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(54) Title: IMPROVED EDIBLE COMPOSITIONS COMPRISING AN IMPROVED *CHLAMYDOMONAS REINHARDTII* COMPONENT

(57) **Abstract:** The subject invention pertains to a composition for humans or animals that provides effective amounts of algal protein for nutrition or functionality, or algal pigment for functionality, without concurrently adding algal starch that leads to increasing the composition's glycemic index or unwanted calories. The invention provides for a foodstuff comprising wholemeal biomass of *Chlamydomonas reinhardtii* that is higher in protein and substantially starchless. This provides for flexibility in food compositions, ingredient proportions, and algal inclusion rates to attain a flavor, texture, shape, viscosity, rheology, color, ingredient substitutions, enhanced palatability, or other desirable attribute in uncooked or cooked products. The invention provides a process for producing an edible product comprising the *C. reinhardtii* component.

DESCRIPTION

TITLE

IMPROVED EDIBLE COMPOSITIONS COMPRISING AN IMPROVED *CHLAMYDOMONAS REINHARDTII* COMPONENT

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims the benefit of U.S. Provisional Application Serial No. 63/507,826, filed June 13, 2023, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

There is a growing global call for affordable, natural, safe, and efficacious products that benefit consumer long-term health while protecting the environment. Recurring droughts, heatwaves, and farmable land losses are diminishing or even devastating crop yields, while livestock are also suffering the ill effects of climate change and bad weather. Such trends can impact the long-term availability of nutritious and functional food components, especially proteins, plant-derived antioxidants and vitamins, and healthy fatty acids. Production of microalgae using fermentation technology can replace unsustainable or problematic products or ingredients currently used in the marketplace and thereby address the vulnerabilities of our food systems. With high algal cell titers in vertical production tanks enabled by aerobic fermentation cultivation, the volume requirements for irrigation water, drinking water, and for farm and grazing land areas are greatly reduced. Microalgae are distinguished by their potential to generate numerous components desirable in foodstuffs and beverages, human and pet dietary supplements, and other animal foods (feeds). As a nutrient dense component of edible compositions, microalgae can be considered a “superfood” containing proteins and amino acids, fats including omega-polyunsaturated fatty acids and phospholipids, fiber, pigments, antioxidants, terpenes, flavonoids, vitamins, minerals, flavorings, steroids/sterols, and other nutritional and functional compounds. Nevertheless, it is the protein value of microalgae that is considered paramount (Caporgno *et al.*, 2018). The global algae proteins market is estimated to reach USD 1.1 Billion by 2026.

Given the need for a variety of sustainable and healthy sources of non-animal protein and pigments in our diets using ingredients, there remains a need for an improved composition

of a microalgae foodstuff for practical deployment purposes in foods, which includes pet foods, beverages, and food supplements.

BRIEF SUMMARY OF THE INVENTION

The present invention pertains to food, beverage, and food supplement compositions for organisms, including, for example, humans and animals, comprising improved biomass components derived from microalgae in combination with one or more other edible components.

In one embodiment, the improved microalgal component of the food compositions is derived from algae, such as, for example, *Chlamydomonas reinhardtii*, grown heterotrophically comprising protein exceeding a concentration of about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or about 90%, and with a concentration of starch of less than about 2.25%, about 2.0%, about 1.75%, about 1.5%, about 1.25%, about 1%, about 0.75%, about 0.5%, about 0.25%, about 0.2%, about 0.15%, about 0.1%, or 0% by weight.

In certain embodiments, the subject composition comprises a novel composition of *Chlamydomonas reinhardtii* biomass with a protein concentration exceeding 50% by weight and a starch concentration of less than about 2.25% by weight and at least one other edible component combined in a food, beverage or food supplement.

In certain embodiments, *Chlamydomonas reinhardtii* biomass is present in an effective amount to deliver algal protein, pigment or other algal component for nutrition or other functional aspects without also adding algal starch. The improved *Chlamydomonas reinhardtii* biomass can be present in an effective amount to deliver a nutrient, a color, a palatant, or an antioxidant.

In certain embodiments, the subject invention further pertains to methods of producing *Chlamydomonas reinhardtii* strains that have the preferred phenotypic characteristics exhibited in biomass produced under heterotrophic cultivation conditions by genetic crossing. In one embodiment, both zygotes and non-zygotic cells are desiccated after promoting agglutination through proximity to yield progenies wherein the non-zygotic cells die from desiccation and the zygotes survive desiccation. Some embodiments describe obtaining substantially starchless cell types from zygotes with a range of colors.

In some embodiments, the biomass is comprised of cells with a phenotype of protein above about 50% dry cell weight and being substantially starchless that originates from zygotes from genetic crossings in which one parent is unable to accumulate detectable starch (e.g., less

than about 2.25%). In some embodiments, the biomass is comprised of cells with a phenotype of protein above about 50% dry cell weight and being substantially starchless that originates from a mutation (through mutagenesis that is actively performed or occurs in the cell as a random event or "sport") in any genetic loci or regulatory elements for the loci that affect biosynthesis or storage of starch. In some embodiments, the cell phenotype arises through epigenetic changes. In some embodiments, the higher protein composition of the algal biomass is obtained by chemical removal of starch, leaving a biomass with concentrated protein. In certain embodiments, the baseline protein content of biomass exceeds 50% with no starch content at the outset of growth under nutrient replete cultivation, including any lag phase and the exponential growth phase. In some embodiments, culture medium with excess nutrients results in biomass with relatively lower protein amounts but which are still in excess of 50%. In some embodiments, the methods for determining starch content, protein content, and pigment content as well as other component fractions are provided. In one embodiment, protein digestibility is assessed.

In certain embodiments, provided herein are methods for producing *C. reinhardtii* algae with higher amounts of protein and negligible amounts of starch (e.g., less than about 2.25%) or no starch. In some embodiments, the algae with higher protein and negligible starch also produce pigments. The pigments can be carotenoids, chlorophyll, heme, or other protein-binding pigments. The algae can be cultivated under aerobic heterotrophic conditions. In some embodiments, the growth conditions and processes for production of the *C. reinhardtii* are provided. In various embodiments, the heterotrophic production culture producing the *C. reinhardtii* biomass that is higher protein and substantially starchless uses an added carbon source for energy that is not a sugar, preferably an acetic acid or acetate. In various embodiments, the heterotrophic production culture results from the inoculation of growth media with a substantially pure culture of *Chlamydomonas* with an improved phenotype of protein levels above about 50%, above about 60%, above about 70%, and above about 80% and being substantially starchless. The process can entail a seed train to scale up the production cultures to the final scale, which is then harvested to yield the biomass with the preferred features for an edible composition. The described process also entails a final scale of production that can be maintained in a draw and fill, semi-continuous, or continuous mode of operation for producing biomass for harvest.

In certain embodiments, *Chlamydomonas reinhardtii* can be produced under heterotrophic cultivation conditions to synthesize protein at higher rates and starch at lower rates. In some embodiments, the synthesis of pigments is also produced at a higher rate with

the subject methods. The wholemeal of the improved higher purity phenotype of *Chlamydomonas reinhardtii* is more efficiently and sustainably produced in terms of utilizing carbon provided in the form of acetic acid in the fermentation feedstock. The carbon nutrient is selectively partitioned into higher nutritional value protein or pigment rather than into lower nutritional value starch compared to the wholemeal of non-improved phenotype of *Chlamydomonas reinhardtii* with protein subceeding 50% and containing starch.

In certain embodiments, the blockage of starch biosynthesis due to genetics and/or epigenetics (i.e., genomics and/or epigenomics) that results in the absence of measurable starch in a biomass of the instant invention also results in the presence of elevated protein exceeding about 50% dry weight of the biomass for algae grown under nutrient replete cultivation in heterotrophic fed-batch fermentation. Under these cultivation conditions, biomass is produced by cell growth in fermentation supplied with fed carbon in the form of acetic acid and cells that do not assimilate carbon directly via uptake of CO_2 using photosynthesis.

In certain embodiments, for phenotypes of *Chlamydomonas* in which starch subceeds about 2.25% or is undetectable in its biomass due to genetics and/or epigenetics blocking starch biosynthesis, and in which protein exceeds about 50% under nutrient replete cultivation, the carbon provided from acetic acid as the exogenous source of carbon for heterotrophic algal culture in fed-batch fermentation is preferentially directed into protein production. In certain embodiments, this is measured by a more efficient (i.e., lower) acetic acid usage rate for production of protein in substantially starchless biomass containing protein exceeding about 50% by weight in biomass compared to wildtype below about 50% protein by weight and having starch above about 2.25% in biomass sampled throughout nutrient replete algal cultivation. In certain embodiments, protein is consistently high already at the outset of a cultivation including lag phase while there is little or no change measured in storage compounds of starch and lipids during cultivation compared to wildtype. For the latter, lipid content falls within the range measured in biomass of the wildtype parent (having starch above about 2.25%) grown under the same heterotrophic nutrient replete fed-batch fermentation cultivation conditions.

In certain embodiments the baseline protein content of *Chlamydomonas* biomass is at least about 50% by weight and the starch content is less than about 2.25%. In particular embodiments, the protein content is at least about 70% and starch is less than about 2.25%. In particular embodiments, the baseline protein content is at least about 70% and starch is less than about 0.1%. In particular embodiments, the baseline protein content exceeds about 70%

and starch is less than about 0.1%. In particular embodiments, the baseline protein content is at least about 70% and starch is undetectable.

In several embodiments, carbon flux is directed towards protein biosynthesis due to genetic and/or epigenetic changes compared to wildtype *Chlamydomonas*. In several embodiments this produces a wholemeal that is high in protein and lacking starch for use in the instant invention. In certain embodiments, less carbon in the form of acetic acid or acetate feedstock is required to be supplied during fermentation to produce a biomass is at least about 50% by weight and the starch content is less than about 2.25%.

In certain embodiments, about one-half to one-third the amount of acetic acid and/or acetate is required to be supplied during fermentation cultivation to produce nutritive protein in substantially starchless biomass with about 70% to about 80% protein content compared to starch-containing biomass with about 30% to about 40% protein from wildtype algae. In certain embodiments, on a protein basis, the improved high protein starchless phenotype has a much more efficient acetic acid and/or acetate usage, as seen by acetic acid/acetate feedstock conversion when compared to wildtype *Chlamydomonas*. In certain embodiments, no supplied carbon in the form of added acetic acid and/or acetate is partitioned into non-nutritive starch, since starch is not biosynthesized in the substantially starchless biomass with about 70% to about 80% protein content compared to starch-containing biomass with about 30% to about 40% protein from wildtype *Chlamydomonas*. In some embodiments, about one-third of the supplied carbon in the form of acetic acid/acetate is partitioned into a non-nutritive component of biomass, namely starch, for wildtypes with about 31% starch content.

In certain embodiments, the supplied carbon in the form of added acetic acid and/or acetate converts more efficiently per unit protein during biomass production, with an acetic acid and/or acetate conversion ratio of about 3.4 to about 3.75 for about 80% and about 72% protein content in substantially starchless biomass, respectively, compared to a ratio of 7.5 to 10 for a 40% to 30% protein content in starch containing biomass (e.g. wildtype *Chlamydomonas*), respectively. In certain embodiments, the high protein substantially starchless *Chlamydomonas* phenotypes use about 2 to about 3 times less acetic acid and/or acetate per unit protein, resulting in a more efficient, less wasteful, and thus more sustainable fermentation production.

In certain embodiments, an algal biomass ingredient that results from selective partitioning of carbon into more nutritional protein rather than into less nutritional starch yields a biomass that is produced more sustainably by using less nutrients during algal growth compared to nutrients provided to other *Chlamydomonas* that accumulate starch.

In several embodiments, the algal ingredient used in the instant invention is produced more sustainably by being less nutritionally wasteful of carbon nutrient provided as feedstock during algal growth compared to ingredients comprised of other *Chlamydomonas* cells that accumulate starch. The algal ingredient of the instant invention is produced using *Chlamydomonas* that directs fed carbon via acetic acid into an abundance of protein, which is more nutritious than starch.

In certain embodiments, the high protein, substantially starchless biomass ingredient used in compositions of the instant invention is more sustainable than biomass with a lower protein containing starch due to its more efficient use of energy provided by the acetic acid feedstock via its conversion into acetyl-CoA and metabolic intermediates used in biosynthesis of nutritive protein rather than non-nutritive starch.

In certain embodiments, usage of carbon in the form of added acetic acid and/or acetate occurs into both proteins and pigments during growth to produce the improved wholemeal rich in protein and pigments for use in the instant invention.

In several embodiments, any resulting *Chlamydomonas* phenotypes that display starch content below about 2.25%, along with protein content above about 50%, above about 60%, more preferably above about 70%, are suited for use in the compositions of the present invention.

In certain embodiments, the acetic acid feedstock providing the carbon and energy fed to the algal culture uses carbon recycled from CO_2 emissions.

In several embodiments, the algal ingredient used in the instant invention is produced into a more nutritious protein more sustainably by metabolism of recycled carbon in the form of acetic acid fed during fermentation.

In certain embodiments, sustainability metrics of water and land use for the cultivation phase of protein production from the *Chlamydomonas reinhardtii* are calculated and compared to other protein sources of terrestrial plants or livestock. Efficient, selective carbon metabolism into nutritionally valuable compounds is described. In one embodiment, biomass is produced through closing the carbon cycle for the fermentation process by recycling the carbon from carbon dioxide emissions into an acetic acid/acetate chemical intermediate prior to the selective metabolism into the protein of the biomass rather than into starch.

In certain embodiments, the improved *Chlamydomonas reinhardtii* material can be combined with at least one other edible component in any number of different conventional food compositions, including, for example, pet foods. In several embodiments, the improved algal ingredient is used for protein fortification of a composition without adding to the calories

or to the glycemic index with algal starch. In several embodiments, the incorporation of the improved *Chlamydomonas reinhardtii* ingredient enhances the nutritional value of conventional food compositions without concurrently adding algal starch-derived sugars. The compositions include, for example extrudates, batters, doughs, milks, broths/soups, meat-like products, dressings, snacks, drinks, and pet food or treats. In some embodiments, the compositions are food supplements in the form of, for example, capsules and tablets.

In some embodiments, the food is a dry mixture composition. The dry composition can be used for producing a batter by addition of liquid. In one embodiment, the food is a pour batter for pancakes. Pour batters also include waffles, popover, and Yorkshire pudding. Other batters include coating batter and drop batter. In one embodiment, the food composition is a dough. The dough includes a Chinese scallion pancake or scallion pancake, with scallion filling fortified in protein and with lower gluten without adding algal sugars.

In certain embodiments, an even further embodiment provides a food in an alternative meat, cultured meat (e.g., *in vitro* cultured meat cells), or meat alternative (e.g., plant-based meat) product category. In one embodiment, the food is a vegan meat-less meatball. In certain embodiments, the food is a pasta or bread. In certain embodiments, a pasta filling using the improved *Chlamydomonas reinhardtii* ingredient for a ravioli or dumpling as, for example, an alternative to meat-containing fillings. In some embodiments, the food is a type of extruded food product. Extruded foods can range from pasta, snacks, breakfast cereals, texturized protein, premade doughs, baby food, and pet food. In one embodiment, the food is a vegan, egg-less version of an extruded egg noodle, known as Spaetzle. A cooker-extruder method is employed in preparation of dry pet foods.

In certain embodiments, the preparation of meat-containing compositions with *Chlamydomonas reinhardtii* derived compositions for pet food or pet treats is provided. In one embodiment the algal wholemeal is used as a palatant in cat food. In some embodiments the algal wholemeal replaces animal protein entirely or partially in a dog food or cat food. In some embodiments the algal wholemeal contains glycine.

In certain embodiments, flavor modification in food and beverage compositions using the improved *Chlamydomonas reinhardtii* wholemeal is provided. In one embodiment, a novel use of vanillin during algal fermentation cultivation as a process aide to manage oxygen demand adds aroma to the wholemeal.

In certain embodiments, the *Chlamydomonas reinhardtii* composition can be used to produce algae milk with no starch-based sugars and calories being contributed from the algal wholemeal irrespective of the effective protein levels in the milk.

In certain embodiments, the *Chlamydomonas reinhardtii* composition can be used in a food emulsion. In one embodiment, the food emulsion is an oil-in-water emulsion. In one embodiment, the food emulsion is an algae-protein fortified version of mayonnaise and thus a product that is a dressing. In one embodiment, the composition is fortified in algal protein without the addition of algal starch-based sugars.

