The second pair of primers (SEQ ID NOs. 24 and 25) amplifies a constant, or control region that is not targeted by the expression vector, so should produce a product of expected size in all cases. This reaction confirms that the absence of a PCR product from the endogenous locus did not result from cellular and/or other contaminants that inhibited the PCR reaction. Concentrations of the primer pairs are varied so that both reactions work in the same tube; however, the pair for the endogenous locus is 5X the concentration of the constant pair. The number of cycles used was >30 to increase sensitivity. The most desired clones are those that yield a product for the constant region but not for the endogenous gene locus. Desired clones are also those that give weak-intensity endogenous locus products relative to the control reaction. Results from this PCR are shown in FIG. 5, panels A, B, and C.

[0077] To determine if the FPP synthase gene led to expression of the FPP synthase and if the bisabolene synthase gene led to expression of the bisabolene synthase in transformed algae cells, both soluble proteins were immunoprecipitated and visualized by Western blot. Briefly, 500 ml of algae cell culture was harvested by

centrifugation at 4000xg at 4°C for 15 min. The supernatant was decanted and the cells resuspended in 10 ml of lysis buffer (100 mM Tris-HCl, pH=8.0, 300 mM NaCl, 2% Tween-20). Cells were lysed by sonication (10x30sec at 35% power). Lysate was clarified by centrifugation at 14,000xg at 4°C for 1 hour. The supernatant was removed and incubated with anti-FLAG antibody-conjugated agarose resin at 4°C for 10 hours. Resin was separated from the lysate by gravity filtration and washed 3x with wash buffer ((100 mM Tris-HCl, pH=8.0, 300 mM NaCl, 2% Tween-20). Resin was mixed 4:1 with loading buffer (XT Sample buffer; Bio-Rad), samples were heated to 95°C for 1 min, cooled to 23°C, and insoluble proteins were removed by centrifugation. Soluble proteins were separated by SDS-PAGE, followed by transfer to PVDF membrane. The membrane was blocked with TBST + 0.5% dried, nonfat milk at 23°C for 30 min, incubated with anti-FLAG, alkaline phosphatase-conjugate antibody (diluted 1:2,500 in TBST + 0.5% dried, nonfat milk) at 4°C for 10 hours, washed three times with TBST. Proteins were visualized with chemifluorescent detection. Results from multiple clones (FIG. 5D) show that expression of the FPP synthase gene led to expression of the FPP synthase and expression of the bisabolene synthase gene led to expression of the bisabolene synthase.

[0078] Cultivation of *C. reinhardtii* transformants for expression of FPP synthase and bisabolene synthase was carried out in liquid TAP medium at 23°C under constant illumination of 5,000 Lux on a rotary shaker set at 100 rpm, unless stated otherwise. Cultures were maintained at a density of 1×10^7 cells per ml for at least 48 hr prior to harvest.

[0079] To determine whether bisabolene synthase produced in the algae chloroplast is a functional enzyme, sesquiterpene production from FPP was examined. Briefly, 50 mL of algae cell culture was harvested by centrifugation at 4000xg at 4°C for 15 min. The supernatant was decanted and the cells resuspended in 0.5 mL of reaction buffer (25 mM HEPES, pH=7.2, 100 mM KCl, 10 mM MnCl₂, 10% glycerol, and 5 mM DTT). Cells were lysed by sonication (10x30sec at 35% power). 0.33 mg/mL of FPP were added to the lysate and the mixture was transferred to a glass vial. The reaction was overlaid with heptane and incubated at 23°C for 12 hours. The reaction was quenched and extracted by vortexing the mixture. 0.1 mL of heptane was removed and the sample was analyzed by gas chromatography - mass spectrometry (GC-MS). Results are shown in FIG. 6.

TABLE 2

SEQ ID NO.	Use	Sequence
21	psbA 5' UTR forward primer	GTGCTAGGTAACCTTTGATTTTT
22	psbA 5' UTR forward primer (wild-type)	GGAAGGGGACGTAGGTACATAAA
23	psbA 3' reverse primer (wild-type)	TTAGAACGTGTTTTGTTCCCAAT
24	Control forward primer	CCGAAGTGAAGTTGGGTTTA
25	Control reverse primer	GGGGGAGCGAATAGGATTAG
26	FPP synthase reverse primer (163)	CGTTCTTCTGAGAAATGGCTTA
27	Bisabolene synthase reverse primer (307)	GACGTTCTTGACGTTTTGTTTG

Example 3 Expression of Phytase in *S. dimorphus*

[0080] In this example a nucleic acid encoding a phytase (SEQ ID NO. 28 [FD6] or SEQ ID NO. 29 [FD7]) is introduced into *S. dimorphus*. Transforming DNA is shown graphically in Fig. 4D. In this instance the DNA segment labeled "Transgene 1" is a phytase encoding gene (FD6 or FD7), the promoter and 5' UTR for the *psbD* gene from *S. dimorphus*, and the 3' UTR for the *psbA* gene from *S. dimorphus*. The segment labeled "Selection Marker" is the

chloramphenicol acetyl transferase gene (CAT) from *E. coli*, which is regulated by the promoter and 5' UTR sequence for the *tufA* gene from *S. dimorphus* and the 3' UTR sequence for the *rbcl* gene from *S. dimorphus*. The segment labeled "Transgene 2" is the xylanase from *T. reesei* (BD11 SEQ ID NO: 31), the promoter and 5' UTR for the *psbD* gene from *S. dimorphus*, and the 3' UTR for the *psbA* gene from *S. dimorphus*. In another experiment, the vector was prepared without Transgene 2. The transgene expression cassette and selection marker are targeted to the *S. dimorphus* chloroplast genome via the segments labeled "5' Homology" and "3' Homology" which are approximately 1000 bp fragments homologous to sequences of DNA adjacent to nucleotide 071,366 (Site 1; nucleotide locations according to the sequence available from NCBI for *S. obliquus*, NC_008101) on the 5' and 3' sides, respectively. All DNA segments were subcloned into pUC 18 (gutless pUC). All DNA manipulations carried out in the construction of this transforming DNA were essentially as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press 1989) and Cohen et al., *Meth. Enzymol.* 297, 192-208, 1998.

[0081] Transforming DNA was introduced into *S. dimorphus* via particle bombardment with DNA carried on 550 nm gold particles at 500psi and a shooting distance of 4cm. Transformants were selected by growth on TAP-CAM agar medium under constant light 50-100uE at room temperature for approximately 2 weeks. Transformants were streaked onto TAP-CAM agar medium to ensure single colony isolation and grown for 4 days under constant light. Transformants were analyzed by PCR screening for homoplasmy.

[0082] To ensure that the presence of the endoxylanase- and phytase- encoding genes led to expression of the endoxylanase and the phytase proteins, a Western blot was performed. Briefly, algae cells were collected from TAP agar medium and suspended in BugBuster solution (Novagen). Cells were lysed by bead beating using zirconium beads. Cell lysates were clarified by centrifugation and the supernatants were normalized for total soluble protein (Coomassie Plus Protein Assay Kit, Thermo Scientific). Samples were mixed 1:4 with loading buffer (XT Sample Buffer with β -mercaptoethanol, Bio-Rad), heated to 98°C for 5 min, cooled to 23°C, and proteins were separated by SDS-PAGE, followed by transfer to PVDF membrane. The membrane was blocked with Starting Block T20 Blocking Buffer (Thermo Scientific) for 15 min, incubated with horseradish peroxidase-linked anti-FLAG antibody (diluted 1:2,500 in Starting Block T20 Blocking Buffer) at 23°C for 2 hours, washed three times with TBST. Proteins were visualized with chemiluminescent detection. Fig. 7 shows the expression of the phytase gene alone (columns labeled "FD6") or both the phytase gene and the endoxylanase gene (column labeled FD6/BD11) in *S. dimorphus* cells resulted in production of the protein. Wild type cells (in column labeled SE70) did not express either the phytase or the endoxylanase product. Likewise, Fig. 8 shows that expression of the a phytase gene (columns labeled FD7) in *S. dimorphus* cells resulted in the production of protein in 3 clones (middle columns)

[0083] One of skill will appreciate that many other methods known in the art may be substituted in lieu of the ones specifically described or referenced.