In certain embodiments, the *Chlamydomonas reinhardtii* composition can provide a pigmented composition with high protein and no added algal starch for a variety of applications, including, for example, for food and beverages. In certain embodiments, the *Chlamydomonas reinhardtii* composition for nutritional supplementation for protein and antioxidants is provided. In certain embodiments, the *Chlamydomonas reinhardtii* composition can be use in savory broths and soup stocks. In certain embodiments, the *Chlamydomonas reinhardtii* composition can be used in compositions to simulate meat in appearance, flavor, and color, texture, advantageously with an extended shelf-life by virtue of control of free water content and activity of the product. In certain embodiments, the *Chlamydomonas reinhardtii* composition can facilitate rehydration of previously dehydrated food compositions. In certain embodiments, the *Chlamydomonas reinhardtii* composition has higher water absorbance capacity compared to wildtype *Chlamydomonas* containing lower protein and containing starch. In certain embodiments, the *Chlamydomonas reinhardtii* composition can be used in a recreational drink.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 Manufacturing flow outlining the overall steps for producing improved edible compositions using improved higher purity *Chlamydomonas reinhardtii*. The preferred mode of algae cultivation to produce the algal product for the composition is in heterotrophic production (aerobic fermentation). A microalgal cell (**10**) is provided in culture (such as on plates or in liquid) for sequential scale up in flasks, bottles, and bioreactors (can include smaller and larger tanks as needed) as part of the seed train (**20**). This preferentially uses dark heterotrophic growth, although optional growth under light may be used during the seed train. Thereafter, algal biomass formation is obtained in a destination bioreactor under dark heterotrophic growth (**30**), with an optional transfer to a second vessel or additional vessels for fermentation in a draw and fill mode of operation (**40**). The culture in (**30**) or (**40**) may undergo an optional step of exposure to illumination for a light treatment of the algal biomass (**50**), followed by harvesting and dewatering into a wet mass or a dried mass (**60**), resulting in the output of a novel algal product with the desired phenotype (**70**). Optionally, the output can be

further processed, such as with a pre-treatment prior to food formulation (**80**). Thereafter the algal wholemeal is formulated into an edible composition of a food, beverage, or food supplement for humans and animals in an amount effective for the desired composition (**90**). The carbon feedstock or aeration input (**100**) into the seed train bioreactors (**20**) and into the destination bioreactors (**30**) or additional tanks for continuous mode, such as draw and fill (**40**), can include carbon that is captured from the air or from industrial carbon sources such as, but not limited to, CO, CO_2, methane, or carboxylic acid (**110**) and upgraded into acetic acid/acetate. Recycled carbon can also derive from CO_2 emissions from the fermentation of the *Chlamydomonas reinhardtii* (**120**) to produce renewable acetic acid/acetate. The aeration input (**100**) of air can include supplemental oxygen (**110**) that is a waste stream of oxygen (O_2) or process oxygen generated by catalytic processes or other process oxygen sources as known in the art to be supplied into the algae fermentation process for oxygen enrichment.

FIG. 2 Various applications of improved higher purity *Chlamydomonas reinhardtii* of different colors and hues in foods and beverages. **Panel A**) Aqueous wholemeal mixture to illustrate a cocktail color. **Panels B - E**) Images of algal colonies growing on solid agar plates to illustrate various colors. **Panel C**) Includes image on right of yellow liquid culture illustrating noticeable presence of carotenoids amid diminution or absence of chlorophylls in heterotrophically grown, yellow, higher protein substantially starchless *Chlamydomonas reinhardtii* during exponential growth phase.

FIG. 3 Image of a cooked airy pancake with chartreuse interior produced from a batter composition containing a dry matter mixture (i.e., not including water weight) comprising 8% weight dried algae. The algal component is an improved higher purity *Chlamydomonas reinhardtii* wholemeal with protein content exceeding 50% and is substantially starchless.

FIG. 4 A protein fortified food composition that is baked algae-mushroom vegetarian meatballs with creamy red pepper sauce. The 'meatballs' are produced by incorporation of the improved *Chlamydomonas reinhardtii* at 3% inclusion that provides 37% of the total protein value (uncooked basis). Inclusion of the alga component increases the calculated protein content of the food by almost 70%, from 3% to 5%.

FIG. 5 Panel A) Neutral colored biomass of *Chlamydomonas reinhardtii* can be produced from green biomass by heat treatment. **Panel B)** Chartreuse (a color between green and yellow) flakes and powder are converted to cream color by heating, in this case in a liquid at starting pH 7. The color change is associated with pigment denaturation, primarily of chlorophyll.

FIG. 6 Example of a food composition that is an oil-in-water emulsion using improved higher protein substantially starchless *Chlamydomonas reinhardtii* wholemeal for protein enrichment. One-day-old "mayonnaises" (dressings) after overnight chilling are identical in gloss, texture and stiffness. **Panel A)** An avocado-green 3% w/w algae dressing with double the protein content of the control; **Panel B)** A cream-white unfortified control dressing lacking algae.

FIG. 7 Cooked Spaetzle pasta, showing (**Panel A**) the traditional pale yellow egg noodle and (**Panel B**) an algae-containing egg-less version with equivalent protein content comprised of an improved high protein substantially starchless *Chlamydomonas* wholemeal component. In **Panel B**, a green algal wholemeal was used resulting in a green noodle; use of a yellow wholemeal instead results in a pale-yellow noodle.

DETAILED DISCLOSURE OF THE INVENTION

As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having", "has", "with", or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term "comprising". The transitional terms/phrases (and any grammatical variations thereof) "comprising", "comprises", "comprise", "consisting essentially of", "consists essentially of", "consisting" and "consists" can be used interchangeably.

The phrases "consisting essentially of" or "consists essentially of" indicate that the claim encompasses embodiments containing the specified materials or steps and those that do not materially affect the basic and novel characteristic(s) of the claim.

The term "about" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which depends in part on how the value is measured, *i.e.*, the limitations of the measurement system. In the context of compositions containing amounts of ingredients where the term "about" is used, these compositions contain the stated amount of the ingredient with a variation (error range) of 0-10% around the value (X ± 10%). In other contexts, the term "about" is used provides a variation (error range) of 0-10% around a given value (X ± 10%). As is apparent, this variation represents a range that is up to 10% above or below a given value, for example, X ± 1%, X ± 2%, X ± 3%, X ± 4%, X ± 5%, X ± 6%, X ± 7%, X ± 8%, X ± 9%, or X ± 10%.

In the present disclosure, ranges are stated in shorthand to avoid having to set out at length and describe each and every value within the range. Any appropriate value within the

range can be selected, where appropriate, as the upper value, lower value, or the terminus of the range. For example, a range of 0.1-1.0 represents the terminal values of 0.1 and 1.0, as well as the intermediate values of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and all intermediate ranges encompassed within 0.1-1.0, such as 0.2-0.5, 0.2-0.8, 0.7-1.0, etc. Values having at least two significant digits within a range are envisioned, for example, a range of 5-10 indicates all the values between 5.0 and 10.0 as well as between 5.00 and 10.00 including the terminal values. When ranges are used herein, combinations and subcombinations of ranges (e.g., subranges within the disclosed range) and specific embodiments therein are explicitly included.

By "reduces" is meant a negative alteration of at least 1%, 5%, 10%, 25%, 50%, 75%, or 100%.

By "increases" is meant as a positive alteration of at least 1%, 5%, 10%, 25%, 50%, 75%, or 100%.

All percentages refer to refer to percentage by weight (wt% or % dry weight or %DW or % dry matter or w/w) unless otherwise stated. When the term %DW is used with dried algal biomass, it is understood that the algal biomass may still have slight moisture such as around 5% or less moisture of total mass.

As used herein, the term "photoautotrophs" refers to an organism capable of synthesizing its own food from inorganic substances using light as an energy source. Examples of photoautotrophs include green plants and photosynthetic bacteria.

As used herein, the term "facultative" refers to an organism that is capable of but not restricted to a particular mode of life. For example, a facultative anaerobe can synthesize ATP by aerobic respiration if oxygen is present but is capable of fermentation or anaerobic respiration if oxygen is absent.

As used herein, the term "facultative heterotroph" refers to a photoautotrophic organism that is also capable of utilizing organic compounds for growth and/or maintenance and/or survival when light energy is not sufficient or is absent. The term also encompasses facultative heterotrophs and descendants thereof that lose their capability to perform photosynthesis, or acquire defects that result in their inability to grow as phototrophs, or are enabled to grow in the dark through genetically engineering, including for trophic conversion or for utilization of the preferred carbon feedstock. Some representative facultative heterotrophs that can grow in the dark in the presence of acetate as a carbon source are *Chlamydomonas reinhardtii* and *Chlamydomonas dysosmos*. *Chlamydomonas* spp. may be exemplified but not limited to species and strains of the Chlamydomonas Resource Center (see worldwide website:

Chlamy.org/) and those listed in Algaebase. *Chlamydomonas nivalis* is a collective name for red/orange pigmented *Chlamydomonas* and *Chloromonas.*

As used herein, the term "Chlamydomonadales" refers to the order of green algae that includes taxa formerly placed in Volvocales and Dunaliellales and as typified by *Chlamydomonas* (Lewis and McCourt, 2004). Members of the order Chlamydomonadales have life cycles with distinct cell types for vegetative reproduction, resting phases, and sexual reproduction.

As used herein, the term "*Chlamydomonas"* refers to a genus of microalgae classified in the Eukaryota domain, Viridiplantae kingdom, Chlorophyta phylum or division, Chlorophyceae class, Chlamydomonadales order, and *Chlamydomonadaceae* family.

As used herein, the term "axenic" refers to the state of a culture in which only a single species, variety, or strain of an organism is present and wherein the culture is free of all other organisms.

As used herein, the term "biomass" refers to a mass of living or non-living biological material and its derivatives and includes both natural and processed, as well as natural organic materials more broadly. Thus, "microalgal biomass," and "algal biomass" refers to material produced by growth and/or propagation of microalgal cells.

As used herein, the term "wholemeal" refers to algal biomass that is predominantly intact and/or homogenized or micronized. The term is used interchangeably with "whole biomass" or "wholemeal biomass". It is distinct from fractionated, separated component macronutrients of protein or lipids originating from biomass. The wholemeal can be in dried or wet forms over a range of moisture contents. It generally has some or most of its cell water weight removed through drying or else it is in the form of a slurry that is a concentration of algal cells.

As used herein, the term "micronized" refers to a substance reduced to a fine powder such as by pulverization of a solid material's particles to reduce the size compared to the un-micronized substance.

As used herein, the term "biomass production" or "biomass accumulation" means an increase in the total number or weight of the cells of the organisms that are present in a culture over time. Biomass is typically comprised of cells; intracellular contents as well as extracellular material that may be secreted by a cell; and can also be processed such that a fraction of the biomass is removed leaving residual biomass.

As used herein, the term "biological carbon" in the context of CO_2 emissions is that which is generated by a biological organism, such as during microalgal aerobic fermentation,

alcohol fermentation or other microbial fermentation. Non-biological carbon is produced by non- biological processes, such as, for example, in cement factories, steam generation, or other industries with carbon dioxide emissions of sufficient quality for carbon capture, as is known in the art.

As used herein, the term "recycled carbon" refers to carbon derived from non-renewable waste streams (such as fossil fuel, natural gas, gaseous wastes, flue gas, other industrial process emissions and process byproducts) but also refers to carbon derived from renewable waste streams when the recycled carbon is one or more of carbon that is biological carbon from fermentation emissions or from other biological emissions, such as from *C. reinhardtii* fermentation emissions. Recycled carbon can also refer to carbon derived from a mixture of biological and non-biological CO_2 as in direct air capture. For the instant invention, carbon is captured from these sources and converted by chemical synthesis into acetic acid or acetate by means known in the art for use in aerobic fermentation of *C. reinhardtii*, thereby helping close the carbon cycle in production of *C. reinhardtii* biomass.

As used herein, the term "fed-batch fermentation" refers to fermentation in which one or more nutrients are supplied to the bioreactor during cultivation and in which the product remains in the bioreactor until the end of the fermentation run. In some cases, a volatile or gaseous product can be removed in part during the fed-batch fermentation run.

As used herein, the term "product of interest" refers to a substance synthesized by a cell. Examples of a product of interest include but are not limited to, proteins, lipids, carbohydrates, biogases, volatile materials, sugars, amino acids, isoprenoids, terpenes, or precursors thereof. Such substances may be synthesized constitutively by the organisms throughout growth and the amount of the substance in the culture may increase simply due to an increase in the number of organisms. Alternatively, the synthesis of such substances may be induced in response to culture conditions or other environmental factors, for example, nitrogen starvation or elevated ammonium levels.

The amount of a product of interest accumulated over time relative to the culture volume and relative to their original amount is considered as "product accumulation" that can be measured by specific productivity.

As used herein, the term "protein productivity" refers to the amount of crude protein produced over time.

As used herein, the term "concentration" refers to the process of enriching a material in a substance or otherwise purifying the substance. Concentration of protein can be through fractionation of the protein component from biomass or by a delipidation step that leaves a de-

fatted biomass residue with an effectively higher protein content than before delipidation. Protein concentration by delipidation can utilize an organic solvent such as food-grade renewable ethanol but may concurrently concentrate other water-soluble components such as carbohydrates. Concentration of biomass can be through removal of water.

As used herein, the term "cultivated", "cultivation" or "culturing" refers to the purposeful fostering of growth (increases in cell size, cellular contents, and/or cellular activity) and/or propagation (increases in cell numbers via mitosis) of one or more microbial or microalgal cells by use of intended culture conditions. The combination of both growth and propagation may be termed proliferation. Examples of intended conditions include the use of a defined medium (with known characteristics such as pH, ionic strength, and carbon source), specified temperature, oxygen tension, and growth in a fermentor or bioreactor. The term does not refer to the growth of microorganisms in nature or otherwise without intentional introduction or human intervention, such as natural growth of an organism.

As used herein, the term "fermentor", "bioreactor", "fermentation vessel" or "fermentation tank" refers to an enclosed vessel or partially enclosed vessel in which cells are cultivated or cultured, optionally in a liquid suspension. A fermentor or bioreactor includes non-limiting embodiments, such as, for example, an enclosure or partial enclosure that permits cultured cells to be exposed to light or which allows the cells to be cultured without the exposure to light. As used herein, the term "port", in the context of a vessel that is a fermentor or bioreactor, refers to an opening in the vessel that allows influx or efflux of materials, such as, for example, gases, liquids, and cells. Ports are usually connected to tubing leading from the fermentor or bioreactor.

As used herein, the term "fermenter" when referring to a biological organism refers to an organism that causes fermentation.

As used herein, the term "final destination bioreactor" or "destination production tank" refers to the vessel from which the cultured cells are harvested. This can be in batch or continuous mode (including draw and fill) for complete or partial harvest of cells, respectively.

As used herein, the term "seed train" refers to the scaling of the algal culture from a small volume of cells in a cell bank vial, plate, or flask (at least about 25 mL, about 50 mL, or about 125 mL to about 250 mL flasks for the cell bank) to a larger volume of cells (in about 1 L to about 3 L flasks, about 5 to about 20 L carboys, or any size fermentor such as about 1 L to about 13 L, about 20 L to about 40 L to about 100 L, to about 500 L to about 1000 L depending on the production facility) that is used to inoculate the main production reactor.

As used herein, the term "fixed carbon source" means a compound containing carbon that can be used as a source of carbon and/or energy by an organism. Typically, a fixed carbon source exists at ambient temperature and pressure in a solid or liquid form.

As used herein, the term "organic acid" refers to one or more molecules that are organic compounds with acidic properties. The most common organic acids are the carboxylic acids. A "carboxylic acid" contains a carboxyl group distinct from sugar carbohydrates, such as, for example, glucose, which is commonly used in algal fermentation. Acetic acid is a two-carbon carboxylic acid, CH_3COOH, commonly used in chemical manufacturing. In contrast, the organic salt, sodium acetate, CH_3COONa, can be anhydrous or as a trihydrate sodium salt of acetic acid. Propionic acid (propanoic acid) is a carboxylic acid with the chemical formula CH_3CH_2COOH. The anion $CH_3CH_2COO^-$ as well as the salts and esters of propionic acid are known as propionates (or propanoates). Other such acids can include but are not limited to citric, fumaric, formic, glycolic, lactic, malic, pyruvic, and succinic acids.

As used herein, the terms "heterotrophic conditions", "heterotrophic fermentation", and "dark heterotrophic cultivation" or "dark heterotrophic culture" refer to the presence of at least one fixed carbon source and the absence of light during fermentation.

As used herein, the term "process oxygen" refers to oxygen generated from a process that otherwise goes unused and can be directed into fermentors to aid in oxygenation of the fermentor. It is handled as supplemental oxygen to supplement the air entering fermentors (that by default contains some portion of oxygen). Sources of process oxygen can be numerous. Among these is from water that is split via electrolysis to make hydrogen and oxygen for syngas production, a process in the pathway for acetic acid production. Other sources can include, for example, carbon that is recycled and upgraded including from the fermentation of algae or from other industrial carbon sources including CO_2, carbon monoxide, syngas, or methane.

As used herein, the term "aeration input" refers to air supplied into fermentors for aeration and for mixing.

As used herein, the terms "manufacturing efficiency" or "efficient production" refer to a process requiring high productivity to benefit cost efficiency. During cultivation of algae this requires rapid accumulation of the product of interest, such as algal biomass or total protein, and high volumetric concentrations of the desired product.

As used herein, the term "carotenoid" refers to a compound composed of a polyene backbone which condensed from a five-carbon isoprene unit. A "carotenoid" can be acyclic, or one (monocyclic) or two and it can be terminated by cyclic end-groups of the number (bicyclic). The term "carotenoid" may include both carotenes and xanthophylls.

As used herein, the term "carotene" refers to a hydrocarbon carotenoid. "Xanthophylls" are oxygenated carotenoids. Modification of pyrophosphate and phosphate groups of isoprene derivatives include, for example, oxidations or cyclizations to yield acyclic, monocyclic, and bicyclic terpenes, including, for example, monoterpenes, diterpenes, triterpenes, or sesquiterpenes.

As used herein, the term "microorganism" refers to microscopic unicellular organisms, including microalgae. The microorganisms usable in the fermentation according to the present invention can include mutants, naturally occurring strains selected for a specific characteristic, or genetically engineered variants of a naturally occurring strain.

As used herein, the term "microalgae" refers to a eukaryotic microorganism that contains a chloroplast, and optionally is photosynthetic, or a prokaryotic microorganism capable of being photosynthetic. Microalgae include obligate photoautotrophs, which are incapable of metabolizing an organic carbon source as energy, as well as obligate or facultative heterotrophs, which can metabolize an organic carbon source. Microalgae as obligate heterotrophic microorganisms include those that have lost the ability of being photosynthetic and may or may not possess a chloroplast or chloroplast remnant. Microalgae can divide to produce populations of cells and can be scaled-up or enter a production phase to produce biomass, and this process can be continued indefinitely until a maximum productivity is achieved.

As used herein, the term "hybridized" refers to lines of progeny produced by mating of plus (mt+) and minus (mt-) mating types of *Chlamydomonas* or to the process of being mated, also known as "hybridization".

As used herein, the term "recombinant" when used in reference to a cell, nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein, or vector, has been modified from its natural state. For example, a recombinant cell comprises an exogenous nucleic acid or protein or the alteration of a native nucleic acid or protein, or is derived from a cell or organism or micro-organism.

As used herein, the terms "robust" or "robust culture", in the context of selected strains or lines of a species, refer to a population of organisms that contain a desired phenotype and equal or greater growth characteristics, especially under heterotrophy, when compared to the original strain. Maintaining the robust growth characteristic is highly problematic when using mutagenesis and chemical selection to obtain mutants. Novel selections and selected subpopulations resulting from methods that do not rely on mutagenesis, such as adaptive laboratory evolution, are more likely to retain the robust growth characteristics along with the

improved phenotype of interest. A "robust" culture is also one that is resistant to oxidative stress. In high density fermentation production, the ability to resist oxidative stress is beneficial for industrial production.

As used herein, the term "selected population" or "selected line" refers to isolating improved strains with preferred phenotypes. For example, the phenotypes can be generated through adaptive laboratory evolution that subjects algae over time to stress conditions or to adverse or altered cultivation conditions. The basis is stable spontaneous mutations or epigenetic effects to confer an adaptive advantage or an associated phenotype.

As used herein, the term "cell-cultured meat" and "cell-cultured seafood" refers to animal cells grown in the laboratory or otherwise outside the whole animal.

As used herein, the term "% Daily Value (DV)" refers to much of a nutrient in a serving of food contributes to a daily diet. The value of 2,000 calories in a day is used for general nutrition advice. Adult men and women should consume about 46 g to 56 g protein daily. As an example, a serving of meatballs with 19 g protein provides about 38% of the % DV for protein.

As used herein, the term "animal" is used in a general sense and means a human or other animal that consumes an edible composition, including pets. Pets or companion animals may be avian, bovine, canine, equine, feline, lupine, murine, ovine, and porcine animals.

As used herein, the term "food" refers to any nutritious substance that people or animals eat or drink or that algae take up in order to maintain life and growth. Food may include snacks and supplements, including, for example, for pet foods.

As used herein, the term "conventional food product" or "conventional food composition" refers to a composition intended for consumption by people or animals that lacks algal wholemeal or other algal components and includes ingredients ordinarily associated with the food product, particularly a vegetable protein, mycoprotein, animal protein, egg, flour, vegetable oil, and/or animal fat together with other edible ingredients. Conventional food products can include plant-based or other non-animal derived meat-like products, such as, for example, vegetarian or vegan meatballs. Conventional food products are found in stores, in restaurants and are made at home. Conventional food products are often made by following conventional recipes that specify inclusion of protein, egg, flour, oil, or fat from a non-algal source together with other edible ingredients.

As used herein, the term "food additive" refers to a substance or mixture of substances that is added to food to improve the quality, consistency, and shelf-life or shelf stability of the prepared food. This includes any substance or mixture of substances used in the production,

processing, treatment, packaging, transportation, or storage and longevity of a food. Further, a food additive is defined by the US Department of Health and Human Services as "any substance the intended use of which results or may reasonably be expected to result (directly or indirectly) in its becoming a component or otherwise affecting the characteristics of any food". The term applies to the algae substance in the present invention without regard to the quantity of the algae substance used in any given formulation. Thus, for the purposes of the present invention the terms "food additive" and "food ingredient" are not differentiated and are interchangeable.

As used herein, the term "food supplement" is defined as a product intended to supplement the diet, be it for humans or animals including companion animals. The term food supplement is interchangeable with "dietary supplement" and "nutritional supplement". Such supplements are generally taken by mouth as a tablet, capsule, gummy, or a liquid.

As used herein, the term "recreational drink" refers to a drink or cocktail, alcoholic or non-alcoholic, consumed during socializing or in a social setting.

As used herein, the terms "edible composition", "edible component", "edible algae composition", and "edible food composition" means any substance, ingredient, or composition which is fit to be eaten.

As used herein, the term "excipient" means an edible, inert substance.

As used herein, the term "effective amounts" refers to using an ingredient (such as algal wholemeal) in an amount that will deliver a similar or superior nutrition for one or more specified nutrients of interest or a similar or superior functionality of interest. The point of reference can be what is found in an existing commercial product category for which the algal wholemeal is replacing/substituting an ingredient (including partial replacement); or to which the algal ingredient is being added in the absence of substitutions. The nutrient objective may be to inhibit nutrient inferiority in a food that replaces a traditional food in the diet, such as when removing animal-based components in a formulation. The functionality objective may be to inhibit loss of technical aspect (e.g., binding) when an ingredient is being replaced (e.g., egg replacement to lower cholesterol or make it vegetarian). An "effective amount" can also be determined by cost factors or sustainability factors. It can also refer to using the ingredient (algal wholemeal) in an amount that fortifies a food composition such as in protein or antioxidant content. The effective amount for fortification relates to the nutrient objectives and should follow applicable regulatory guidelines.

As used herein, the term “fortified food” refers to a food that has extra nutrients added to it or has nutrients added that are not otherwise present. This can help boost their nutritional value and benefit our health.

As used herein, the term “enriched food” refers to nutrients that were lost or reduced during processing or formulation are added back in. The term “enriched food” can refer to partial or complete ingredient substitution, such as reduction in gluten by reducing flour, wherein the reduced component is replaced in certain nutrients by adding another component. The terms “enriched”, “fortified” or similar terms are used interchangeably herein.

As used herein, the term “protein fortification” refers to enrichment or improvement of a food by adding a protein source. In the instant invention it can refer to addition of non-extracted (wholemeal) algae that will provide more protein on a weight basis using the substantially starchless biomass with its higher protein content than wholemeal of algae that contains starch and has lower protein. It can also refer to addition of isolated protein obtained from algal biomass.

As used herein, the term “protein” in a composition of algae or in the “proximate composition” of algae refers to crude protein as measured as described in Example 2, unless otherwise specified.

As used herein, the term “complete protein” is a protein that contains all the essential amino acids in appropriate amounts to allow normal growth and tissue maintenance when adequate energy is provided in the diet of an animal.

As used herein, the term "nutritionally complete", "nutritionally balanced" in the context of food or subgroup of food categories refers to containing all known required nutrients for the intended animal, in appropriate amounts and proportions based on, for example, recommendations of authorities in the field of human and companion animal nutrition.

As used herein, the term “dietary fiber” refers to non-starch carbohydrates found in plants and other organisms containing cell walls, including microalgae. Dietary fiber can be soluble (i.e., dissolved in water) or insoluble (i.e., not able to be dissolved in water). Soluble and insoluble fiber makes up total dietary fiber.

As used herein, the term “crude fiber” refers to a measure of the quantity of indigestible cellulose, pentosans, lignin, and other non-starch carbohydrates components of this type that are present in food or a biomass. A proximate composition measures crude fiber, per the examples of the instant invention.

As used herein, the term “foodstuff” refers to a substance suitable for consumption as food or to make food.

As used herein, the term "functional food" refers to a food or processed food consumed in the normal food pattern but provides a beneficial effect beyond what is required to meet basic nutritional needs.

As used herein, the term "bulking agent" in food refers to a non-nutritive additive that increases the bulk (volume or weight) of a food without affecting its taste and keeping its utility and functionality intact. As used herein, an "additive" is a substance added to something in small quantities to improve or preserve it.

As used herein, the term "water holding capacity", "WHC", "water-binding capacity", "water absorption ratio" or "water-absorption capacity", is a measure of the total amount of water that can be absorbed per unit of weight expressed as a percentage or as gram water per gram algal biomass or per gram protein powder. The property of WHC is an important determinant of food texture. Selection of proteins with an appropriate WHC is vital in food formulation. This attribute is based on the direct interaction of protein molecules with water and other minor components in an aqueous solution and is an essential factor in the formulation of food.

As used herein, the term "low starch digestion rate" means lower starch hydrolysis by digestive enzymes in a certain period of time. Therefore, blood glucose level will not increase drastically after the food is digested and metabolized by the body. Food components with a moderate or high starch digestion rate will yield a moderate or high glycemic index level of about 56 (i.e., moderate glycemic index) to about 69 (i.e., high glycemic index) or about 56 to about 70 and higher. Conversely starchless or low starch food components will yield a low glycemic index (of 55 or less). The term "glycemic index" refers to a rating system that ranks foods on a scale from 1 to 100 based on their effect on blood-sugar levels.

As used herein, the term "satiety index" or "satiety value" refers to a measure of which foods make us feel the fullest or the degree at which a food gives a human the sense of food gratification, the contrast being the feeling of hunger. "Satiety" in nutrition is the absence of hunger; a satiating food composition leaves you satisfied for longer. It is a useful tool for choosing the right foods for weight loss. High-satiety foods are rich in protein while foods with high-fat content create almost instant cravings for more. An algal ingredient with relatively high protein and moderate to low fat content contributes to higher satiety than an algal ingredient with relatively lower protein and higher fat content used in a food composition.

As used herein, the term "meat analogues" refers to food products that lack animal protein but possess meat-like (includes poultry-like and fish-like) characteristics in appearance, texture, color, flavor, or any combination thereof. Algae-based or plant-derived material can

be structured into fibrous forms or layered structures, with texturization commonly achieved through extrusion or extrusion cooking.

As used herein, the term "texturized meat proteins" refers to the extrudates produced from vegetable proteins along with meat to stimulate the texture of whole-muscle meat structures. These products may be applied to pet and human foods.

As used herein, the term "extrusion cooking" or "cooker-extruder method" refers to a continuous process by which materials, such as proteins and starches, are mixed and plasticized to form a fluid melt in a chamber or barrel from exposure to heat, pressure, and shear forces, causing the material to be conveyed and forced to flow through a perforated plate or die of specific shape. This process can be used to manufacture pasta, processed meats, and fillings. A single screw cooking extruder can be used to produce, for example, dry and semi moist pet foods, expanded snacks, breakfast cereals, puddings, soup and drink bases, gelatinized starch, and texturized vegetable proteins.

As used herein, the terms "substantially starchless", "very low starch" and "negligible starch" are used interchangeably. They refer to a starch content that is undetectable, or "undetectable starch", depending on the lowest measurable amount of the analytical method used. "Undetectable starch" is less than or "subceeding" about 2.25% in some cases (when glucose from starch is measured with a glucose meter) and less than about 0.18%, and specifically less than about 0.09%, in other cases when measured spectrophotometrically by AOAC Method 996.11. Substantially starchless also applies to, "zero starch", in a proximate composition of algal biomass. "Undetectable starch" encompasses the terms of having "no starch" or "0% starch" or "zero starch" or being "starchless" or "eliminating starch". It does not include an intentionally added amount of the starch or starch-containing component to an algal preparation, be it part of the algal or food processing or part of a food composition. Starch, including its starch-based or starch-derived component sugars, is less nutritive than protein on a weight basis.

As used herein, the term "proximate composition" in reference to an algal biomass refers to moisture, ash, fat, protein (crude), fiber (crude), and carbohydrate (including starch) contents expressed as a percentage in the sample.

As used herein, the term "mayonnaise" refers to a thick dressing comprising egg yolks, vinegar or lemon juice, oil, and seasonings. In some regions, such as the European Union, a true mayonnaise must be at least 70% oil and contain egg.

As used herein, the term “oil-in-water emulsion” refers to emulsified sauces and dressings. This includes mayonnaise that is thick and salad dressing or emulsified sauces that are thinner than a mayonnaise and pourable.

As used herein, the term "palatability" refers to a relative preference of an animal for one food composition to another. It can also apply to pet beverages. Such preference typically is related to one or more of, for example, aroma, smell, taste, aftertaste, flavor, texture, and/or mouth feel or any of the animal’s senses. It can be ascertained by different means, including, for example, via a "two-bowl test" or "versus test" as is known in the art. "Initial appeal" is an aspect of palatability that induces an animal to initially taste or try a food, as can be measured by the criterium of "first choice" or "first food consumed". "Continued consumption" is an aspect of palatability that induces an animal to continue consuming a food that has been initially only tasted or tried.

As used herein, the terms "palatability enhancer", "palatant", "flavor", "flavor enhancer", and any other similar terms refer to any material that enhances the palatability of a food or beverage composition to be appealing or pleasing to an animal of interest.

As used herein, the terms “sustainable manufacturing”, “produced sustainably”, and any other similar terms refer to the creation of manufactured products through economically-sound processes that minimize negative environmental impacts while conserving energy and natural resources. The terms “sustainable manufacturing”, “produced sustainably”, and any other similar terms also refer to the creation of manufactured products through economically-sound processes that are less wasteful of inputs, that eliminate output waste and pollution, are regenerative or that recycle process outputs into process inputs, and/or are energy efficient. The term “sustainable” can be applied to a product that is an ingredient produced sustainably or to a product that is a composition comprising the sustainable ingredient. As examples, metrics of water and land use for the cultivation phase of protein production can be compared among protein sources (plant, algae, animal) to assess which protein is relatively more sustainable. Use of fertilizer, including, for example, hydrocarbon-containing nutrients for the cultivation of algal biomass in heterotrophic fermentation can be compared based on the nutritional composition of that resulting biomass to assess which alga is relatively more efficacious and thus more economically sound and more sustainable. Protein is more nutritive than starch on a weight basis. Heterotrophically grown *Chlamydomonas* algae use fed organic acids, such as, for example, acetic acid, as their source of carbon (and energy) for cellular metabolism. More efficient carbon use for making more proteins rather than non-nutritive starch in a biomass can

be considered a feature of higher sustainability. A more sustainable ingredient or composition is a valued differentiator for the food and beverage industry.

As used herein, the term "energy efficient" refers to relative use of input energy in the form of power for equipment used in a process. The term also refers to relative use of energy produced during metabolism for biological synthesis into cellular components. Heterotrophically grown *Chlamydomonas* algae use fed acetic acid as their source of energy for cellular metabolism. Acetic acid fed into an algal fermentation culture is metabolized into acetyl-CoA, a molecule used in biochemical reactions in protein, starch, and lipid metabolism. The acetyl-CoA enters the TCA cycle (tricarboxylic acid, citric acid, or Krebs cycle) where it is oxidized for energy production. For a given amount of acetic acid provided as feedstock in a fermentation, the more protein produced compared to starch in a biomass reflects more efficient and thus sustainable use of the energy derived from the feedstock for producing nutritious biomass.

The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Algae Compositions and Methods of Use

The subject invention pertains to novel algae-based compositions, particularly of *Chlamydomonas,* more particularly of *Chlamydomonas reinhardtii*, that feature a protein content exceeding about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or about 90%, and with a concentration of starch that less than about 2.25%, about 2.0%, about 1.75%, about 1.5%, about 1.25%, about 1%, about 0.75%, about 0.5%, about 0.25%, about 0.2%, about 0.15%, about 0.1% by weight or substantially starchless including zero or 0% starch. In certain embodiments, the algae compositions can be suited for use in food, pet food, beverage, or nutraceutical compositions. The description encompasses algae wholemeal. The description encompasses an edible algal composition as food additive or foodstuff in numerous recipes to enrich the product with protein without adding starch-based sugars or to add some other algae component without adding starch-based sugars. In certain

embodiments, the foods can be fortified with biologically active phytonutrients derived from algae as well as the use of a range of desired colors without the use of synthetic dyes. In certain embodiments, methods for the selection of microalgal lines that yield a novel phenotype are provided. In certain embodiments, the presence of a cell wall of the algae allows for high agitation for high cell density fermentation production.

In certain embodiments, microalgae from the eukaryotic genus *Chlamydomonas* Ehrenberg, of the chlorophyte family Chlamydomonadales, can be useful in the compositions of foods, beverages, and food supplements of the present invention. *Chlamydomonas* is unicellular, polar in shape, with paired apical flagella and a basal chloroplast surrounding one or more pyrenoids, and most have a prominent eyespot. Vegetative cells are generally motile in liquid medium but may be non-flagellated on solidified medium. Some *Chlamydomonas* can also form asexual resting spores or as zygospores during sexual reproduction. Vegetative cells of *Chlamydomonas* share a distinct multilayered proteinaceous cell wall that is composed of hydroxyproline-rich glycoproteins rather than cellulose/hemicellulose and that lacks a resistant outer stratum of algaenan or sporopollenin.

In several embodiments, *Chlamydomonas* requires no cell wall pre-treatment prior to cooking or to producing a food, beverage, or food supplement formulation to attain high digestibility in the gastrointestinal tract of animals. In certain embodiments, *Chlamydomonas* cell wall pre-treatment prior to performing standard laboratory digestibility evaluation assays is not required.

In some embodiments, the *Chlamydomonas* cell may have a wall-deficient phenotype. This wall deficiency may be due to a genetic mutation. In such cells, the walls are absent or reduced compared to a walled wildtype. The wall thickness of a cell does not predict its relative growth rate in heterotrophic production with sufficient aeration and nutrition. A *Chlamydomonas* cell line (i.e., strain) that is wall-deficient generally can be recognized by its flat liquid colony that forms on agar plates compared to a colony of a walled phenotype that appears solid, dry and is not pipettable. In some embodiments, cells of a wall-deficient phenotype lyse very easily in detergents used below 1%.