Example 4: Expression of Phytase in *C. reinhardtii*

[0084] A nucleic acids encoding xylanase from *T. reesei* (BD11 SEQ ID NO. 31) or a phytase (FD7 SEQ ID NO. 29 or FD11 SEQ ID NO. 30) are introduced into *C. reinhardtii*. Transforming DNA is shown graphically in FIG. 4. In FIG. 4A the segment labeled "Transgene" is a phytase encoding gene (FD7 SEQ ID NO. 29 or FD11 SEQ ID NO. 30), the 5' UTR and promoter sequence for the *psbD* gene from *C. reinhardtii*, and the 3' UTR for the *psbA* gene from *C.*

reinhardtii. The segment labeled "Selection Marker" is a kanamycin resistance encoding gene from bacteria, which is regulated by the 5' UTR and promoter sequence for the *atpA* gene from *C. reinhardtii* and the 3' UTR sequence for the *rbcL* gene from *C. reinhardtii*. The transgene cassette is targeted to the 3HB locus of *C. reinhardtii* via the segments labeled "Homology C" and "Homology D," which are identical to sequences of DNA flanking the 3HB locus on the 5' and 3' sides, respectively. In another instance, the transforming DNA is shown graphically in Fig. 4D. The segment labeled "Transgene 1" is a phytase encoding gene (FD6 SEQ ID NO. 28), the 5'UTR and promoter sequence for the *psbD* gene from *C. reinhardtii*, and the 3' UTR for the *psbA* gene from *C. reinhardtii*. The segment labeled "Selection Marker" is a kanamycin resistance encoding gene from bacteria, which is regulated by the 5' UTR and promoter sequence for the *atpA* gene from *C. reinhardtii* and the 3' UTR sequence for the *rbcL* gene from *C. reinhardtii*. The segment labeled "Transgene 2" is the xylanase from *T. reesei* (BD11 SEQ ID NO. 31), the promoter and 5' UTR for the *psbD* gene from *C. reinhardtii*, and the 3' UTR for the *psbA* gene from *C. reinhardtii*. All DNA manipulations carried out in the construction of this transforming DNA were essentially as described by Sambrook, Fritsch, Maniatis, Molecular Cloning, A Laboratory Manual, 2nd edition, vol. 1, 2 & 3 Cold Spring Harbor Press, 1989, New York and Cohen et al., *Meth. Enzymol.* 297, 192-208, 1998.

[0085] For these experiments, all transformations were carried out on *C. reinhardtii* strain 137c (mt+). Cells were grown to late log phase (approximately 7 days) in the presence of 0.5 mM 5-fluorodeoxyuridine in TAP medium (Gorman and Levine, *Proc. Natl. Acad. Sci., USA* 54:1665-1669, 1965, which is incorporated herein by reference) at 23°C under constant illumination of 450 Lux on a rotary shaker set at 100 rpm. Fifty ml of cells were harvested by centrifugation at 4,000xg at 23°C for 5 min. The supernatant was decanted and cells resuspended in 4 ml TAP medium for subsequent chloroplast transformation by particle bombardment (Cohen et al., *supra*, 1998). All transformations were carried out under kanamycin selection (150 µg/ml) in which resistance was conferred by the gene encoded by the segment in Figure 1 labeled "Selection Marker".

[0086] PCR was used to identify transformed strains. For PCR analysis, 10⁶ algae cells (from agar plate or liquid culture) were suspended in 10 mM EDTA and heated to 95°C for 10 minutes, then cooled to near 23°C. A PCR cocktail consisting of reaction buffer, MgCl₂, dNTPs, PCR primer pair(s) DNA polymerase, and water was prepared. PCR was conducted as described in Example 1 with the addition of SEQ ID NO. 32, SEQ ID NO. 33. and SEQ ID NO. 34 as the reverse primers for FD6, FD7 and FD11, respectively (Table 3). Algae lysate in EDTA was added to provide template for reaction. Magnesium concentration is varied to compensate for amount and concentration of algae lysate in EDTA added. Annealing temperature gradients were employed to determine optimal annealing temperature for specific primer pairs.

[0087] To ensure that the presence of the transgenes led to expression of the enzyme proteins, a Western blot was performed. Approximately 1x10⁸ algae cells were collected from TAP agar medium and suspended in 0.5 ml of lysis buffer (750 mM Tris, pH=8.0, 15% sucrose, 100 mM beta-mercaptoethanol). Cells were lysed by sonication (5x30sec at 15% power). Lysate was mixed 1:1 with loading buffer (5% SDS, 5% beta-mercaptoethanol, 30% sucrose, bromophenol blue) and proteins were separated by SDS-PAGE, followed by transfer to PVDF membrane. The membrane was blocked with TBST + 5% dried, nonfat milk at 23°C for 30 min, incubated with anti-FLAG antibody (diluted 1:1,000 in TBST + 5% dried, nonfat milk) at 4°C for 10 hours, washed three times with TBST, incubated with horseradish-linked anti-mouse antibody (diluted 1:10,000 in TBST + 5% dried, nonfat milk) at 23°C for 1 hour, and washed three times with

TBST. Proteins were visualized with chemiluminescent detection. Results from multiple clones (FIG. 9) show that expression of the phytase genes xylanase genes FD6 and FD11 in *C. reinhardtii* cells resulted in production of the protein. In this experiment, production of the FD7 xylanase was not observed.

Table 3.

SEQ ID NO	Use	Sequence
32	<i>E.coli</i> 4-phytase reverse primer	AGCTGGATTTGGATATGGCATTAC
33	<i>C. crescentus</i> phytase reverse primer	TCAAATTCAGCTTGATGAAGGAAA
34	Alpha galactosidase reverse primer	GCTGCACGAGTTAAACCTTGACTT

Example 5 Generation of an Improved feedstock Comprising an NVPO expressing Biomass Degrading Enzymes

[0088] An improved feedstock is generated by first obtaining alfalfa, barley, grass, spent brewer's grain, and potatoes and mixing the ingredients into a composition. The composition may not be considered an improved feedstock until after the addition of a genetically modified NVPO. In this case, *C.reinhardtii* is genetically modified to produce three biodegradable enzymes, subtilisin, α -amylase and chymotrypsin utilizing the recombinant techniques described in the Examples above. The genes encoding the three enzymes can be obtained from any suitable species using techniques known in the art. The genetically modified *C. reinhardtii* is selected for expression of all three proteins using suitable markers and protein expression as confirmed by Western blot analysis. The activity of each enzyme can be confirmed by FRET analysis using diluted cell lysates and suitable FRET substrates. The genetically modified *C.reinhardtii* is then grown into a biomass at a density of about 500 million cells per ml using a suitable growing system. The biomass is then added to the feedstock composition to generate an improved feedstock. The genetically modified algae are added to the feedstock at a ratio of 1:20. The improved feedstock composition is then exposed to natural sunlight for a holding period of three days with active mixing and mulching 3-4 times a day. The improved feedstock is suitable for providing nutrients to ruminants.