In certain embodiments, exemplary *Chlamydomonas* species that can be used in the subject invention include species that have known mating types. Mating types are known for at least eight species of *Chlamydomonas* to produce zygotes when complementary mating types are mixed, these species including *C. elliptica* Korshikov, *C. frankii* Pascher, *C. minutissima* Korshikov, *C. moewusii* Gerloff, *C. eugametos* Moewus, *C. monoica* Strehlow, *C. reinhardtii* P.A. Dangeard, and *C. smithii* Hoshaw & H. Ettl. Some species are interfertile such as *C.*

smithii with *C. reinhardtii*, and *Chlamydomonas eugametos* with *C. moewusii*. Hybrids can be backcrossed to a recurring parent to transfer a selectable trait of interest into a genetic background of interest.

In certain embodiments, biomass from a cell line resulting from an interspecific cross of *C. reinhardtii* with a complementary mating type of a different *Chlamydomonas* species, which cell line has the features of protein exceeding 50% by dry weight and being substantially starchless below 2.25%, is suitable to be employed in the compositions of this invention. In some embodiments, biomass from a cell line resulting from an intraspecific cross of *C. reinhardtii* with a complementary mating type of a different *C. reinhardtii*, which cell line has the features of protein exceeding about 50% by dry weight and being substantially starchless below about 2.25%, is suitable to be employed in the compositions of this invention.

In some embodiments, a *Chlamydomonas* cell line that has the features of protein exceeding about 50% by dry weight and being substantially starchless below about 2.25%, is suitable for use in the compositions of this invention.

In some embodiments, the microalgal biomass used in the compositions of the invention is from *Chlamydomonas*. In preferred embodiments, the microalgal biomass used in the compositions of the invention is from *C. reinhardtii* including *C. reinhardtii* intraspecific hybrids) or from interspecific hybrids with *C. reinhardtii*. In several preferred embodiments, the particularly preferred species is *C. reinhardtii* for generating biomass for use in the compositions of the invention due to its regulatory status of generally regarded as safe ("GRAS") for consumption and its proven production in aerobic fermentation at industrial scale. Use of biomass from the particularly preferred *C. reinhardtii* in the compositions of the invention are exemplified in the examples below.

Some species of *Chlamydomonas* are naturally facultatively heterotrophic. In certain embodiments, the biomass used in the edible compositions is generated under heterotrophic cultivation. In some embodiments, the biomass is generated in a combination of heterotrophic and illuminated cultivation. Illumination generally results in higher chlorophyll content of a biomass which may be desirable for certain applications such as recreational drinks, pet food palatability, or novelty or "healthy" appearance of a food such as tortilla or pasta. In other embodiments lower or no chlorophyll is a preferred attribute for a food stuff since chlorophyll may confer a color, an unwanted taste, or a diminished shelf-life for a food formulation. Use of heterotrophically-produced biomass from the particularly preferred *C. reinhardtii* in the compositions of the invention are exemplified in the examples below.

In some embodiments, the considerations affecting the selection of *Chlamydomonas* for use in the invention, in addition to having suitable fatty acids, lipids, amino acids, or protein for edible compositions, are

(1) high protein exceeding about 50% by dry weight;

(2) being substantially starchless with undetectable starch (below about 2.25%) by dry weight;

(3) ease of growth, preferably in heterotrophic fermentation conditions;

(4) preferred growth on organic acids, more preferably salt-free acetic acid, for optional circular usage of carbon; and

(5) high protein digestibility exceeding about 0.8 without requiring cell wall breakage by milling or other disruption prior to use in food, beverage, or food supplement compositions.

Ease of growth for industrial applications is that growth rate which supports the economics of the business producing the algal ingredient. While heterotrophic growth rates for *Chlamydomonas* below 1/day may apply in certain circumstances, generally speaking growth rates about 1/day to 1.3/day, and more preferably about 1.7/day and higher are preferred in heterotrophic fermentation conditions.

In certain embodiments the *Chlamydomonas* cell may have a starch-deficient phenotype wherein the phenotype is substantially starchless below about 2.25% to 0% starch by dry weight. This may be due to a starch-deficient mutation or to an epigenetic change, either being transmissible between generations.

In certain embodiments, the *Chlamydomonas sta6* starchless mutation that results in accumulating less than 0.01% starch compared to the wildtype amount of starch has a disrupted STA6 gene locus affecting the small catalytic subunit of ADP-glucose phosphorylase (AGPase-SS), an essential enzyme in the starch biosynthetic pathway. This mutation at the STA6 locus results from random integration of an expression cassette of the argininosuccinate lyase enzyme (pARG7), and inactivates ADP-glucose phosphorylase activity, producing *sta6-1*. In certain embodiments, a cell wall deficient starchless mutant also with a mutation in the STA6 locus designated *sta6-2* results from a single base deletion in the gene's intron3/exon4 border via TALEN targeted mutagenesis. Both the *sta6-1* and *sta6-2* mutants can also be cell wall deficient (i.e., cell walls are absent or produced in greatly reduced quantity compared to wild type) if they carry the *cw15* mutation or similar wall-deficient mutations. In certain embodiments, the *cw15* mutation refers to strains with a cell wall deficient mutant phenotype, as described in Davies, D., & Plaskitt, A. (1971), Genetical and structural analyses of cell-wall formation in Chlamydomonas reinhardi. *Genetics Research, 17*(1), 33-43. This and similar

mutants arise from chemical or irradiation mutation. Mutants can produce a normal amount of wall material, but it is not attached to the plasma membrane (i.e., *cw1, 2, 4, 6, 7, 9, 14, 17, 19)* or can produce minute amounts of wall also not attached to the plasma membrane (*cw3, 10, 15*). These have the capacity to synthesize and secrete the major glycoproteins of the cell wall, but, due to the lack of the W2 wall layer, are unable to assemble these components into a coherent, crystalline wall as described in Zhang and Robinson, 1990 (Zhang, YH., Robinson, D.G. Cell-wall synthesis in *Chlamydomonas reinhardtii*: an immunological study on the wild type and wall-less mutants *cw2* and *cw15* . *Planta* **180**, 229–236 (1990), which is hereby incorporated by reference in its entirety). Starch deficient mutants that have been backcrossed into a wildtype background (21gr also known as CC-1690) are available from the Chlamydomonas Resource Center (see worldwide website: chlamycollection.org/) collection and are of mt+ or mt- mating types.

In wildtype *Chlamydomonas* this AGPase enzyme is activated by 3-phosphoglyceric acid (3-PGA). In certain embodiments, strains with mutations in the STA1 locus when grown photosynthetically may accumulate about 5% to 10% of the wildtype amounts of starch because of a lowered sensitivity of AGPase to 3-PGA activation. In certain embodiments, the mutation at the STAl locus results from insertion of the pARG7 expression cassette into the gene coding for the large subunit (regulatory subunit) of ADP-glucose pyrophosphorylase. In certain embodiments, a *sta7* starchless mutant has a disrupted isoamylase gene locus, which leads to a large reduction of starch content and its replacement by a water-soluble polysaccharide phytoglycogen. In certain embodiments, the mutation at the STA7 locus can result from random integration of the pARG7 expression cassette into the nuclear gene encoding isoamylase. Mutation in the STA11 locus, such as, for example *sta11-1*, is defective in the enzyme 1,4-glucanotransferase.

While recombination by random plasmid integration was used in these starch-deficient and starch-reduced strains, other genetic techniques may produce the same results of disrupting loci affecting activities of ADP-glucose phosphorylase and/or isoamylase to block starch synthesis at different levels. Such techniques may include hybridization (as illustrated in this invention), somatic fusion, genetic modification such as using the CRISPR/Cas system or TALENs (transcription activator-like (TAL) effector nucleases) on the structural gene or on its regulatory elements (such as gene promoter), mutagenesis by UV irradiation, chemical mutagenesis (such as ethyl methanesulfonate (EMS) or N-methyl-N'nitro-N-nitroguanidine (NTG), or, such as, for example, by insertional mutation by a plasmid or PCR product, spontaneous mutations (i.e., botanical sports), or epigenetic modifications (such as DNA

methylation or histone modifications) including post-transcriptional control mechanisms. Genetic mutation and epigenetic variation may be linked to yield the starch-deficient phenotype of interest.

In *Chlamydomonas*, impairment of starch biosynthesis can increase oxidative stress and autophagy. This can be undesirable for fast growth rate especially at higher cell densities. In some embodiments, and for reasons that are not well understood, growth for some lines carrying the *sta6* mutation in fermentation is faster than for other lines carrying the same mutation, and among the fastest or faster compared to wildtype *Chlamydomonas*. These can be selected for further propagation, beginning already in multi-well plates or in flasks and then verified in fermentation vessels. In some preferred embodiments, the selection of relatively fast-growing lines is useful for continued ease of growth under conditions of oxidative stress as cell densities increase in a culture. In some preferred embodiments, the added feature of ease of growth, especially under conditions of oxidative stress, are suited for industrial applications for best economics.

In certain embodiments, methods of producing a food, animal food, including, for example, petfood, a beverage product or food supplement product, wherein the method comprises:

(i) obtaining or producing the subject algae composition; and

(ii) mixing or combining the algae composition with a food component, beverage component, or supplement component to produce the food or beverage product or otherwise adding the algae composition to the finished food or beverage product.

In certain embodiments, the algal biomass of the instant invention produced by a manufacturer under Good Manufacturing Practice (GMP) is used for food-grade products, such as via business-to-business sales to a food or beverage manufacturer including pet food or pet beverage manufacturer, or via business-to-consumer sales for use in a restaurant or for an individual consumer's use. In certain embodiments, the biomass, as a digestible wholemeal in its largely intact form, can be further processed for more homogeneity by sieving or by comminution or micronization into different quality powders or can be flaked by using different drying methods as is known in the art. Packaging of dried powders or flakes employs a sealed bag and is preferably airtight and optionally under vacuum to minimize oxidation during storage. Packaging of wholemeal as a slurry can be in a food-grade pail. Storage under cool, dark conditions is preferred, with refrigeration if available especially once the original packaging is opened or if a wet slurry/paste. Quality control includes passing the standards for microbial and heavy metals. Labeling or specifications can include directions for use in a

recipe, although the user may want to employ their own ratios or applications of the alga wholemeal to other ingredients in a recipe beyond that disclosed in the examples of the instant invention. The wholemeal can also be packaged as a pre-mix with other dry ingredients. Dry mixtures are generally amenable to a multi-year shelf-life without refrigeration. In certain embodiments, the mixture can then be transformed into an edible form by a restaurant or individual consumer by adding liquid, such as water, milk, or oil, according to instructions, blending, and served without cooking (such as a smoothie beverage, sports shake, or pet beverage) or with cooking before serving. In some embodiments, a liquid can be added to reconstitute a dried wholemeal and optionally be pre-treated with heat or by protease digestion. Cooking is performed using common kitchen appliances. In addition to beverages, such mixtures of edible compositions are used for making pancakes, batters, doughs, cakes, breads, sauces, broths/soups, and the like.

The instant invention provides methods of providing algae components, particularly from *Chlamydomonas,* and more particularly components derived from *C. reinhardtii*, to a food, beverage or food supplement that serve to maintain health with health-associated factors or to improve one or more indicators of health in a person or other animal, such as, for example, improved digestive health, providing a source of selenium, providing antibacterial activity against gram-negative microbes or pathogens, and/or providing flavonoids, such as, for example, quercetins and kaempferols for potential anticancer (cytotoxicity) activity.

The invention is directed to improved edible compositions of food and beverages comprising improved compositions of algae biomass in the form of wholemeal. Inclusion of the improved algae biomass in the edible compositions provides an effective amount of wholemeal, algal protein or pigment for its nutritional or functional value without concurrently providing the less valued algal starch. The wholemeal is improved in purity by lacking measurable starch content resulting in a simplified chemical composition while also possessing higher protein content compared to wholemeal that contains starch. Such wholemeal improved in purity is generated from algal strains with phenotypes that do not contain measurable starch, defined as 2.25% or above, and do contain protein exceeding 50% by weight compared to algal phenotypes that contain starch and have protein less than 50% by weight that have not been generated using the subject methods (i.e., unimproved algae). The edible compositions are thereby improved when compared to using unimproved algae biomass in the food composition. The edible compositions are also improved in other aspects described herein. The invention also pertains to improved higher purity wholemeal of algae being produced by cost effective, sustainable and efficient manufacturing using dark heterotrophic fermentation. The invention

of producing improved, edible compositions and the algal wholemeal therein can be practiced using equipment that is commercially available.

In one embodiment, the invention provides a method to generate algal phenotypes with an improved compositional profile that is above 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, or about 90% protein by weight and being substantially starchless, resulting from the process of mating of heterotrophically grown parental cells of *Chlamydomonas* algae, including, for example, *C. reinhardtii*.

In some embodiments, the first parental alga with features of protein below 50% dry weight and containing detectable starch (i.e., 2.25% or above per the detection range of the glucose meter test, as described in Example 2) is crossed with a second parental alga with the feature of being substantially starchless. In some embodiments, the first parental alga with features of protein below 50% dry weight and containing starch at about 2.25% or above grows relatively slower than the second parental alga. In some embodiments, the first parental alga with features of protein below 50% dry weight and containing starch at about 2.25% or above grows relatively faster than the second parental alga.

In some embodiments, the first parental alga with features of protein below 50% dry weight and containing starch 2.25% or above that also has optional features of being yellow, walled, and able to quickly reach high density in fermentation is crossed with a second parental alga that is starchless with optional additional features of being green and wall-less. In some embodiments, the color is determined visually by eye when observing colonies on a plate, a liquid culture in a flask or other vessel, or a dried biomass. In some embodiments, the color is determined by a color chart such as the Pantone Formula Guide. In some embodiments, "yellow" or "green" is a term that appears as a quality or verbal description of the numerical values of the Pantone Formula Guide reading. In some embodiments, "yellow" is described as "yellow, warm red" without black and "green" is described as "yellow, green, black". In some embodiments, "yellow" has a Pantone reading of about 113 U or about 114 U. In some embodiments, "green" has a Pantone reading of about 2307 U or about 7496 U. In some embodiments, the feature of quickly reaching a certain density is determined by relative measures of growth rates among lines of algae or is determined by economics of an industrial production operation. Further embodiments regarding growth rates are provided in sections below.

In some embodiments, one parent is starchless, i.e., undetectable starch below about 2.25% starch by weight by assay or zero by proximate composition, and the other parent contains starch 2.25% or above. In some embodiments both parents are of the starchless

phenotype. The starchless phenotype can be obtained by any number of methods and is further described herein. Any *Chlamydomonas* parent with the starchless phenotype and ability to hybridize can be used in the invention. Progenies are then screened to ensure they have a phenotype of undetectable starch (i.e., subceeding 2.25%) and protein exceeding 50% to be suited for generating wholemeal useful for the food and beverage compositions of the instant invention.

In some embodiments, the first parental alga is a member of a group of *C. reinhardtii* algae with robust features for heterotrophic production used to mate with a second parental alga of a group of starchless *C. reinhardtii* of oppositive mating type. In certain embodiments, robustness includes resistance to oxidative stress. In some embodiments. a robust production line is tolerant of conditions, such as, for example, agitation, bubbling, or vessel pressure, that increase aeration or oxygenation levels as cell densities increase. In some embodiments, a robust production line requires less oxygen to maintain a preferred growth rate or to attain preferred densities. In some embodiments, a robust production line is tolerant of swings in nutrient levels and temperatures with nominal impact on growth rates. In some embodiments, a robust production line has walled cells or has wall-less cells. In some embodiments, one parental line can be robust, both parental lines can be robust, or neither parental line is robust.

In some embodiments, the process of hybridization of algae is improved to produce successful mating and recovery of viable progenies. In some embodiments, agglutination of mating types is promoted through cell proximity followed by desiccation of both zygotes and non-zygotic cells. In certain embodiments, the step of desiccation is performed in multi-well plates in the dark in a sterile laminar flow cabinet. In certain embodiments, any flowable liquid is pipetted out of the wells, removing most non-zygotic cells with the liquid in the process, leaving behind zygotes that stick to the plate wells. Zygotes are clearly visible and distinguished from non-zygotic cells by being larger, rounded and less translucent (i.e., darker). Plates are allowed to dry for several hours, such as, for example at least about 1 hour to about 24 hours. In some embodiments, non-zygotic cells die from desiccation while zygotes survive desiccation. In certain embodiments, Tris-acetate-phosphate (TAP) culture medium can be added and the plate can be incubated in light for about three days to germinate the zygotes. In certain embodiments, these cultures can be plated onto agar plates to generate colonies for screening. Illustratively, the improved process for hybridization of *C. reinhardtii* in the subject methods can be used generally in algae strain development programs.

In some embodiments, a microbial cell used to breed the desired progeny phenotype can have a specific heterotrophic mass cultivation, preferably with a known growth rate. In

certain embodiments, a growth rate can be about 0.8/day to about 1.3/day. In some embodiments, it is also useful to know the heterotrophically produced protein content and heterotrophic cell color in order to verify hybrids. Protein content of parental lines grown under nutrient replete conditions can range from about 25% to about 80%. Colors of parent lines can be green, yellow, 'white' (no yellow from lutein, with relatively low chlorophyll), orange/brown, or red. In certain embodiments, the breeding pedigree includes a facultative heterotrophic parental cell line that is yellow with a protein content of about 40%. In some embodiments, the protein content of the parental line is between about 30% to about 40%. In some embodiments, the breeding pedigree includes a facultative heterotrophic parental cell line with a protein content between about 25% to about 50%.