Example 6 Generation of an Improved feedstock Comprising an NVPO secreting a Biomass Degrading Enzyme

[0089] An improved feedstock is generated by first obtaining alfalfa, barley, grass, spent brewer grain, and potatoes and mixing the ingredients into a composition. The composition may not be considered an improved feedstock until after the addition of a genetically modified NVPO. In this case, *C.reinhardtii* is genetically modified to produce and secrete α -amylase by incorporating a nucleic acid sequence comprising a secretion signal into the α -amylase gene. This can be accomplished using any suitable secretion signal sequence known in the art. The gene encoding α -amylase can be obtained from any suitable species using techniques known in the art. The α -amylase gene comprising the secretions signal sequence is transformed into *C. reinhardtii* utilizing the recombinant techniques described in Examples 1-4. The genetically modified *C. reinhardtii* is selected for expression and secretion of α -amylase using a suitable marker. Amylase secretion is confirmed by Western blot analysis of the culture medium in which the genetically modified *C. reinhardtii* is grown. The activity of α -amylase can be confirmed by FRET analysis using diluted cell lysates and a suitable FRET substrate. The genetically modified *C.reinhardtii* is then grown into a biomass at a density of about 500

million cells per ml. The biomass and wet media used to culture the biomass is then added to the feedstock composition to generate an improved feedstock. The genetically modified wet algae are added to the feedstock at a ratio of 1:5. The improved feedstock composition is then exposed to natural sunlight for a holding period of three days with active mixing and mulching 3-4 times a day. The improved feedstock is then suitable for providing nutrients to ruminants.

5

Example 7 Generation of an Improved feedstock Comprising an NVPO expressing Biosynthetic Enzymes

[0090] An improved feedstock is generated by first obtaining alfalfa, barley, grass and spent brewers grain and mixing the ingredients into a composition. The composition may not be considered an improved feedstock until after the addition of a genetically modified NVPO. In this case, *C.reinhardtii* is genetically modified to produce four biosynthetic enzymes, HMG-CoA reductase, Mevalonate kinase, Phosphomevalonate kinase and Mevalonate-5-pyrophosphate decarboxylase. The genes encoding the four enzymes can be obtained from any suitable species using techniques known in the art. The genetically modified *C. reinhardtii* is selected for expression of all four proteins using suitable markers and protein expression is confirmed by Western blot analysis. The activity of all four enzymes can be confirmed by adding HMG-CoA as a substrate to lysates of the genetically modified *C.reinhardtii* and detecting the presence of Isopentenyl-5-pyrophosphate using mass spectrometry analysis. The genetically modified *C.reinhardtii* is then grown into a biomass to a density of about 500 million cells per ml. The biomass is then added to the feedstock composition to generate an improved feedstock. The genetically modified algae are added to the feedstock at a ratio of 1:5. The improved feedstock is suitable for providing nutrients to ruminants.

20 Example 8 Generation of an Improved feedstock Comprising an NVPO expressing a Biodegrading Enzyme and a Biosynthetic Enzyme

[0091] An improved feedstock is generated by first obtaining alfalfa, barley, grass and spent brewers grain and mixing the ingredients into a composition. The composition may not be considered an improved feedstock until after the addition of a genetically modified NVPO. In this case, *C.reinhardtii* is genetically modified to produce four biosynthetic enzymes, HMG-CoA reductase, Mevalonate kinase, Phosphomevalonate kinase and Mevalonate-5-pyrophosphate decarboxylase and three biodegrading enzymes subtilisin, α -amylase and chymotrypsin. The genes encoding the seven enzymes can be obtained from any suitable species using techniques known in the art. The genetically modified *C. reinhardtii* is selected for expression of all seven proteins using suitable markers and protein expression is confirmed by Western blot analysis. The activity of all four biosynthetic enzymes can be confirmed by adding HMG-CoA as a substrate to a lysate of the genetically modified *C.reinhardtii* and detecting the presence of Isopentenyl-5-pyrophosphate using mass spectrometry analysis. The activity of each biodegrading enzyme can be confirmed by FRET analysis using diluted cell lysates and suitable FRET substrates. The genetically modified *C.reinhardtii* is then grown into a biomass to a density of about 500 million cells per ml. The biomass is then added to the feedstock composition to generate an improved feedstock. The genetically modified algae are added to the feedstock at a ratio of 1:5. The improved feedstock is suitable for providing nutrients to ruminants.

Example 9 Generation of an Improved feedstock Comprising a portion of an NVPO expressing a Biodegrading Enzyme and a Biosynthetic Enzyme

[0092] An improved feedstock is generated by first obtaining alfalfa, barley, grass and spent brewers grain and mixing the ingredients into a composition. The composition may not be considered an improved feedstock until after the addition of a genetically modified NVPO. In this case, *C.reinhardtii* is genetically modified to produce four biosynthetic enzymes, HMG-CoA reductase, Mevalonate kinase, Phosphomevalonate kinase and Mevalonate-5-pyrophosphate decarboxylase and three biodegrading enzymes subtilisin, α -amylase and chymotrypsin. The genes encoding the seven enzymes can be obtained from any suitable species using techniques known in the art. The genetically modified *C.reinhardtii* is selected for expression of all seven proteins using suitable markers and protein expression is confirmed by Western blot analysis. The activity of all four biosynthetic enzymes can be confirmed by adding HMG-CoA as a substrate to a lysate of the genetically modified *C.reinhardtii* and detecting the presence of Isopentenyl-5-pyrophosphate using mass spectrometry analysis. The activity of each biodegrading enzyme can be confirmed by FRET analysis using diluted cell lysates and suitable FRET substrates. The genetically modified *C.reinhardtii* is then grown into a biomass using a suitable growing system at a density of about 500 million cells per ml. The biomass is then subjected to a refining process wherein the biomass is dried, crushed and the lipid content of the biomass is extracted with hexane yielding a defatted biomass. The defatted biomass is then subjected to an evaporation step to remove residual hexane from the biomass. The resulting biomass is then added to the feedstock composition to generate an improved feedstock. The genetically modified algae are added to the feedstock at a ratio of 1:1. The improved feedstock is suitable for providing nutrients to ruminants.

CLAIMS

WHAT IS CLAIMED IS:

1. A composition, comprising:
 - 5 a feedstock; and
 - a genetically modified non-vascular photosynthetic organism, or a cellular component thereof, wherein the organism comprises at least one exogenous nucleic acid encoding a biomass-degrading enzyme or exogenous nucleic acid resulting in increased expression of an endogenous biomass-degrading enzyme compared to said organism without said genetic modification.
- 10 2. The composition of claim 1, wherein said organism is at least partially depleted of at least one of a lipid, fatty acid, isoprenoid, carotenoid, carbohydrate, or selected protein.
3. The composition of claim 1, wherein the biomass-degrading enzyme is at least one of a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase or phytase.
4. The composition of claim 3, wherein the biomass-degrading enzyme is a carbohydrase.
- 15 5. The composition of claim 4, wherein the carbohydrase is at least one of an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase.
6. The composition of claim 3, wherein the biomass-degrading enzyme is a protease.
7. The composition of claim 6, wherein the protease is at least one of a subtilisin, bromelain, or fungal acid-stable protease.
- 20 8. The composition of claim 1, wherein the biomass-degrading enzyme is a phytase.
9. The composition of claim 8, wherein the phytase is of bacterial or fungal origin.
10. The composition of claim 1, wherein the biomass-degrading enzyme is an enzyme other than a phytase.
11. The composition of claim 1, wherein the non-vascular photosynthetic organism is a non-vascular photosynthetic organism other than *Porphyridium*.
- 25 12. The composition of any one of claims 1-10, wherein the non-vascular photosynthetic organism is *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*.
13. The composition of any one of claims 1-10, wherein said genetic modification impairs photosynthetic capability of said organism.
14. The composition of any one of claims 1-10, wherein the genetically modified non-vascular photosynthetic
 - 30 organism has a higher lipid, fatty acid, or isoprenoid content relative to an unmodified non-vascular photosynthetic organism of the same species.
15. The composition of any one of claims 1-10, wherein the genetically modified non-vascular photosynthetic organism is prokaryotic.
16. The composition of any one of claims 1-10, wherein the genetically modified non-vascular photosynthetic
 - 35 organism is eukaryotic.
17. The composition of any one of claims 1-10, wherein the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts,

potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

18. The composition of any one of claims 1-10, wherein said genetically modified organism further comprises at least one exogenous nucleic acid encoding enzyme in an isoprenoid biosynthesis pathway or exogenous nucleic acid which results in the increased production of an endogenous isoprenoid at higher levels relative to an unmodified organism of the same species.

19. The organism of claim 18, wherein the at least one isoprenoid biosynthesis pathway enzyme is an enzyme in a mevalonate pathway.

20. The organism of claim 18, wherein the at least one isoprenoid biosynthesis pathway enzyme is a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase.

21. A method for producing an improved feedstock composition, comprising:
combining a genetically modified non-vascular photosynthetic organism or a cellular component thereof with a feedstock to generate the improved feedstock composition, wherein the organism comprises at least one exogenous nucleic acid encoding a biomass-degrading enzyme or exogenous nucleic acid resulting in increased expression of an endogenous biomass-degrading enzyme compared to said organism without said genetic modification.

22. The method of claim 21, further comprising at least partially removing at least one of a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified non-vascular photosynthetic organism prior to combining said organism with said feedstock.

23. The method of claim 21, wherein the biomass-degrading enzyme is at least one of a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, or phytase.

24. The method of claim 23, wherein the biomass-degrading enzyme is a carbohydrase.

25. The method of claim 24, wherein the carbohydrase is at least one of an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase.

26. The method of claim 21, wherein the biomass-degrading enzyme is a protease.

27. The method of claim 26, wherein the protease is at least one of a subtilisin, bromelain, or fungal acid-stable protease.

28. The method of claim 21, wherein the biomass-degrading enzyme is a phytase.

29. The method of claim 21, wherein the organism further comprises at least one exogenous nucleic acid encoding an enzyme in an isoprenoid biosynthesis pathway.

30. The method of claim 29, wherein the at least one enzyme in an isoprenoid biosynthesis pathway is in a mevalonate pathway.

31. The method of claim 30, wherein the at least one enzyme in an isoprenoid biosynthesis pathway is a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase.

32. The method of any one of claims 21-31, wherein the genetically modified non-vascular photosynthetic organism is prokaryotic.

33. The method of any one of claims 21-31, wherein the genetically modified non-vascular photosynthetic organism is eukaryotic.

34. The method of any one of claims 21-31, wherein the non-vascular photosynthetic organism comprises *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*.

35. The method of any one of claims 21-31, further comprising lysing the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock.

36. The method of any one of claims 21-31, further comprising drying the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock.

37. The method of any one of claims 21-31, wherein the genetically modified non-vascular photosynthetic organism is grown under heterotrophic conditions.

38. The method of any one of claims 21-31, wherein the genetically modified non-vascular photosynthetic organism is grown in darkness.

39. The method of any one of claims 21-31, wherein the genetically modified non-vascular photosynthetic organism is grown under autotrophic conditions.

40. The method of any one of claims 21-31, wherein the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

41. A method for increasing nutrient availability in a feedstock, comprising:
combining a genetically modified non-vascular photosynthetic organism or a cellular component thereof with a feedstock to generate an improved feedstock composition, wherein the organism comprises at least one exogenous nucleic acid encoding a biomass-degrading enzyme or exogenous nucleic acid resulting in increased expression of an endogenous biomass-degrading enzyme compared to said organism without said genetic modification;
and

feeding the improved feedstock composition to an animal, wherein the improved feedstock has an increased nutrient availability as compared to the feedstock prior to combining with the genetically modified non-vascular photosynthetic organism.

42. The method of claim 41, further comprising at least partially removing at least one of a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified non-vascular photosynthetic organism prior to combining said organism with said feedstock.

43. The method of claim 41, wherein the biomass-degrading enzyme is at least one of a galactanase, xylanase, protease, carbohydrase, lipase, reductase, oxidase, transglutaminase, or phytase.

44. The method of claim 43, wherein the biomass-degrading enzyme is a carbohydrase.

45. The method of claim 44, wherein the carbohydrase is at least one of an α -amylase, β -amylase, endo- β -glucanase, endoxylanase, β -mannanase, α -galactosidase, or pullulanase.

46. The method of claim 41, wherein the biomass-degrading enzyme is a protease.

47. The method of claim 46, wherein the protease is at least one of a subtilisin, bromelain, or fungal acid-stable protease.

48. The method of claim 41, wherein the biomass-degrading enzyme is a phytase.

49. The method of claim 41, wherein the organism further comprises an exogenous nucleic acid encoding at least one of an enzyme in an isoprenoid biosynthesis pathway.

50. The method of claim 49, wherein the enzyme in an isoprenoid biosynthesis pathway is in a mevalonate pathway.

51. The method of claim 49, wherein the enzyme in an isoprenoid biosynthesis pathway is at least one of a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase.

52. The method of any one of claims 41-51, wherein the genetically modified non-vascular photosynthetic organism is prokaryotic.

53. The method of any one of claims 41-51, wherein the genetically modified non-vascular photosynthetic organism is eukaryotic.

54. The method of any one of claims 41-51, wherein the non-vascular photosynthetic organism comprises *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvialis*.

55. The method of any one of claims 41-51, further comprising lysing the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock.

56. The method of any one of claims 41-51, further comprising drying the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock.

57. The method of any one of claims 41-51, wherein the genetically modified non-vascular photosynthetic organism is grown under heterotrophic conditions.

58. The method of any one of claims 41-51, wherein the genetically modified non-vascular photosynthetic organism is grown in darkness.

59. The method of any one of claims 41-51, wherein the genetically modified non-vascular photosynthetic organism is grown under autotrophic conditions.

60. The method of any one of claims 41-51, wherein the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

61. The method of any one of claims 41-51, wherein the animal is a ruminant.

62. The method of claim 61, wherein said ruminant is a bovine, sheep, oxen, goat, deer, or elk

63. The method of any one of claims 41-51, wherein the animal is a non-ruminant.

64. The method of claim 63, wherein the non-ruminant is a monogastric animal.

65. The method of claim 63, wherein the non-ruminant animal comprises a pig, horse, avian species or aquatic species.

66. The method of claim 65, wherein the avian species comprises a chicken, duck, goose, turkey, pheasant, partridge, grouse, dove, or quail.

67. The method of claim 64, wherein the monogastric animal is an aquatic species.

68. The method of claim 67, wherein the aquatic species comprises trout, salmon, tilapia, catfish, perch,
5 bluegill or carp.