In one embodiment, the pedigree includes a cell line that is white. In several embodiments, color of a cell line is measured by the appearance of a liquid culture or a dried biomass using the Pantone Formula Guide color chart. In one embodiment, a white cell line is assessed as being visually pigmentless when growing as cell colonies on solidified medium. In one embodiment, the pedigree includes a cell line that carries a mutation in a starch-related locus. In several embodiments, a cell line that carries a mutation in the STA6, STA1, STA7, or STA11 loci are useful for producing biomass for use in the compositions of the instant invention as long as the resulting phenotype of the mutant are substantially starchless with undetectable starch (i.e., subceeding 2.25% per Example 2 or zero in a proximate composition) and protein exceeding 50%.

In some embodiments, some substantially starchless (e.g., less than about 2.25%) lines recovered after the process of hybridization vary in color. In some embodiments, yellow lines, light green lines, normal green lines, pink lines, white lines, chartreuse lines, gray lines, cream lines, red lines, or brown lines are recovered.

In some embodiments, some substantially starchless lines have a high protein content exceeding 50%, 60%, 70%, or 80% by weight. In some embodiments, the substantially starchless, high protein lines contain carotenoids, pigments, or are colorless.

In some embodiments, microalgal cell lines can be screened and selected during the strain improvement process for development of desired phenotypes for use in food and beverage compositions. In some embodiments, initial screening identifies lines that are substantially starchless. In some embodiments, initial screening of starch content is concurrent with screening for color of the cell lines. In some embodiments, yellow or white cell lines lacking starch are preferred over green cell lines lacking starch.

In some embodiments, initial screening of starch content is followed by optional screening of nitrogen usage (such as in a 96-well plate with differing concentrations of components in media to observe growth patterns) and/or the presence of a cell wall.

In some embodiments, the cell lines are screened for relative growth rate in flask and then in the fermentor. Thereafter, in some embodiments, the biomass composition is assessed using flask, or fermentor, grown biomass to identify cell lines with relatively high protein content above 50% by dry weight.

In some embodiments, the outcome of screening selects a cell line that combines the features of higher protein content above 50% and undetectable starch content with efficient heterotrophic growth, efficient heterotrophic protein productivity, and the presence of a cell wall.

In some embodiments, these considerations affecting the selection of the microalgal line omit the requirement for presence of a cell wall if the line can be produced under conditions providing sufficient oxygenation with dissolved oxygen of about 5% or higher preferably sufficient oxygenation for high productivity.

In some embodiments, *C. reinhardtii* cell lines improved in composition with greater than 50% protein and being substantially starchless resemble the walled, starch-containing origin parent in cell size and shape during cultivation, including loss of flagella by cells in fermentation production, but vary in their capability of metabolizing nitrogen in multiple forms, such as, for example, urea, nitrate, and ammonium in heterotrophic culture.

In some embodiments, cell lines of *C. reinhardtii* can possess a unique combination of characteristics described herein as represented by algal strains whose composition of biomass is greater than 50% protein by weight and are substantially starchless. In certain embodiments, biomass of all of these cell lines exceed 70% protein content, have fat content between about 12% to about 15%, and are substantially starchless when grown under nutrient replete conditions.

In some embodiments, all cell lines of *C. reinhardtii* are considered substantially starchless when their starch content is undetectable by common starch assays. In some embodiments, starch assays have lower limits of detection, below which starch is undetectable. In some embodiments, all substantially starchless algal lines produce a composition of biomass which measures below 2.25% starch (i.e., is undetectable below 2.25%) when assaying a 10 g/L biomass quantity by one assay method and also measures below 0.18% or below 0.09% starch when assaying a 10 g/L or a 20 g/L biomass quantity, respectively, by a more sensitive assay method.

In some embodiments, all algal samples tested for starch with readings that are below detectable when measured using a glucose meter are also below detectable when measured using a spectrophotometer for analysis by the AOAC 996.11 method.

In some embodiments, all substantially starchless algal samples analyzed for proximate composition indicate zero or no starch.

In some embodiments, all algal samples that exceed 70% protein by weight are also substantially starchless with an undetectable starch content.

In some embodiments, cell lines of *C. reinhardtii* possess a unique combination of characteristics described herein as represented by, but not limited to, algal strains KAS2010 (including yellow and green forms), KAS2013, KAS2014, and KAS2015. These share the features of having undetectable starch, having protein exceeding 70% and lipids between about 12 to about 15% when biomass is produced under nutrient replete conditions, but differ from each other in ability to grow on differing forms of nitrogen; in their relative growth rates; and in color.

In some embodiments, cell lines that have undetectable starch and high protein exceeding 50% along with lipid of about 12% to about 15% can have differences in nitrogen metabolism. In some embodiments, cell lines, such as, for example, KAS2010, KAS2013, and KAS2015, are capable of metabolizing nitrogen in the form of urea and ammonium but nitrate only poorly. In some embodiments, KAS2014 grows well on urea, nitrate, and ammonium. In some embodiments, growth on nitrogen types is used to confirm hybrid status of colonies resulting from the crossing of a parent that grows very slowly in heterotrophic cultivation on nitrate with a second parent that grows comparatively better in heterotrophic cultivation on nitrate.

In some embodiments, a range of colors from olive green to yellow on plates and in flasks is obtained from the substantially starchless, high protein progeny lines. In some embodiments, this is exemplified by KAS2010, KAS2014, and KAS2015. In some embodiments, the progeny are yellow. In some embodiments the progeny line KAS2013 is yellow. In some embodiments, a progeny line of one color becomes a different color in subsequent generations. In one embodiment, KAS2010 of olive green gives rise to yellow cells in subsequent generations.

In some embodiments, a substantially starchless line can differ from another substantially starchless line by relative growth rate. In some embodiments, KAS2013-yellow grows slower, at a specific growth rate of about 0.8/day to about 0.9/day in a 10-L fermentor, than KAS2010 with about at least 1.3/day in same fermentor conditions as KAS2013-yellow.

In some embodiments, KAS2014 and KAS2015 grow relatively slower in a flask than KAS2010.

In some embodiments, *Chlamydomonas* phenotypes that display undetectable starch content along with protein content above 50%, above 60%, more preferably above 70%, irrespective of their genotype, are suited for use in the food compositions of the instant invention. In certain embodiments, *Chlamydomonas* phenotypes that display undetectable starch content along with protein content above 50%, above 60%, more preferably above 70%, irrespective of which form of nitrogen they can metabolize, of being wall-less or possessing a cell wall, of their color, or of their cell growth as clumps or not, produce a biomass suited to use in the edible compositions of the instant invention.

In certain embodiments, *Chlamydomonas* phenotypes whose composition exceeds 70% protein content have fat content between about 12% to about 15% and are substantially starchless when produced under nutrient replete heterotrophic conditions.

In some embodiments, a *C. reinhardtii* cell line is cultivated to produce a biomass. In some embodiments, the biomass is characterized for its chemical composition. In some embodiments, the chemical characterization assesses the biomass as an ingredient for use in an edible composition. In some embodiments, the *C. reinhardtii* ingredient is improved by an absence of starch content. The absence of starch results in a simplified chemical composition of higher purity. In some embodiments, the absence of starch is due to elimination of starch biosynthesis during biomass (wholemeal) production. In some embodiments, the elimination of starch in the algal wholemeal results in improving (i.e., lowering) the relative glycemic index of a composition due to the absence of starch-derived sugars from the *C. reinhardtii* ingredient as a protein source when compared to using starch-containing alga wholemeal as a protein source. In some embodiments, improving the purity of an ingredient by eliminating its starch minimizes unwanted or non-nutritive calories. In some embodiments, a healthy diet that avoids foods with non-nutritive calories and higher glycemic index helps address causes of metabolic syndrome. In some embodiments, the effects of minimized starch content on the protein content of a *C. reinhardtii* cell for nutrition purposes can be quantified.

In some embodiments the improved microalgal component of a food composition is derived from an alga, such as, for example, *Chlamydomonas reinhardtii*, grown heterotrophically that is improved in purity by comprising protein exceeding about 50%, exceeding about 60%, exceeding about 70%, or even exceeding about 80% and with starch subceeding about 2.25%, subceeding about 0.18%, subceeding about 0.1%, or even subceeding about 0.09% or being substantially starchless by weight.

In one embodiment, the proximate composition of algal biomass, such as, for example, *C. reinhardtii*, grown under nutrient replete heterotrophic conditions shows about 73.45% protein, about 12.61% fat, about 12.89% ash, about 3.75% moisture, and about 0.49% fiber. Carbohydrate content is none by calculation and starch is undetectable; thus, the biomass is deemed substantially starchless. In one embodiment, the caloric value is about 3643 Kcal/kg.

In one embodiment the proximate composition of the algal biomass grown under nutrient replete heterotrophic conditions shows a proximate composition of about 72.1% protein, about 14.92% fat, about 11.32% ash, about 4.75% moisture, and about 0.45% fiber. Carbohydrates is none by calculation and starch is undetectable.

In one embodiment the proximate composition of the algal biomass, such as, for example, *C. reinhardtii*, grown under nutrient replete heterotrophic conditions shows a proximate composition of about 72.1% to about 73.45% protein, about 12.89% to about 14.92% fat, about 11.32% to about 12.89% ash, about 3.75% to about 4.75% moisture, about 0.45% to about 0.49% fiber, and undetectable starch.

In some embodiments, the ash content of the algal biomass grown under nutrient replete heterotrophic conditions can be about 5% to about 12.89%, about 5% to about 12.6%, or about 5% to about 11.32% and protein content be equal to or exceed about 72% protein, about 73.5% protein or 79.5% protein.

In some embodiments, substantially starchless cell lines of *C. reinhardtii* produce biomass with lower protein (but still in excess of 50%) by overfeeding one or more other nutrients, such as, for example, urea, ammonium, iron, sulfate, or any combination thereof, compared to cultures that are not overfed and have higher protein. In some embodiments, nutrients that are three times or more in concentration compared to the initial concentration in growth medium used at the start of a fermentation are considered overfed. For example, urea at a concentration of about 0.11 g/L would be overfed if delivered to the organism at a concentration of about 0.33 g/L. Magnesium sulfate heptahydrate at a concentration of about 0.05 g/L would be overfed when delivered to the organism at a concentration of about 0.15 g/L. In one embodiment, that overfed heterotrophically grown biomass of *C. reinhardtii* contains about 58% crude protein with about 19% fat. In another embodiment, an overfed biomass contains about 62% protein and about 15% fat. In one embodiment, overfed biomass contains about 55% to about 65%, about 56% to about 64%, about 57% to about 63%, about 58% to about 62%, about 59% to about 61%, or about 60% crude protein with about 10% to about 25%, about 11% to about 24%, about 12% to about 23%, about 13% to about 22%, about 14% to about 21%, about 15% to about 21%, about 15% to about 20%, or about 15% to about

19% fat. In some embodiments, the medium comprising an over-supply of nutrients that results in biomass with lower protein content, at about 62% protein or at about 58% protein, has a relatively lower C:N (carbon to nitrogen) ratio compared to those ratios in medium yielding about 73% protein and about 12% fat. In one embodiment, the lower protein (e.g., 58%) and higher fat (e.g., 19%) biomass can a higher caloric value of, for example, about 3912 Kcal/kg than the higher protein (e.g., 73.5%), lower fat (e.g., 12.6%) biomass with caloric value of, for example, 3643 Kcal/kg. In certain embodiments the range of caloric value is from about 3600 Kcal/kg to about 3920 Kcal/kg.

In certain embodiments, a relatively lower C:N ratio due to acetate depletion (from withholding acetic acid feed but not nitrogen or other nutrients in heterotrophic fermentation) results in fat content decreasing by about 22% while biomass decreases by about 11% compared to nutrient replete cultivation conditions. In certain embodiments, the algal compositions can have an amino acid profile expressed as amino acid percentage of total amino acids comprising: methionine at a concentration of about 1.75% to about 2.25% or about 2.03% to about 2.15% relative to the total amino acids; cystine at a concentration of about 0.50% to about 2.25% or about 1.10% to about 1.70% relative to the total amino acids; lysine at a concentration of about 5.50% to about 8.00% or about 6.16% to about 7.15% relative to the total amino acids; phenylalanine at a concentration of about 3.50% to about 5.00% or about 4.01% to about 4.49% relative to the total amino acids; leucine at a concentration of about 6.50% to about 9.10% or about 7.53% to about 7.80% relative to the total amino acids; isoleucine at a concentration of about 2.00% to about 4.11% or about 2.79% to about 3.05% relative to the total amino acids; threonine at a concentration of about 4.00% to about 5.50% or about 4.70% to about 4.90% relative to the total amino acids; valine at a concentration of about 4.00% to about 6.33% or about 4.70% to about 5.21% relative to the total amino acids; histidine at a concentration of about 1.25% to about 3.25% or about 1.82% to about 2.75% relative to the total amino acids; arginine at a concentration of about 7.00% to about 17.50% or about 11.21% to about 15.06% relative to the total amino acids; glycine at a concentration of about 4.75% to about 6.00% relative to the total amino acids; aspartic acid at a concentration of about 8.50% to about 10.50% or about 9.01% to about 9.48% relative to the total amino acids; serine at a concentration of about 3.75% to about 6.0% or about 4.35% to about 5.08% relative to the total amino acids; glutamic acid at a concentration of about 10.20% to about 12.00% or about 11.14% to about 11.38% relative to the total amino acids; proline at a concentration of about 4.06% to about 7.00% or about 5.33% to about 6.01% relative to the total amino acids; hydroxyproline at a concentration of about 1.00% to about 3.00% or about

1.44% to about 2.33% relative to the total amino acids; alanine at a concentration of about 7.00% to about 8.50% or about 7.45% to about 7.87% relative to the total amino acids; tyrosine at a concentration of about 2.75% to about 4.25% or about 3.26% to about 3.90% relative to the total amino acids; and tryptophan at a concentration of about 0.75% to about 2.00% or about 1.02% to about 1.19% relative to the total amino acids.

In certain embodiments, the algal composition includes glycine, a pet food palatant and cell health promoter, but excludes starch.

In certain embodiments, the algal compositions contain both protein, comprised of amino acids, and fats, comprised of fatty acids.

In certain embodiments, the algal compositions can have a fatty acid profile comprising: myristic acid (C14:0) at a concentration of about 0.01% to about 1.00% or about 0.38% to about 0.45% relative to the total fatty acids, myristoleic acid (C14:1) at a concentration of about 0.005% to about 0.50% or about 0.05% to about 0.18% relative to the total fatty acids; pentadecanoic acid (C15:0) at a concentration of about 0.005% to about 0.25% or about 0.05% to about 0.06% relative to the total fatty acids; palmitic acid (C16:0) at a concentration of about 20.00% to about 35.00% or about 27.27% to about 28.01% relative to the total fatty acids; palmitoleic acid (C16:1ω7) at a concentration of about 1.00% to about 10.00% or about 3.28% to about 6.32% relative to the total fatty acids; hexadecadienoic (C16:2) at a concentration of about 0.00% to about 0.50% or about 0.01% to about 0.11% relative to the total fatty acids; hexadecatrienoic acid (C16:3) at a concentration of about 0.00% to about 0.50% or about 0.01% to about 0.12% relative to the total fatty acids; hexadecatetraenoic acid (C16:4) at a concentration of about 1.0% to about 10.0% or about 3.46% to about 5.94% relative to the total fatty acids; heptadecanoic acid (C17:0) at a concentration of about 0.01% to about 0.50% or about 0.11% to about 0.12% relative to the total fatty acids; heptadecanoic acid (C17:1) at a concentration of about 0.00% to about 2.00% or about 0.00% to about 0.97% relative to the total fatty acids; stearic acid (C18:0) at a concentration of about 1.00% to about 10.00% or about 3.52% to about 3.55% relative to the total fatty acids; oleic acid (C18:1ω9 & C18:1ω8) at a concentration of about 5.00% to about 15.00% or about 9.7% to about 12.47% relative to the total fatty acids; oleic acid (C18:1ω7) at a concentration of about 2.50% to about 10.00% or about 6.12% to about 7.99% relative to the total fatty acids; linoleic acid (C18:2ω6) at a concentration of about 5.00% to about 25.00% or about 8.34% to about 18.49% relative to the total fatty acids; linoleic acid (C18:2ω4) at a concentration of about 0.00% to about 10.00% or about 0.00% to about 7.83% relative to the total fatty acids; linolenic acid (C18:3ω6) at a concentration of about 0.00% to about 1.0% relative to the total fatty acids; linolenic acid

(C18:3ω3) at a concentration of about 5.00% to about 15.00% or about 9.03% to about 12.01% relative to the total fatty acids; octadecatetraenoic acid (C18:4ω3) at a concentration of about 0.00% to about 1.00% relative to the total fatty acids; arachidic acid (C20:0) at a concentration of about 0.01% to about 0.50% or about 0.0% to about 0.11% relative to the total fatty acids; eicosanoic acid (C20:1ω9) at a concentration of about 0.01% to about 0.50% or about 0.05 to about 0.09% relative to the total fatty acids; eicosanoic acid (C20:1ω7) at a concentration of about 0.01% to about 0.50% relative to the total fatty acids; eicosadienoic acid (C20:2ω6) at a concentration of about 0.01% to about 0.50% or about 0.01% to about 0.05% relative to the total fatty acids; eicosatrienoic acid (C20:3ω6) at a concentration of about 0.01% to about 0.50% relative to the total fatty acids; arachidonic acid (C20:4ω6) at a concentration of about 0.01% to about 0.50% relative to the total fatty acids; eicosapentaenoic (EPA) acid (C20:5ω3) at a concentration of about 0.01% to about 0.50% relative to the total fatty acids; behenic acid (C22:0) at a concentration of about 0.01% to about 0.50% or about 0.05% to about 0.12% relative to the total fatty acids; and lignoceric acid (C24:0) at a concentration of about 0.01% to about 0.50% or about 0.05% to about 0.10% relative to the total fatty acids. In certain embodiments, other fatty acids can be in the algal compositions at a concentration of about 1.00% to about 25.50% or about 5.97% to about 17.48% relative to the total fatty acids.