69. The method of claim 67, wherein the aquatic species is a crustacean.

70. The method of claim 69, wherein the crustacean comprises shrimp, lobster, crab or crayfish.

71. A method for decreasing the amount of phytic acid in animal manure comprising:

10 combining a genetically modified non-vascular photosynthetic organism or a cellular component thereof with a feedstock to generate an improved feedstock composition, wherein the organism comprises at least one exogenous nucleic acid encoding a phytase; and

feeding the improved feedstock composition to an animal, wherein the manure of the animal has a lower phytic acid content than the manure of an animal not fed the improved feedstock composition.

72. The method of claim 71, wherein the genetically modified non-vascular photosynthetic organism is
15 prokaryotic.

73. The method of claim 71, wherein the genetically modified non-vascular photosynthetic organism is eukaryotic.

74. The method of claim 71, wherein the non-vascular photosynthetic organism comprises *S. dimorphus*, *S. obliquus*, *C. reinhardtii*, *D. salina*, or *H. pluvalis*.

20 75. The method of any one of claims 71-74, further comprising lysing the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock.

76. The method of any one of claims 71-74, further comprising drying the genetically modified non-vascular photosynthetic organism prior to combining with the feedstock.

77. The method of any one of claims 71-74, wherein the genetically modified non-vascular photosynthetic
25 organism is grown under heterotrophic conditions.

78. The method of any one of claims 71-74, wherein the genetically modified non-vascular photosynthetic organism was grown in darkness.

79. The method of any one of claims 71-74, wherein the genetically modified non-vascular photosynthetic organism was grown under autotrophic conditions.

30 80. The method of any one of claims 71-74, wherein the feedstock comprises one or more of alfalfa, barley, blood meal, beet, bone meal, brewer grain, brewer's yeast, broom grass, carrot, cattle manure, clover, coffee, corn, corn gluten meal, distiller grains, poultry fat, grape, hominy feed, hop leaves, spent hops, molasses, oats, algae, peanuts, potato, poultry litter, poultry manure, rape meal, rye, safflower, sorghum, soybean, soy, sunflower meal, timothy hay, or triticale.

35 81. The method of any one of claims 71-74, wherein wherein the organism further comprises an exogenous nucleic acid encoding at least one of an enzyme in an isoprenoid biosynthesis pathway.

82. The method of claim 81, wherein the enzyme in an isoprenoid biosynthesis pathway is in a mevalonate pathway.

83. The method of claim 81, wherein the enzyme in an isoprenoid biosynthesis pathway is at least one of a farnesyl pyrophosphate synthase, geranyl geranyl phosphate synthase, squalene synthase, thioesterase, or fatty acyl-CoA desaturase.

84. The method of any one of claims 71-74, further comprising at least partially removing at least one of a lipid, fatty acid, isoprenoid, or carbohydrate from a genetically modified non-vascular photosynthetic organism prior to combining said organism with said feedstock.

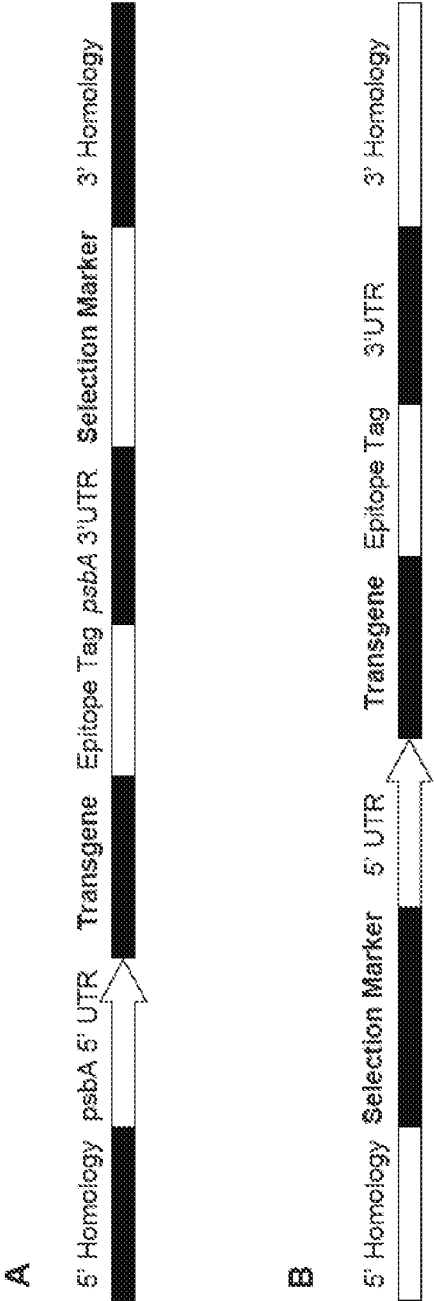
85. The method of any one of claims 71-74, wherein the animal is a non-ruminant.

86. The method of claim 85, wherein the non-ruminant is a monogastric animal.

87. The method of claim 85, wherein the non-ruminant animal comprises a pig, horse, dog, cat, rabbit, rodent or avian species.

88. The method of claim 83, wherein the avian species comprises a chicken, turkey, pheasant, partridge, or quail.

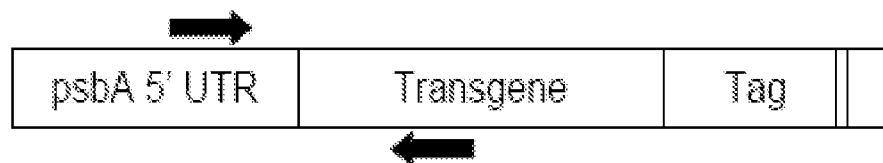
Figure 1



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Figure 2

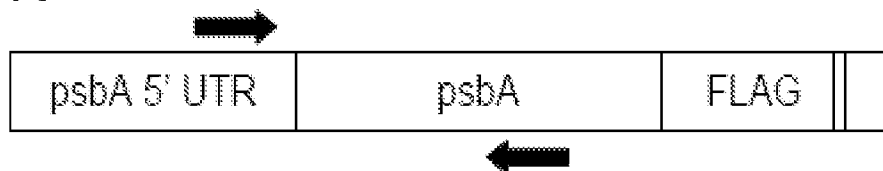
Gene:



Control:

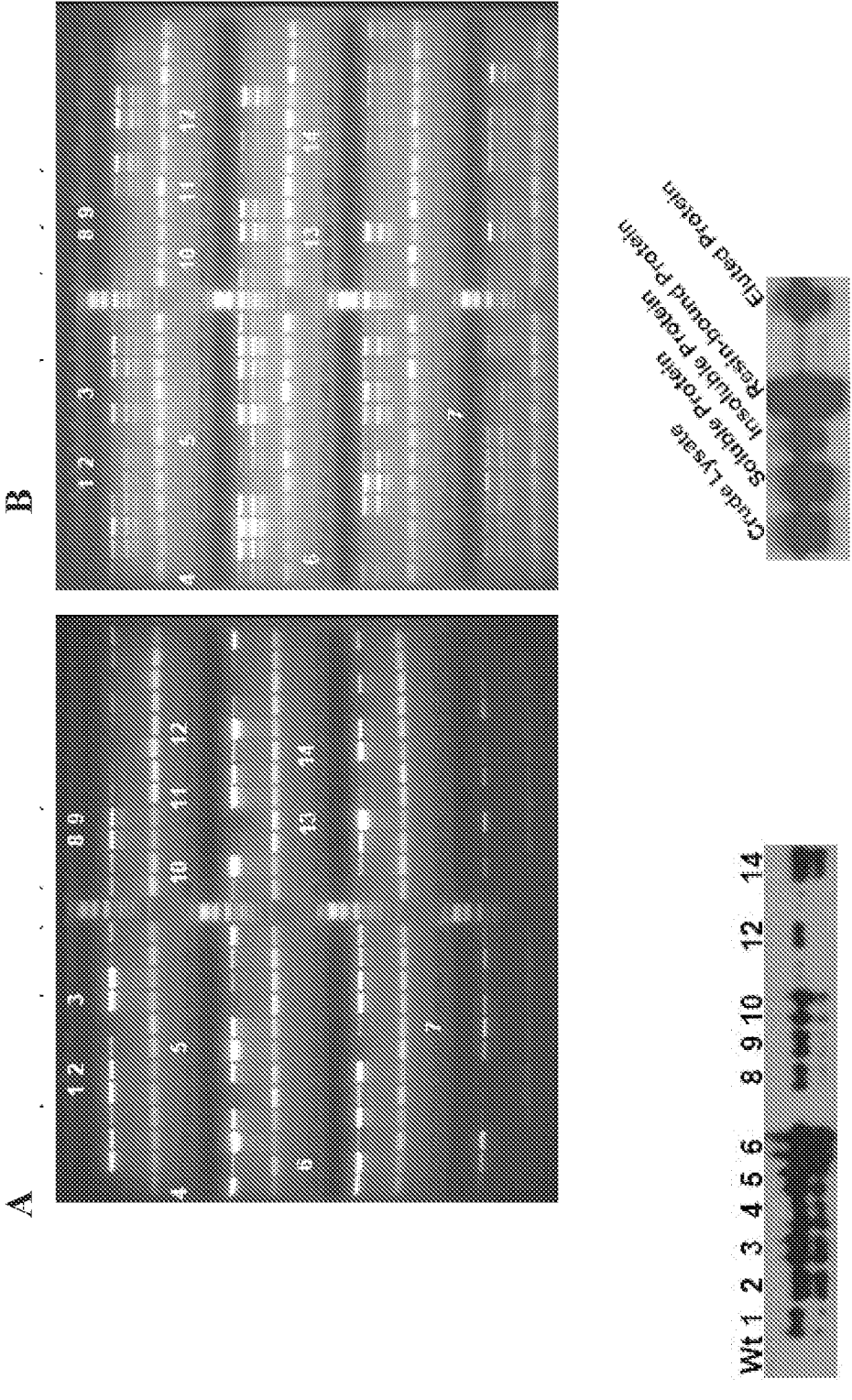


WT:



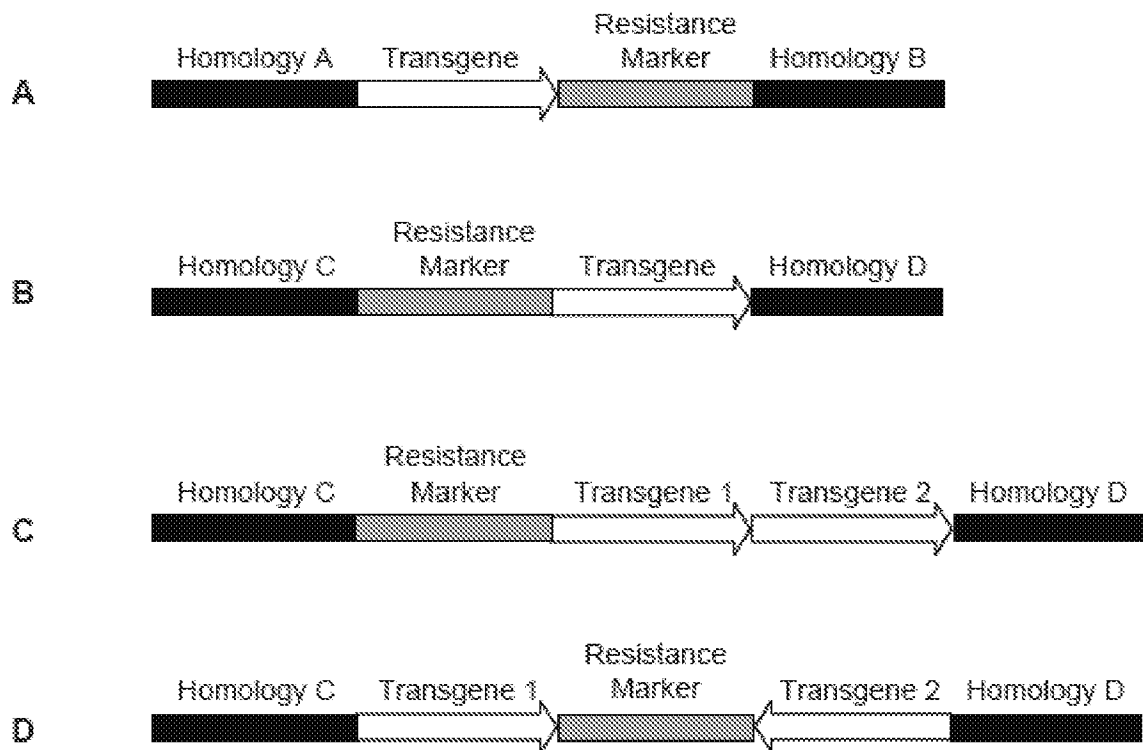
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Figure 3