In certain embodiments, the high protein starchless biomass contains a fatty acid that is absent in the starch-containing parent, namely C18:2ω4.

In certain embodiments, the high protein starchless biomass contains palmitoleic acid (16:1ω7) with known anti-inflammatory properties and benefits to help inhibit metabolic syndrome.

In certain embodiments, the invention provides a method that enables the generation of heterotrophic biomass from facultative heterotrophic algae cells, such as, for example, *C. reinhardtii* cells, with higher protein above 50% by weight, above 60% by weight, above 70% by weight, and even above 80% by weight and being substantially starchless. The biomass can be generated in a fermentor at a high growth rate and over a period meaningful for industrial application.

In one embodiment, the culture pH is maintained at about 7.3 to about 7.8. In one embodiment, the culture pH is maintained at about 6.5 to about 7.0. In some embodiments, the ranges of operational pH for the growth phase are about 5.5 to about 8.5.

In certain embodiments, the fermentation culture is sustained for a period sufficient to produce growth whereby at least about 24 hours, about 36 h, about 48 h, about 60 h, or about 72 h or more. In some embodiments, the total fermentation cycle time is about 72 h, about 96,

about 120 h, about 144 h, or about 168 h or by any duration falling within the range of 24 hours to 168 hours or a duration that is economically justified. In certain embodiments, those numbers apply to cultures that are completely harvested.

In certain embodiments, using the draw and fill method, the fermentation culture can be 24 hours or less depending on how much of the culture is drawn, such as, for example, an 80% culture draw, in which the same volume that is removed is added back as new media. In one embodiment using the continuous culture method, fermentation culture will extend beyond about 168 hours, such as, for example, at least about 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days.

In some embodiments, the specific growth rate of the *C. reinhardtii* phenotype that contains detectable starch (i.e., is above 2.25% by weight) is between 1/day to about 2/day, between 1.1/day to about 1.8/day, between 1.3/day to about 1.4/day, or at least about 1.34/day.

In some embodiments, biomass productivity of the algae with a starch content of less than about 2.25% by weight is as productive as an alga with a starch content of greater than about 2.25% by weight. In some embodiments, the biomass productivity of algae with a starch content of less than about 2.25% by weight can be somewhat lower than the algae with a starch content of greater than about 2.25% by weight, preferably if the protein productivity is much higher than the algae with a starch content of greater than about 2.25% by weight. In some embodiments, the biomass productivity of algae with a starch content of less than about 2.25% by weight can be about 5% to 50% lower than the algae with a starch content of greater than about 2.25% by weight if the protein productivity is 1.4-times to 4.4-times higher than the algae with a starch content of greater than about 2.25% by weight.

In certain embodiments, the invention is directed to improved higher purity wholemeal that is also improved with more efficient production. In some embodiments, a higher productivity of biomass, protein, and pigments over a period is preferred for industrial manufacturing. Productivity is the amount of a target compound such as biomass, or a component of the biomass such as protein or pigment in the total biomass, produced over a fixed time. For biomass components such as protein, the productivity is calculated as final density of dried biomass over cultivation time multiplied by the protein content (% of biomass). Higher productivity can be a result of increased final density, decreased cycle time, or higher protein content in biomass. Faster growth rate can help with the density and cycle time but is not required as starting density can be increased to make slower growing lines as productive as faster ones.

In some embodiments, the rate of biomass production is independent of the protein content above 50% dry weight and being substantially starchless. In some embodiments, the rate of protein production depends on the rate of biomass production, such that cell cultures with a given protein content have higher protein productivity if the cell line grows faster in a given period of time.

In some embodiments, the productivity of algae during fermentation production of the improved higher purity phenotype that is substantially starchless surpasses that of the unimproved parent that contains starch. In some embodiments the specific growth rate of *C. reinhardtii* phenotype that is substantially starchless is between 0.8/day to about 2/day, between 1.4/day to about 1.8/day, or at least about 1.41/day.

In some embodiments, the protein productivity during fermentation production of the algae that yield improved higher purity biomass that is substantially starchless surpasses that of the algae that contains starch above 2.25% by weight. In one embodiment, the protein productivity is about 500 mg/L-hour to about 1000 mg/L-hour, about 702 mg/L-hour to about 770 mg/L-hour, or about 760 mg/L-hour for about 70% to about 85%, about 73.45% to about 80.5% or about 79.5% protein, in the substantially starchless algae. For lower protein lines, the protein productivity can be less than 702, 600, or 500 mg/L-hour but still exceed that of the algae that contains starch above 2.25% which has a protein productivity of about 229 mg/L-hour, about 305 mg/L-hour, to about 343 mg/L-hour as calculated for 30%, 40%, to 45% protein, respectively.

In one embodiment, the protein productivity is, for the draw and fill mode of fermentation production, about 1500 mg/L-hour to about 2000 mg/L-hour or about 1766 mg/L-hour to about 1788 mg/L-hour for about 70% to about 85% or about 79.5% to about 80.5% protein content by weight.

Notably this source of biomass for algal protein without the added burden of algal starch of the instant invention is obtained from manufacturing using a carbon feedstock that is not a sugar (e.g., glucose). In several embodiments, the biomass is produced from fermentation utilizing acetic acid as carbon feedstock. In some embodiments, alternatives to acetic acid include, for example, carboxylic acids, sugar acids, and chlorogenic acids, formic acid, propionic acid, citric, fumaric, glycolic, lactic, malic, pyruvic, and succinic acids.

In some embodiments, the improved higher protein substantially starchless algae phenotype has an intermediate acetic acid/acetate (carbon feedstock) use rate between its two mating parents of about 2 g acetic acid/g biomass to about 4 g acetic acid/g biomass, about 2.4

g acetic acid/g biomass to about 3.0 g acetic acid/g biomass, or about 2.7 g acetic acid/g biomass.

In some embodiments, the pigment productivity during fermentation production of the improved higher purity phenotype algae that is substantially starchless surpasses that of algae that contains starch above 2.25% by weight. In one embodiment, the pigment productivity is above about 5.5 mg/L-hour. In some embodiments, the pigment productivity is about 5.74 mg/L-hour for biomass with 0.6% carotenoid pigments by weight or 9.6 mg/L-hour with 1% carotenoid pigments by weight. In some embodiments, the pigment productivity ranges from about 5.5 mg/L-hour to about 9.6 mg/L-hour.

In certain embodiments, to grow the algae of the subject invention, flasks are incubated at about 25°C to about 35°C or about 28°C in the dark. In certain embodiments, the algae can be shaken, agitated, or other moved, with shaking at about 1 rpm to about 1000 rpm, about 10 rpm to about 500 rpm, or about 115 rpm. In certain embodiments, flask heterotrophic growth media consist of about 0.05 g/L to about 0.25 g/L urea or about 0.11 g/L urea, about 0.025 g/L to about 0.1 g/L magnesium sulfate heptahydrate or about 0.05 g/L magnesium sulfate heptahydrate, about 0.025 g/L to about 0.1 g/L calcium chloride dihydrate or about 0.05 g/L calcium chloride dihydrate, about 0.01 g/L to about 0.04 g/L potassium phosphate or about 0.02 g/L potassium phosphate, about 0.005 g/L to about 0.02 g/L iron-EDTA or about 0.01 g/L iron-EDTA, about 0.003 g/L to about 0.015 g/L iron chloride hexahydrate or about 0.0063 g/L iron chloride hexahydrate, about 11 mg/L to about 44 mg/L tetrasodium EDTA or about 22 mg/L tetrasodium EDTA, about 0.15 mg/L to about 0.6 mg/L cobalt sulfate or about 0.3 mg/L cobalt sulfate, about 3 mg/L to about 12 mg/L manganese sulfate or about 6 mg/L manganese sulfate, about 0.4 mg/L to about 1.6 mg/L zinc sulfate or about 0.8 mg/L zinc sulfate, about 0.1 mg/L to about 0.4 mg/L copper sulfate or about 0.2 mg/L copper sulfate, about 0.35 mg/L to about 1.4 mg/L ammonium molybdate or about 0.7 mg/L ammonium molybdate, and about 0.2 mg/L to about 0.8 mg/L boric acid or about 0.4 mg/L boric acid. Additionally, the flask medium contains either about 1.25 g/L to about 6.0 g/L Tris base or about 2.78 g/L Tris base with about 0.05 mL/L to about 2.5 mL/L acetic acid or about 1.15 mL/L acetic acid or it may initially contain about 0.75 g/L to about 3.5 g/L sodium acetate or about 1.6 g/L sodium acetate to allow for maintenance growth of low-density flask cultures.

In certain embodiments, the culture can then be transferred to fermenter heterotrophic growth media, which is the same media as the flask heterotrophic media but additionally supplied with 0.7 g/L Tris base and an initial 0.29 mL/L acetic acid or 0.4 g/L sodium acetate, ranging up to 2.78 g/L Tris base and 1.15 mL/L acetic acid or 1.6 g/L sodium acetate, optionally

with an initial biomass density of at least about 0.01 g/L, about 0.1 g/, or about 0.2 g/L. Cultivation of the algae preferably occurs in darkness; optionally it can be mixotrophic under ambient or other lighting if desired such as, for example, about 10 μE/m^2-sec (10 μmol photons/m^2-sec) (m^{-2} s^{-1}) to about 400 μE/m^2-sec). In certain embodiments, the culture can be incubated at about 25°C to about 35°C or about 28°C with gas exchange provided by 7.2 L/min sparged air (about 6 to about 8 L/min) and an impeller at about 100 rpm to 650 rpm at a pH about 7.3 to about 7.8. In certain embodiments, throughout the duration of the fed-batch fermentation, pH-triggered additions of 20% acetic acid supplied at 5% pump speed and other nutrients (all excluding the acetic acid) are frequently supplied every about 0.5 to about 4 hours at about 10% pump speed throughout the fermentation to keep nutrient levels near starting concentration of fermenter heterotrophic growth media, particularly to maintain a pH of about 5.5 to about 8.5, 6.5 to about 7.0, or about 7.3 to about 7.8, and thus the pH set points for triggering acetic acid/acetate feeds can vary accordingly as is known in the art and are not limited to maintaining pH 7.3 to 7.8. Alternatively, macronutrients, micronutrients and organic carbon source, such as, for example, acetic acid, are supplied together in one feed. In certain embodiments, samples can be collected every about 24 hours for dry weight analysis to determine growth rate.

In some embodiments, the pigment content of the substantially starchless algae is sufficient for functional food compositions when used at about 1% or higher wholemeal inclusion. In some embodiments, the antioxidant features imparted by the antioxidant-containing composition of xanthophylls and carotenoids, such as, for example, lutein and beta-carotene, are imparted to an uncooked product. In some embodiments, the antioxidant functionalities imparted by the antioxidant-containing composition are imparted to the cooked product.

In some embodiments, efficient production of substantially starchless algae with acceptable biomass and protein productivity rates is suited for commercial manufacturing. Illustratively improved productivity translates to improved cost efficiency for practical deployment purposes in foods and beverages.

A critical feature is to provide edible compositions improved also in sustainability by using a more water-conservative, land-conservative, and nutrient partitioning-efficient protein or pigment in the wholemeal of the substantially starchless algae component compared to using land grown plant-derived protein or pigment or animal protein or algae with starch greater than 2.25% by weight; and optionally also by using recycled carbon in the fermentation feedstock.

In certain embodiments, the higher protein, substantially starchless algae-containing compositions reduce ecological and environmental impact over plant-based proteins or animal derived proteins. In some embodiments water usage for cultivation of biomass is lowered by about 99% when using the improved higher purity algae compared to plant and animal protein sources.

In certain embodiments, edible compositions can be improved in sustainability by using a wholemeal of the higher protein substantially starchless algae with a more water-conservative, land-conservative protein or pigment compared to using land grown plant-derived protein or pigment or animal protein.

In certain embodiments, edible compositions can be improved in sustainability by using a wholemeal of the higher protein substantially starchless algae that is more sustainably produced compared to lower purity algae with less protein or pigment and containing more starch. In one embodiment, the more sustainable production is from more efficient partitioning of carbon into nutritionally valued protein instead of into less nutritionally valued starch under nutrient replete conditions.

In certain embodiments, the more sustainable production is by using recycled carbon in the fermentation feedstock via an acetic acid or acetate chemical intermediate. In certain embodiments, the more sustainable production is by using process oxygen in the fermentation.

Edible Algal Compositions

The present invention provides microalgae, such as, for example, *Chlamydomonas reinhardtii*, as described herein when used in the production of a product to provide nutritive or functional property of the algae component without concurrently increasing the level of starch or negatively impacting the glycemic index (GI) or non-nutritive calories of said product.

Accordingly, the present description provides for the use of an algal ingredient with improved higher purity as described herein, produced according to a process described herein, such as, for example, a wholemeal biomass in the production of a food, petfood, beverage or food supplement product.

In some embodiments, the algae or part thereof is in the form of, for example, a food product, petfood product, a beverage, a pet beverage, a food supplement, or a petfood supplement composition. Several embodiments pertain to food, beverage, and food supplement compositions for humans and for pets comprising improved biomass components as foodstuff from microalgae in combination with one or more other edible component.

The edible component can be selected from all the major food groups. These include, without limitation, fruits, vegetables (dark green vegetables, red and orange vegetables, starchy vegetables, roots and tubers, and legumes), grains (whole, cracked, or milled into flour), proteins (fish, poultry, meats, eggs, microbial, insect, nuts and seeds, and alternatives), dairy and alternatives, and oils and spreads. Water, though not belonging to a food group per se, can be a component of the food materials and is an important component of beverages as sources of hydration. Fiber comes from any food that is plant based, such as fruits, vegetables, grains, legumes, nuts, and seeds. Many components can be multifunctional. For example, nuts and seeds are rich sources of heart-healthy oils as well as protein and fiber.

Other edible components with which the algal component can be combined in accordance with the present invention to produce an edible composition depends on the product to be produced and the desired properties, including, without limitation, components that may provide calories, essential nutrients, other nutrition or fiber, antioxidants, organoleptic features, texture, formulating properties, cooking properties, shelf life properties, and packaging properties. These can include, without limitation, carbohydrates, protein, fats, vitamins, minerals and water, vegetable broth, juice, wine, vinegar, herbs and spices, and food additives.

In certain embodiments, the edible component is any ingredient used to create a food, food supplement, or beverage, such as, for example, grain (e.g., flours including nut flour, corn, rice, wheat, barley, rye, quinoa, oats, cornmeal, soy, or other grains such as used in bread, tortillas, rice, pasta, and breakfast cereals); protein (e.g., eggs as dried or liquid, meats such as poultry, chicken, turkey, duck, beef, lamb; seafood such as fish, prawns and shrimp, tuna, salmon, crab, lobster, oyster, scallop, other shellfish; whey protein, pea protein, soy protein, fungal or insect protein, beans, lentil, nuts, seed, soy products); dairy products and alternatives, full fat, fat free or reduced fat (e.g., milk, yogurt, kefir, butter, cream, coconut milk and cream, cheese, cream cheese, cheese products such as cheese sauces such as nacho, cheese roux, and cream cheese dips; nut, grain and seed milks such as almond, oat, rice, soy); oils and solid fats (polyunsaturated fats with omega-6 fatty acids such as corn, safflower, sunflower and soybean oils, and omega-3 fatty acids such as cod liver oil, canola, flax seed oil, salmon, anchovy, sardines, and walnuts; and monounsaturated fat such as olive, canola, and peanut oils; grapeseed, coconut, palm, soybean, sesame, fat, lard, shortening, margarine/spreads, nut butters, dressings); carbohydrates (grain flour, nut flours, starches, roots, tubers, breadfruit, cassava); dietary fiber; water or other liquids (e.g., bouillon; wine; vinegar; liquid aromatics or essential oils such as vanilla extract, almond extract, rose water; sauces such as fish sauce, oyster sauce, remoulade); fruit (e.g., stone fruits, melons, citruses such as lemon, lime,

tangerine, orange, pomelo; berries such as strawberry, raspberry, blueberry, blackberry; tropical fruits such as banana, lychee, mango, papaya, pineapple, coconut); vegetable (e.g., cucurbits including cucumber and squash; brassicas including broccoli, Brussels sprouts, cabbage, cauliflower, collard greens, kale, and turnips; asparagus; legumes such as garden pea, chickpea, lentil; parsley; leafy greens, onions, garlic, ginger mushrooms; roots and tubers such as potatoes, sweet potatoes, yams, ginger, daikon, cassava, carrots; beets; fermented, brined or pickled vegetables such as caper, olives, pickles, kimchi, radish, sauerkraut); sweetener (e.g., sugar, molasses, agave, honey, artificial sweeteners, sorbitol, mannitol, glycerol), herbs and spices.

In certain embodiments, the edible component is any ingredient used to create a food, food supplement, or beverage, such as, for example, food additives. These include without limitation, thickeners, stabilizers, emulsifiers, and gelling agents (e.g., alginic acid, alginates, agar, agarose, gelatin, microbial gums, vegetable gums, tapioca, pectin, glycerol, polyoxyethylene stearates, polysorbates, celluloses, starches, organic acid esters of mono- and diglycerides of fatty acids, bromated vegetable oils, polyphosphates, oxystearin, lecithin, sodium stearoyl lactylate, stearyl citrate); anti-caking, leavening and raising agents (e.g., mannitol, magnesium and other salts of fatty acids, sodium silicates, carbonates, bicarbonates, baking soda, baking powder, yeast, cream of tartar, hydrochloric acid, calcium phosphates, silicon dioxide); humectants (e.g., glycerin, honey); firming agents (e.g., magnesium chloride, aluminum sulfates, calcium hydroxide); preservatives, antioxidants, acidity regulators, and pH control agents (e.g., ascorbates, tocopherols, tocotrienols, xanthophylls and carotenoids such as astaxanthin, lutein, zeaxanthin, carotenes, and lycopene, polyphenols, bioflavonoids, gallates, lactates, citrates, tartrates, phosphates, succinates, sorbates, nitrates, tartrates, benzoates, propionates, sulfites, acetates, sodium ascorbate, ascorbic acid, citric acid, malic acid, rosmarinic acid, sorbic acid, benzoic acid, levulinic acid, phosphatidic acid, anisic acid, acetic acid, paraben, stannous chloride, butylated hydroxytoluene, butylated hydroxyanisole, phospholipids such as N-acylphosphatidylethanolamine, phosphatidyl ethanolamine, phosphatidylcholine, phosphatidylinositol and lysophosphatidyl choline); sequestrants (e.g., gluconates, disodium EDTA); bulking agents (e.g., guar gum, psyllium husk, starch, polydextrose, inulin); foaming agents (e.g., quillaia extract); anti-foaming agents (e.g., oils); waxes and glazing agents; packaging gases; food colors; and flavor enhancers (e.g., glutamates, inosinates, maltol, zinc acetate, ribonucleotides).

In some embodiments, the algal biomass itself contains components that have antioxidant properties. These properties can be attributed to components naturally present in

the biomass which include but are not limited to chlorophylls, xanthophylls and carotenoids, tocopherols (vitamin E) and tocotrienols, and essential minerals such as magnesium, calcium, zinc, copper, and manganese. In some embodiments, total tocopherols, comprising about 10 to 80 ppm, can have four forms (α, β, γ, and δ) of which α-tocopherol has the highest vitamin E activity and accounts for the largest fraction of about 80%. The invention provides for an improved higher purity foodstuff comprising wholemeal of higher protein and substantially starchless algae that can be used in a food or beverage composition to deliver effective amounts of algal protein for nutrition or functionality, or to deliver algal pigment for functionality, and thereby improve the overall food or beverage product.

In certain embodiments, the algal compositions can fortify foods in protein from, for example, *Chlamydomonas reinhardtii*, without concurrently adding sugars derived from algal starch. The improved foodstuff simplifies the composition and formulation of foods using algae since it avoids also bringing in starch with the improved wholemeal material. A beneficial feature is the ability to comprise an edible composition with algal wholemeal without concurrently increasing the glycemic index of the food or adding non-nutritive calories through presence of algal starch.

In certain embodiments, an edible composition comprises algal heme or flavor compounds without added algal starch sugars. In one embodiment, vanillin is added during algal fermentation cultivation as a process aide at a concentration that reduces oxygen demand of the algal culture and adds aroma to the wholemeal as an approved food flavoring agent. In certain embodiments, the vanillin can be added to the algal culture during fermentation and permitted to continue fermentation for about 3 to about 24 hours. In certain embodiments, about 1 mg/L to about 500 mg/L, about 1.9 mg/L to about 100 mg/L, about 20 mg/L to about 50 mg/L, about 20 mg/L to about 40 mg/L, or about 7 mg/L of vanillin can be added to the algal culture medium. Alternatives to vanillin include, for example, wortmannin and cisplatin.

In some embodiments, the edible composition comprising the algae composition is a mixture, food supplement, oil-in-water emulsion, batter, dough, pancake, pasta, pasta filling, mayonnaise/dressing, meat-like meatball, textured meat, pet food, pet treat, cat kibble, soup/broth, algae milk, algae cream, sweet or savory snack, muesli bars, protein bars, jerky, crisps, smoothie, sports drink, or cocktail.

In certain embodiments, the composition includes the algae component to replace egg in an extruded pasta composition and to produce a superior pasta. In certain embodiments, the composition includes the algae component to replace plant-based protein, such as from pulses (e.g., beans (e.g., kidney beans, navy beans, black beans, broad beans, lima beans, butter

beans), chickpeas, lentils, and peas). In certain embodiments, the composition includes texturized algal wholemeal that is a texturized algal protein product with fibrous texture resembling animal meat.

In certain embodiments, the composition includes texturized algal wholemeal that is a texturized algal protein product with fibrous texture resembling animal meat. Texturing can be by extrusion, a mechanical process that uses heat and pressure to transform the appearance. Low-moisture extrusion (LME, moisture <50% during texturization) and high-moisture extrusion (HME, moisture 50-70% during texturization) are well-established modes of extrusion suited to texturize compositions incorporating the high-protein algal component.

In certain embodiments, shear cell technology can also be used for texturing with inclusion of cross-linking agents, such as, for example, calcium and transglutaminase, in the composition. Micro-extrusion is another method using 3D printing with the algal protein or high-protein algal biomass combined with one or more of xanthan gum (e.g., xanthan gum), gelatin, sodium alginate, or xylose for forming whole-muscle type structures.

In certain embodiments, enzyme treatment such as by transglutaminase before or after extrusion or shear cell treatment can help modify the texturization of the algal protein to resemble properties of meat proteins more closely. Fermentation of extruded product, such as by mycelia, can be used to improve taste, texture, and water-holding capacity.

In certain embodiments, texturing can also be by scaffolding to recreate muscle fiber appearance. This can use 3D printed matrices or freeze structuring (such as hydrocolloid matrices created by freeze-thaw processing), to create a scaffold for adding in the high protein algal component of the instant invention. The type of texture of the end-product depends on the type of meat being replicated or the organoleptic or cooking property that is desired. Restructuring types can be for replication of muscle cuts or ground meat, for chewability or hardness, for example.

Other texturing methods include freeze alignment, freeze structuring, spinning, electrospinning. In some embodiments, the composition is a mixture of proteins, hydrocolloids, and multivalent cations. In certain embodiments, the texturized composition containing algal protein has a texture resembling minced meat.

In certain embodiments, the algae-containing composition of textured meat alternative is well suited to post-texturization treatments. Such treatments can include marinating, hydration, breading, coating, or other cooking procedures. The high protein substantially starchless algal material used in the composition is advantaged by low carbohydrate that can

otherwise interfere with breading and oil pickup; and by lack of starch gelatinization from the algal ingredient that similarly interferes with oil transfer during frying.

In certain embodiments, the invention also provides for ingredient substitutions with the improved higher protein and low starch algal wholemeal in order to improve a food composition. This includes substitution of, for example, an egg to create an improved vegan dough and pasta product based on the algal wholemeal properties of binding, higher protein digestibility, and excellent extrusion properties for an improved form of a cooked pasta and without comprising the product's protein nutritive value. This also includes substitution of wheat flour for protein fortification such as, for example, in a dough. In several embodiments, the ingredient substitutions are made without adding algal starch-derived sugars or calories (such as would occur using unimproved algae containing starch).

In certain embodiments, the invention provides for substitution of animal-based protein in meat replacement formulations, such as, for example, pet food.

In certain embodiments, the subject compositions can deliver effective amounts of algal protein without needing to raise inclusion rates or compromising food properties that would otherwise arise using less pure biomass of lower protein value and that also contains starch. This provides flexibility in food compositions and ingredient proportions to attain flavor, texture, shape, ingredient substitutions or other desirable attributes in products.

In certain embodiments, the inclusion of the improved foodstuff comprising the algae composition retains the clarity, texture or consistency of a broth or soup stock. In certain embodiments, the inclusion of the algae composition into a broth or soup stock provides flavoring or accentuates the umami flavor while providing for protein enrichment. In some embodiments, the inclusion of the algae composition provides addition of glutamic acid while being substantially starchless compared to inclusion of algal wholemeal containing starch exceeding 2.25%.

In certain embodiments, the subject algal compositions can fortify foods in protein while also elevating carotenoid content of the food. Carotenoids can enhance a food's color value by pigmentation, nutritional value by antioxidant properties, and monetary value by health benefits of carotenoids for functional foods. In certain embodiments, the subject algal compositions can fortify foods with protein with an acceptable color or without changing product color due to the range of hues available for the improved higher protein, very low starch algal wholemeal. In certain embodiments, the subject compositions can enhance the pigment, antioxidant or monetary value of a food composition without introduction of algal starch.

In certain embodiments, the subject algal compositions can be produced to combine the algae with meat ingredients to produce a meat-containing product. In certain embodiments, the meat may be derived from livestock (e.g., cattle, pig, horse, donkey, zebu, yak, buffalo, goat, reindeer, camel, llama, alpaca or rabbit), poultry (e.g., duck, chicken, goose, emu, or turkey), open nature-sourced or aquaculture-sourced fish, shellfish and crustaceans, or cell-cultured meat and cell-cultured seafood.

In some embodiments, the subject algal composition is an algal biomass containing additional pigment, such as chlorophyll, that imparts a deeper pink or reddish or brownish color to the meat product. This can be for cooked or uncooked product.

In certain embodiments, the subject composition can contain green algal material. In certain embodiments, the composition can lose color and appear gray or whitish once heated. In certain embodiments, the color of the meat product containing the algal composition is unaltered by the addition of the algae in a cooked/heated product.

In certain embodiments, the addition of the subject algal compositions in a meat-containing composition may serve functional roles, such as, for example, benefitting the palatability (aroma/taste) of a composition, water holding capacity of the composition, or to serve as a binding agent.

In certain embodiments, the improved higher protein (72.1% dry cell weight) substantially starchless algal material has almost double (1.7-times) the water absorption capacity of wildtype *Chlamydomonas* material with lower protein (39.9% dry cell weight) and containing starch above 2.25% (namely, 31.3%).

In certain embodiments, the water holding capacity of an ingredient and a food composition comprising the ingredient improves sensory properties, shelf-life properties, nutritional properties, health features, and convenience properties.

In certain embodiments, the algal wholemeal used in the subject compositions is particularly useful in improving the texture of food preparations, such as, for example, pasta. In certain embodiments, the algal wholemeal used in subject compositions is particularly useful in dried food, such as, for example, pasta, to aide in its rehydration due to the water binding properties.

In certain embodiments, the inclusion of algal wholemeal in the subject compositions can improve water retention and shelf stability of cooked or baked foods.

In certain embodiments, the high protein algal wholemeal of the instant invention can increase the satiety of foods.

In certain embodiments, inclusion of high protein substantially starchless algal wholemeal of the instant invention in a food or beverage formulation fortifies that food or beverage composition in protein value without also adding starch-based sugars or increasing its glycemic index or non-nutritive calories. In several embodiments, the protein content of a composition is increased by at least about 22% or about 115%. In certain embodiments, the protein content in increased by at least about 22%, about 36%, about 50%, about 70%, and about 115% in dough, batters, meat-like products, oil-in-water emulsions, and in soups and broths compared to unfortified compositions.

In certain embodiments, a food composition is improved in protein digestibility by replacement of protein sources of egg or flour with algal protein of *Chlamydomonas* wholemeal.

In certain embodiments, the subject compositions can enhance the palatability of a pet food. In some embodiments, pet food comprises the subject algal biomass with one or more amino acids, such as, for example, glycine. In some embodiments the algal wholemeal is pre-treated by heat or enzymatic digestion followed by a step of inactivation.

In certain embodiments, the higher protein, substantially starchless algae is incorporated into a pet food for its palatability value, specifically into a cat food.

In certain embodiments, the subject compositions and methods can be used to fortify foods and supplements with essential amino acids and non-standard non-proteinogenic amino acids. In certain embodiments, the algae wholemeal can enrich an oil-in-water emulsion, such as, for example, a mayonnaise composition, by more than doubling the protein content within acceptable ranges of viscosity. In certain embodiments, a serving size of 13 g (1 tablespoon) contains 0.24 g protein in an unfortified mayonnaise and 0.53 g protein in a protein-fortified 'algae mayonnaise' with about a 3% algal wholemeal inclusion in the composition using the improved algae ingredient with about a 73% protein content. Such an 'algae mayonnaise' has over double the protein content (about a 115% increase) of the unfortified control mayonnaise. In several embodiments, there is no addition of starch-derived sugars in the food composition by inclusion of the algae. In certain embodiments, for protein fortification of an oil-in-water emulsion, algal inclusion raises the total protein (calculated as dry weight protein on wet weight of the finished product) from between the about 1.9% total protein for an algae-free egg mayonnaise up to 4% total protein in the 'algae-fortified egg mayonnaise'. In preferred embodiments, the amount of algal inclusion to obtain the same 4% total protein can be less than 3% algal wholemeal in the composition if using the improved algae ingredient with at least about or equal to 73% protein content.

In certain embodiments, the algae wholemeal can enrich batter in protein content without changing the resulting texture and form of the cooked batter product. In certain embodiments the protein content of the batter is increased without adding starch-derived sugars or calories (such as would occur using unimproved algae containing starch).

In certain embodiments, a serving size of 6 pancakes (from about ½ cup dry mix) contains 15 g protein in an unfortified batter and 20.5 g protein (36% higher protein) in an algae-fortified batter if using wholemeal with 69% protein content. The final dry matter mixture (i.e., not including water weight of a batter) comprises 8% weight dried algae. The same 8% inclusion using wholemeal with higher protein content than about 69%, such as, for example, about 73% to about 80% protein, results in 5.8 g or 6.4 g algal protein added to the 15 g protein in the unfortified batter for totals of 20.8 g and 21.4 g protein, respectively. Inclusion of less than about 8% (dry latter basis) algal wholemeal yields the same 20.5 g total protein in a serving size when using algal wholemeal with higher protein content, such as, for example, about 73% to about 80%, namely inclusion of 7.5% or 6.9% dry matter, respectively.

In certain embodiments, the inclusion rate of a substantially starchless algal wholemeal depends on its protein content to attain a certain total protein in a serving size of a food composition. In certain embodiments, relatively less substantially starchless algal wholemeal with higher protein content can be used compared to using a substantially starchless algal wholemeal with relatively lower protein content. In certain embodiments the fluctuations in algal wholemeal inclusion rates based on protein content proceeds without alteration of starch-based food properties that otherwise occur during varying the inclusion rates of starch-containing algal wholemeal ingredients.

In certain embodiments, the algae wholemeal can enrich a dough in protein content without affecting the rheological properties of the cooked food. Protein is increased by about 22% from a composition containing 30 g protein to one containing 38.55 g protein by partial flour substitution. In certain embodiments, the algal wholemeal can enrich savory broths and soup stocks in protein value by about 50% and impart a richer flavor. In certain embodiments, compositions of plant-based (vegan) or other non-animal derived meat-like products and pasta fillings containing the improved higher protein substantially starchless algal wholemeal have about a 70% higher protein content.

In certain embodiments, the protein content of the batters, doughs, meat-like products, pasta fillings, oil-in-water emulsions, savory broths and soup stocks is increased without adding starch-derived sugars or calories, such as would occur using unimproved algae containing starch.

Compositions Containing Algae

Illustratively, the algal component in the edible compositions of the present invention can be contained in any food or beverage to produce the effect of enrichment of protein, enrichment of antioxidants, substitution of undesirable other protein, fat or animal ingredients, providing functional features and textures, or providing desirable colors or taste. Examples of food forms that can be used include, for example, products in the pasta and bread category; baked, steamed or fried products such pancakes, dumplings, tortilla, pastry, cracker, bagels, pretzels cookies, breakfast cereal, biscuits, cakes, cookies, snack chips, brownie, crackers, breads and buns; products in an alternative meat or seafood category, including, for example, cultured meats or seafoods; products in the emulsion category such as, for example, salad oil, margarine, mayonnaise, fats and oils, such as, for example, dressings; dairy products, such as, for example, alternative "milks" (e.g., cow milk, goat milk, almond milk, oat milk, soy milk), ice cream and yoghurt; liquids, such as, for example, soups and sauces, and processed foods including pizza toppings. Examples of the beverage include, for example, sports drinks, fruit drinks, health food drinks, and carbonated drinks.

In order to produce the composition comprising the subject algal composition in a food, including petfood or beverage, the algal composition can be added and mixed during the manufacturing process of the food, petfood, or beverage, or it can be added by sprinkling it onto the finished food, petfood or beverage. The content of the algal composition in the food, petfood or beverage of the present invention is appropriately selected within a range that imparts an acceptable taste, an improved taste, or does not distort the original taste of the items, generally about 1 g to about 130 g per kg of food or beverage (e.g., about 0.1% to about 13% algal wholemeal inclusion for dry matter or for wet weight, as described in the various examples herein). For food supplements, the algal composition is combined with an edible capsule or with at least one other component that is needed for tableting in an admixture. For supplements swallowed whole and not chewed, the algal composition comprises the majority weight of the supplement and can be greater than 99% by weight. Thus, some edible compositions of the instant invention ranges generally from about 0.1% to about 99% algae inclusion. In certain embodiments, algal inclusion ranges from 0.1% to 4% for petfood and pet beverages, 0.1% to 13% for food and beverages, and 30% to 99% for food supplements.

In certain embodiments, the present invention provides a food product comprising *Chlamydomonas* algal wholemeal with an inclusion of about 13% of total dry matter or 6% of total wet mass of a dough (per Example 12). In particular embodiments, the dough is pasta

dough. In certain embodiments, lower inclusions such as 2%, 1%, or even 0.1% algae per total dry mass of a composition can be employed for different features such as product labeling or for a desired taste or color of a food composition.

In certain embodiments, the algal inclusion in a food composition is for egg replacement. In certain embodiments, the amount of algal biomass used is calculated on a protein basis to replace, partially or totally, the total egg protein value in a food composition.