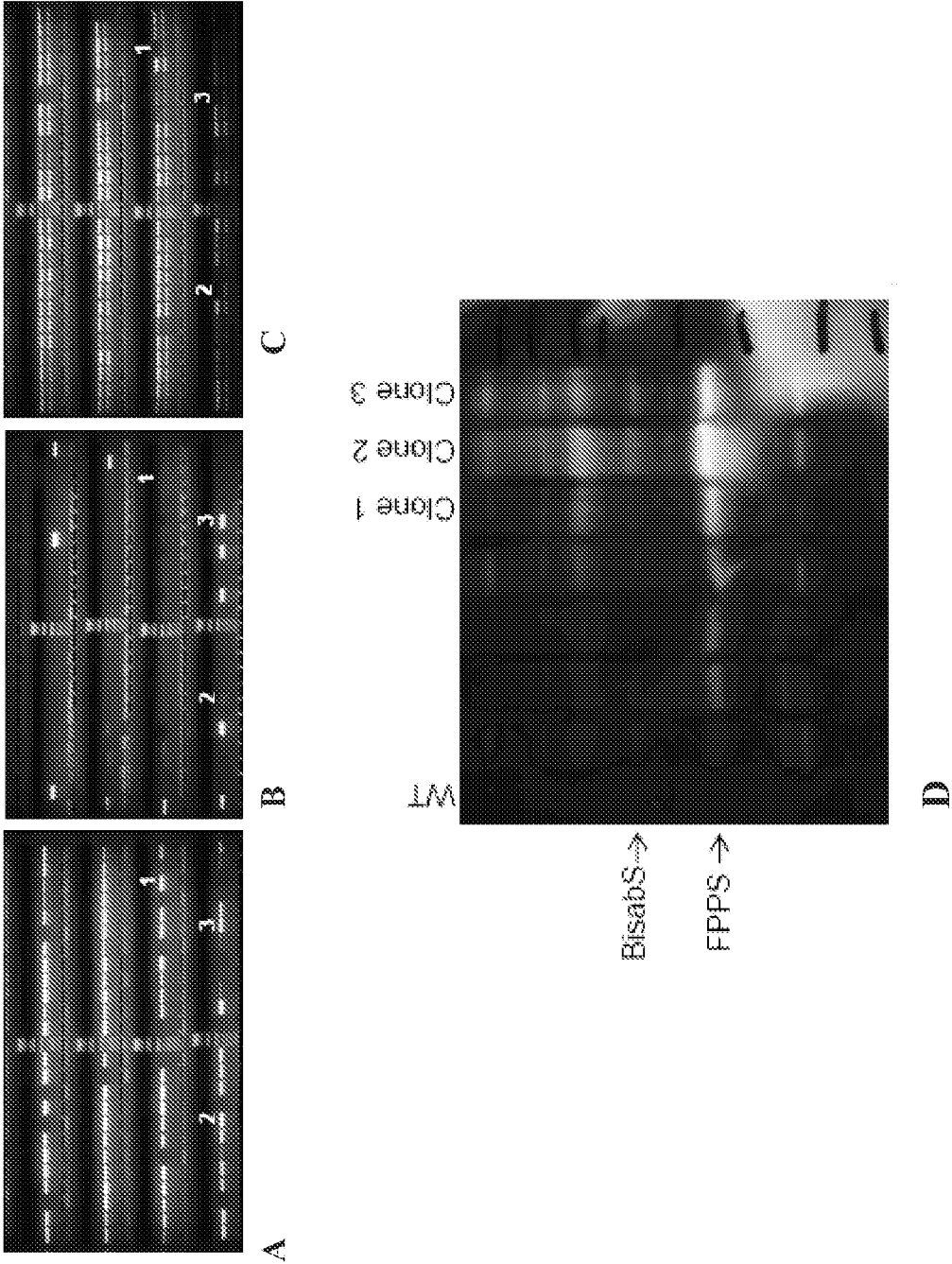


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FIGURE 4



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Figure 5



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Figure 6

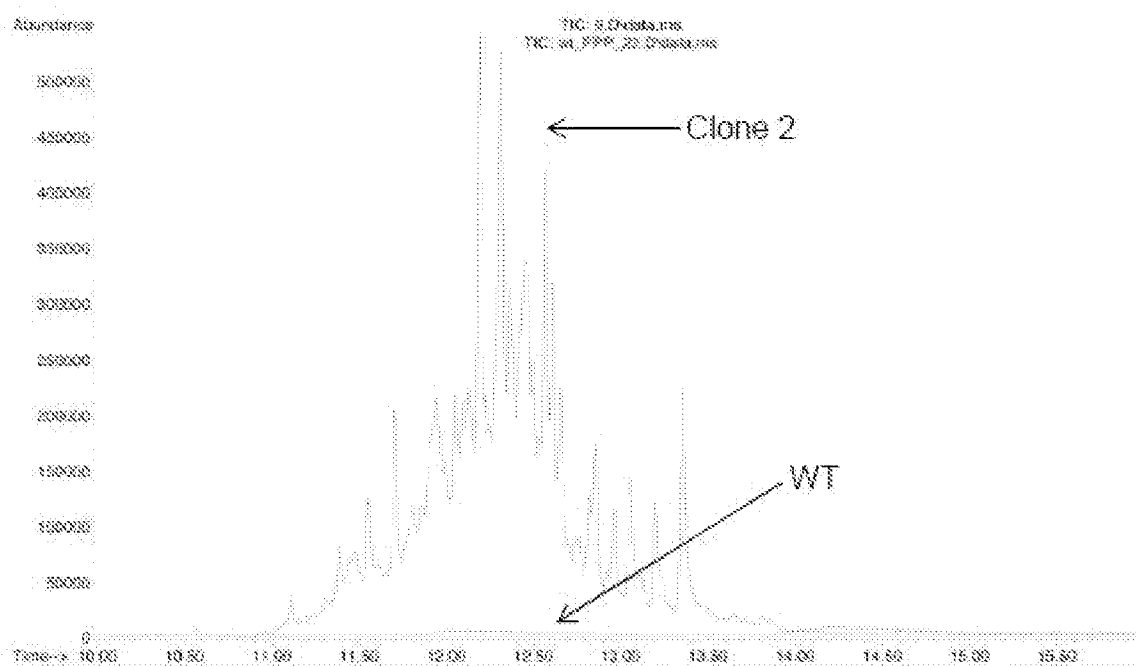
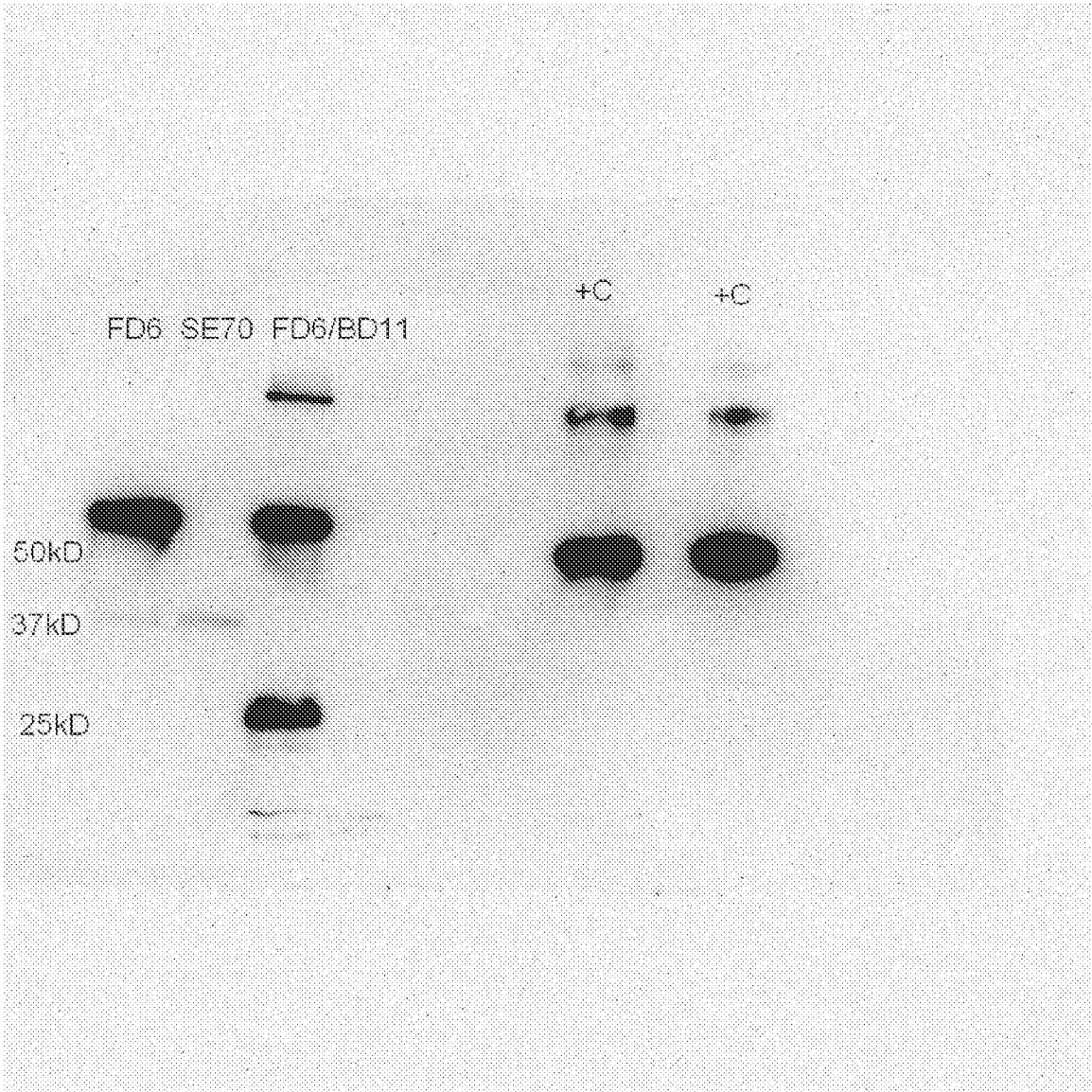


Figure 7



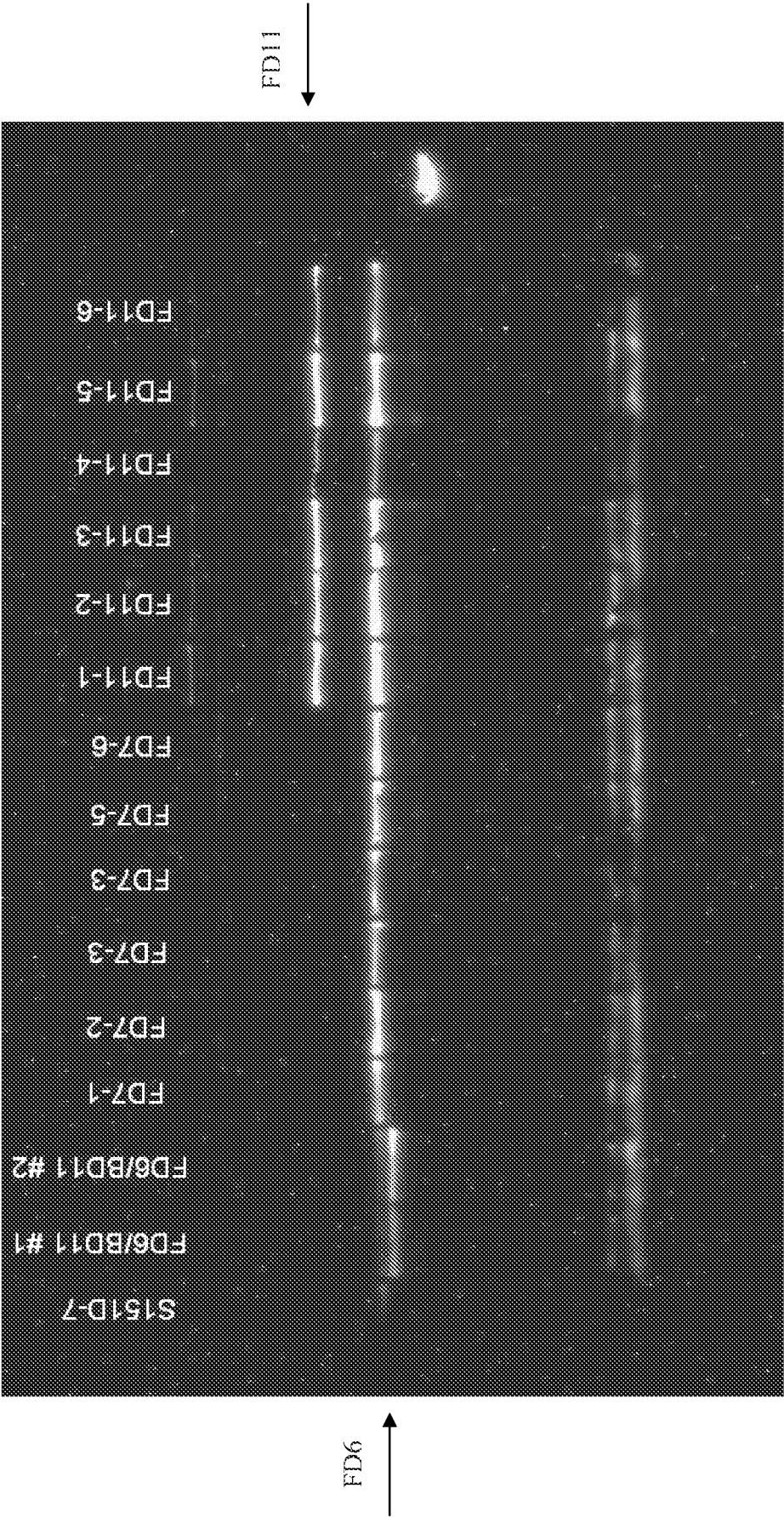


Figure 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2009/062843

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl.		
C12N 1/13 (2006.01)	A01H 13/00 (2006.01)	A23K 1/165 (2006.01) A23L 1/105 (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
Databases: AGRICOLA, BIOSIS, MEDLINE, CAPLUS, EPODOC, WPI; search terms: alga, cyanobacteria, chlorophyta, chlamydomonas, scenedesmus, dunaliella, haematococcus, recombinant, genetically modified, feedstock, foodstuff, biomass degrading enzyme, carbohydrase, alpha-amylase, glucanase, endo-xylanase, mannanase, galactosidase, pullulanase, galactanase, xylanase, cellulase, protease, proteinase, subtilisin, bromelain, phytase, reductase, oxidase, lipase, transglutaminase, similar terms.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 2008/150463 A2 (SAPPHIRE ENERGY et al.) 11 December 2008 whole document, in particular paragraphs [0007] – [0011]; [0013]; [0016]; table 1; examples 1-4, 6, 9	1-40
P, X	WO 2009/132008 A2 (CHROMATIN, INC.) 29 October 2009 whole document, especially paragraphs [0004] – [008]; [0012]; [0025]; [0029] – [0032]; [0171]; table 1	1-40
A	US 2004/0047881 A1 (KYLE, David J.) 11 March 2004 whole document, especially paragraphs [0003]; [0014]; [0017]; [0018]; [0027]; [0055]; [0056]; claims 1, 5, 14, 15	
A	WO 2002/056703 A1 (THE OHIO STATE UNIVERSITY et al.) 25 July 2002 whole document, especially abstract, pg. 1, line 12 – pg. 2, line 17; claims 1 and 2	
☒ Further documents are listed in the continuation of Box C ☒ See patent family annex		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 08 January 2010		Date of mailing of the international search report 12 JAN 2010
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. +61 2 6283 7999		Authorized officer IRENE BAROLI AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No : +61 2 6283 7968

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
 - ☐ on paper
 - ☐ in electronic form
 - b. (time)
 - ☐ in the international application as filed
 - ☐ together with the international application in electronic form
 - ☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

A sequence listing was filed with the application but it was not used for the purposes of this search and opinion.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2009/062843

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2008/0131560 A1 (LANAHAN, Michael B. et al.) 5 June 2008 whole document, especially paragraphs [0021]; [0096]; [0122] – [0129]; [0135]; [0142]; [0145]	
A	US 2003/0103958 A1 (SHORT, Jay M. et al.) 5 June 2003 whole document, in particular paragraphs [0046]; [0061]; [0064]; [0101]; example 4; claims 42 and 96	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2009/062843

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report				Patent Family Member			
WO	2008150463	AU	2008260555	AU	2008260557	US	2009246766
		US	2009253169	WO	2008150461		
WO	2009132008		NONE				
US	20040047881	AU	2002255851	WO	2002076391		
WO	2002056703	US	6932980				
US	20082364266	AU	2002364266	WO	2003057247	EP	1465655
US	20030103958	AU	735371	WO	2000071728	EP	1301592
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.							
END OF ANNEX							