In certain embodiments, the present invention provides algal wholemeal with an inclusion of about 8% of total dry matter of a food product that becomes a batter upon hydration. In particular embodiments, the batter is pancake batter (per Example 6). In certain embodiments the pancake batter is a thick dough-like batter. In certain embodiments, the thick batter comprises 4.8% dry matter algae or 3% algae for total wet weight.

In certain embodiments, the inclusion of algal biomass of substantially starchless *Chlamydomonas* algal wholemeal with more than 50% protein is for flour replacement, partial or complete, that is otherwise commonly used in a food composition.

In certain embodiments, inclusion in a food composition of the substantially starchless *Chlamydomonas* algal wholemeal with more than 50% protein bestows one or more properties of a food composition: a) increased moisture retention compared to use of *Chlamydomonas* with less than 50% protein and containing measurable starch; b) reduction in gluten content without increasing calories or glycemic index due to absence of added starch; and/or c) increased satiety index due to increased protein content without increasing calories or glycemic index due to absence of added starch compared to a composition lacking the algal ingredient or use of *Chlamydomonas* with less than 50% protein and containing measurable starch.

In certain embodiments, the present invention provides a food product comprising a food or beverage ingredient including for pets with an inclusion level of at least about 0.1% on a dry weight basis, wherein the ingredient is an algal composition according to any one of the herein described embodiments, such as a wholemeal mixed with another food or beverage ingredient. In some embodiments, the petfood contains about 3% to about 4% algal wholemeal. In some embodiments, the algal ingredient inclusion of about 0.1% is for palatability, and about 3% to about 4% or more can be used for protein value. In one embodiment, the about 3% to about 4% algal wholemeal can replace chicken meal as a source of protein in a dog food or a cat food.

In certain embodiments, the present invention provides a food supplement product. In some embodiments the higher protein substantially starchless algal biomass can be packaged in capsules and tablets.

In certain embodiments, a food supplement tablet comprising of about 96.3% algae wholemeal, such as, for example, *Chlamydomonas reinhardtii* wholemeal and about 3.7% excipient. In some embodiments, the tablet comprises about 70% to about 99% algae wholemeal combined with a botanical and/or an excipient up to 100%. In some embodiments, excipients are inert substances, such as, for example, magnesium stearate, cellulose, microcrystalline cellulose, stearic acid, gelatin, polyvinylpyrrolidone, polyethylene glycol, mannitol, sucrose, starch, polysorbate, sodium phosphate, sodium chloride as known in the art. In certain embodiments, a food supplement comprising a granulated higher protein substantially starchless algae biomass combined with a food-grade vegetable cellulose gel cap component for an edible composition is provided. Granulation binds the wholemeal particles into granules or larger multiparticle entities using compression or by using a binding agent such as magnesium stearate.

In some embodiments, compositions for tablets or capsules can include an inert bulking agent, such as, for example, microcrystalline cellulose; an antioxidant, such as, for example, tocopherols or sodium ascorbate; an anti-caking, flow, or binder agent, such as, for example, vegetable stearate; a preservative; an emulsifier, such as, for example, soy lecithin; a sweetener, flavoring agent, or flavor maskers; or other botanical material such as mango, fig, date, pure stevia powder, or glucose sweetener. The proportion of algae to other botanical material can be 30%, 40%, 50%, 60% 70%, 80% 90% or 99% algae to the corresponding proportion of botanical to reach 100%. Tablets are formed by compressing with, for example, a compression machine using high pressure, the algal powder mixtures filled into molds.

In certain embodiments, the invention also pertains to compositions useful for maintaining or improving the health of a person or pet. In some embodiments, the invention provides a higher purity algal component in an effective amount, such as, for example, about 0.1% to about 99% (dry weight), about 0.1% to about 4%, or about 3% to about 4% (wet weight), in the absence of starch to improve one or more indicators of health in a person or pet, wherein the process comprises a person or animal consuming a food product composition comprising an improved algal wholemeal, as herein described.

In some embodiments, the edible food compositions can be used to deliver effective amounts of algal protein, wherein the compositions comprise at least one ingredient that is a higher protein, very low starch algae of the instant invention.

In some embodiments, the edible food compositions can be used to deliver effective amounts of algal protein along with desired pigments or colors to a subject, wherein the

compositions comprise at least one ingredient that is a higher protein, very low starch algae of the instant invention.

In certain embodiments, algal carotenoids and heme are quantified spectroscopically, spectrophotometrically, chromatographically or by other methods as known in the art to determine the amount of the chemicals in the algae sample. In certain embodiments, the algal biomass contains the carotenoids, lutein, and beta-carotene. In certain embodiments, the total carotenoids range from about 0.1% to about 1%, more preferably about 0.3% to about 1% and more preferably about 0.6% to about 1% by dry weight. In certain embodiments, the algal biomass contains protoporphyrine IX ranging from about 0.1% to about 5%, more preferably about 2% to about 5%, and even more preferably about 4% to about 5%. The algal biomass can be used at about 0.01% to about 5% by weight in a composition to affect its color.

In certain embodiments, the subject compositions provide for a *C. reinhardtii* material with a higher overall protein content with very low starch for a purpose of making a food composition having algal protein.

Some embodiments illustrate that the subject algal compositions comprising, for example, *Chlamydomonas reinhardtii* strains, with high protein and undetectable starch have a higher protein digestibility over some plant proteins and other algae. In certain embodiments, analyses show a PDCASS score of about 0.846, protein digestibility of about 0.940, and amino acid score of about 0.900.

In some embodiments, the food compositions incorporating the higher protein substantially starchless algae, such as, for example, *C. reinhardtii*, can represent different food categories, including, for example, meat-like products or meat analogues.

In certain embodiments, the algae-containing pet and human food is a texturized protein product. In some instances, the food is a savory snack. In some instances, the food is produced by extrusion cooking. In some embodiments the texturized protein products mimic meat, poultry, and fish in appearance, texture, flavor and color.

In some embodiments the higher protein substantially starchless *C. reinhardtii* component has functional properties, including, for example, gelation, binding, water holding capacity, emulsification capacity, elastic and fibrillar properties, incorporation of antioxidant properties, minerals, and incorporation of natural color.

In certain embodiments, the subject algal compositions can provide numerous benefits in an edible composition including, for example, increased or improved protein nutritional or functional value (in a food) without requiring an increased inclusion rate of algae material; increased or improved protein nutritional or functional value without negatively altering the

technical properties of breading, pasta extrusion, noodle tensile strength, matrix formation, viscosity and the like; increased or improved protein nutritional value without negatively altering textural properties; improved nutritive value of carotenoids in combination with effective protein value; increased or improved protein nutritional value yet with similar lipid content or percent saturated fatty acids and polyunsaturated fatty acids; and higher protein, lower starch algal composition used in the production of a product to increase the level of protein without concurrently increasing the level of starch or increasing the glycemic index (GI) of said product, and also optionally increasing carotenoids or providing certain fatty acids.

The present invention further relates to generating and cultivating microorganisms suited for heterotrophically producing higher yields of protein in biomass that is substantially starchless as well as producing carotenoids.

In preferred embodiments, the microorganism of the invention, *C. reinhardtii*, can be selected for a high protein substantially starchless phenotype for use in the methods described herein. In one embodiment heterotrophic fermentation of *C. reinhardtii* is described.

In certain embodiments, the invention provides improved strains that provide high productivity under dark heterotrophic fermentation. In certain embodiments, the heterotrophic cultivation is with strains of *C. reinhardtii* that are walled. In certain embodiments, the heterotrophic cultivation of *C. reinhardtii* yields a cell density of at least about 30 g/L, at least about 40 g/L, at least about 50 g/ L, at least about 60 g/L, at least about 70 g/L, at least about 80 g/L, or any higher densities for which sufficient oxygenation, such as, about 5% to about 100%, is provided. In certain embodiments, Lower densities than these usually pertain to the seed train.

In certain embodiments, selection performed during log phase (growth phase) for each species increases the efficiency of recovering improved cell lines with superior industrial performance. In one embodiment, pigment production in improved (high protein, substantially starchless) yellow lines can be at a rate no less than wildtype yellow lines with the latter disadvantaged by their lower protein and higher starch contents. In one embodiment, pigment production in non-white (e.g., shades of yellow, orange, green) improved higher purity (high protein, substantially starchless) *C. reinhardtii* lines can be at least about a 50%, about a 75%, about a 100%, about a 200%, or about a 300% increase compared to that of a yellow line with protein less than 50% and starch exceeding 2.25% by weight.

In certain embodiments, the improved higher purity phenotypes can arise from hybridization, somatic fusion, genetic modification, spontaneous mutations or epigenetic effects that result in defective starch biosynthesis to produce starch content subceeding 2.25%.

In a preferred embodiment the novel phenotype is obtained by sexual hybridization and selection against starch accumulation.

In certain embodiments, the methods can enable cells to be easily manageable, easily cultivable with faster crop cycle times, production all during the year and across geographies, and from which the desired product can be obtained economically in high yields.

In certain embodiments, the methods used in harvesting and further processing the biomass for isolating a product of interest are well known in the art. For example, some non-limiting methods of harvesting include, for example, centrifugation, flocculation, and filtration for dewatering, which separates biomass from a cultivation liquid or from water, including any rinse water. Additional removal of moisture into a paste or into a drier (less than about 10% moisture, and preferably less than about 5% moisture) material can be accomplished through evaporation, spray drying, freeze drying, drum drying, UV or heat assisted drying, or vacuum drying. In certain embodiments, the employed to achieve the desired moisture content. The biomass can be used whole, as a wholemeal. In certain embodiments the heterotrophically produced biomass is used directly as a composition consisting of at least one other edible component being a vegetable gel capsule encasing the biomass; or as a composition with at least one other edible component that is needed for tableting in an admixture in tablet form of food (nutritional supplement).

A further embodiment of the invention provides methods for improved cultivation of cells under mixotrophic (i.e., illumination is provided along with organic carbon) conditions for a portion of their culturing (e.g., 30 minutes under illumination up to hours or days until material is harvested). In some embodiments, the mixotrophic conditions may be used to accelerate growth during the seed train. In some embodiments, light can be briefly supplied to increase pigmentation of the algae. Light can range from about 30 to about 400 μmol m^{-2} s^{-1} for about 30 to about 60 minutes and can be based on the desired color.

Examples of general principles and methods for heterotrophic algae cultivation, such as establishing axenic cultures, using a seed train with a plurality of passages prior to addition of final inoculum, the design of the fermentors that inhibit illumination of the microalgae, and cultivation until harvest or partial harvest, are described in the art, for example, in US Pat. No. 8,278,090, which is incorporated herein by reference in its entirety.

The product of interest can be a microalgal biomass comprising the microalgal cells, with high protein and low or no starch, which is optionally white or pigmented. The pigment can be a carotenoid, namely lutein and beta-carotene, or heme.

In certain embodiments, the methods provide a substantially new profile of pigmented biomass with high protein and substantially starchless (along with low ash, vitamins and minerals in the biomass) for a product that confers nutritional or functional benefits for use for food and beverage compositions for animals and humans. These nutrition benefits are comparable to those provided by pulse protein concentrates for delivering high protein but with added benefits of pigment supplement, low glycemic index, and additional algal lipids, vitamins and minerals. By virtue of this composition, it is also attractive in meal replacement beverages or nutrient supplement additive.

In certain embodiments, the methods comprise a process for making an edible composition having algal protein, comprising:

(i) selecting a *Chlamydomonas reinhardtii* algal cell that has a protein content exceeding 50% and has an undetectable starch content (i.e., subceeding 2.25%); and

(ii) using the *Chlamydomonas reinhardtii* selected in step (i) as a foodstuff to produce the composition having an inclusion of at least about 0.1% by weight *Chlamydomonas reinhardtii* or otherwise add to the foodstuff.

Also provided herein is a method of identifying and isolating a microalgal cell that is possesses the preferred characteristics.

The step of culturing the preferred lines of algae to produce the wholemeal ingredient that is high protein and substantially starchless can be performed under mixotrophic conditions, at least for a portion of the culturing step.

The algae used as wholemeal food component provides a high level of complete protein and healthy fats along with pigments, and some micronutrients like vitamins and minerals that overlap with the major benefits of eating nutritious vegetables.

Sustainable Manufacturing

Sourcing efficiently manufactured microalgal proteins is desirable to displace the use of less efficiently produced crop proteins or otherwise provide protein fortification by microalgae in food compositions. Due to availability of large-scale food-grade fermentation facilities and the strain compatibility of the present invention with standard agitated tanks, heterotrophic production of the improved *C. reinhardtii* strains can provide a relatively low cost, high supply volume of algal biomass for cost-effective nutrition. Using the specific growth rate of 1.41/day biomass for the improved *C. reinhardtii* strain from **Example 4** it is calculated that a 20 m^3 tank produces 1 ton of algal biomass from seed flasks in 2 weeks. To

assess sustainability from viewpoint of natural resources, water and land usages are compared to show that this strain and process offers a favorable land and water footprint compared to other plant proteins. Example comparisons are 1664 m^3 water and 17.5 acres required for one ton of soybean protein and only 14 m^3 water on 0.003 acres for one-ton algae protein (over 99% less water and land). Because the algal biomass is used whole as a wholemeal, there is no waste of inedible or non-target plant parts and the loss of their associated virtual water value. When compared to beef production, which uses about 15,500 m^3 water and 350 acres per ton protein, algae production uses about 99.9% less water and land. Thus, algae protein from the improved *C. reinhardtii* uses over 99% less water and land than terrestrial plant and cattle protein and with virtually no waste. Taken together algae-based protein produced using fermentation with the improved *C. reinhardtii* strain eliminates problems faced by protein obtained from land crops and livestock. Fermentation employs software-controlled precision cultivation for best outcomes delivering only as much fertilizer as needed resulting in no fertilizer runoff and pollution as occurs for field crops. Algae fermentation allows high consistency and quality with no heavy metals or pesticide chemical residues, and no exposure to air pollution experienced in outdoor cultivation. Additionally, it removes limitations of seasons and geography that limit nutrition sourced from crops, is deployable all year around, and decouples protein supply from natural resource overuse.

A second major advantage of the higher purity *C. reinhardtii* is that new sources of protein are projected to be in great demand for pet foods as well as human foods. Pet food manufacturers are prioritizing a reliable supply chain which encompasses product sustainability, product quality, and product volume for such new sources. In addition to the factors described above, sustainability extends to overfishing of our ocean resources, with priority given to human consumption over pet food. Reduction in beef production through increasing industry commitments to greenhouse gas reduction will decrease supply available for pet foods. Soy production has been driving deforestation, notably in South America, a contributor of climate change. Soy is also largely a genetically modified crop, which is in disfavor among consumers and thus food manufacturers in several regions. Quality includes nutritional composition of the protein meal, which for pet foods is 10-15% as typical fat content, a minimum of 60% typical for animal protein meal, and negligible crude fiber. Quality features in animal diets also includes being non-hypoallergenic and digestible. Minimum digestible carbohydrates are required especially in cat foods and prescription diets; carbohydrates are considered as cheap fillers and effect pricing/demand. These quality factors are all found to be met by the wholemeal of the instant invention, as described in Example 2.

A third major advantage is a more sustainably produced biomass of the improved higher purity *C. reinhardtii* from use of carbon upcycled or converted from waste or byproduct carbon dioxide (CO_2), air, and other one-carbon and two-carbon sources. The carbon is used to generate acetic acid (or other carboxylic acids as listed elsewhere) feedstock supplied during fermentation as a chemical intermediate that is metabolized during to biomass production. This method of carbon upcycling is not available to make glucose feedstock such as employed for *Chlorella* heterotrophic fermentations. Using emissions for carbon capture and carbon utilization closes the carbon cycle through using the carbon in CO_2 or other carbon sources for synthesis of the preferred algal compounds. Uniquely and more economically, the alga of the instant invention directs recycled carbon into nutritional protein component to the exclusion of starch in the biomass; the carbon also is metabolized into fat, fiber, ash and pigments as the major chemical components of a biomass. Available technology known in the art is used to capture CO_2 in emissions from a variety of sources. Relevant technology for carbon capture and conversion to syngas and acetic acid is described in US. Pub. No. 2017/0321333/A1 (see Table 2), which is hereby incorporated in its entirety. This can be applied to the food-grade bio-based CO_2 in emissions from the fermentation of the Chlamydomonadales algae (see **FIG. 1 embodiment 100**). This and other sources of carbon, e.g., one-carbon molecules such as methane (CH_4), syngas, carbon monoxide (CO), methanol or carboxylic acids (**FIG. 1 embodiment 110**) are converted to two-carbon acetic acid/acetate as a regenerated feedstock input for the algal fermentation (**FIG. 1 embodiment 100**). Anaerobic digestion is another source of one-carbon molecules such as bio-based methane and CO_2 for upgrading. Two-carbon molecules include ethylene, ethane, ethanol, and acetaldehyde for conversion into acetic acid as is known in the art. Acetic acid production methods include but are not limited to the BP Cativa™ process, Monsanto process, Celanese AO Plus™, SABIC process, Showa Denko process, and the BP SaaBre™ process. These chemical synthesis methods are summarized as follows:

(i) The Monsanto process (US. Pat No. 3,769,329A and others such as US. Pat. No. 5,334,755, which are each hereby incorporated in their entirety) for production of acetic acid by carbonylation of methanol at low- pressure conditions, using a homogeneous, aqueous rhodium–based catalyst

(ii) The CelaneseAO Plus™ process (WO2013119275A1, which is hereby incorporated in its entirety) for production of acetic acid by carbonylation of

methanol at low pressure and low-water conditions, using a homogeneous rhodium- based catalyst

(iii) The BP Cativa™ process (US. Pat. No. 6,140,535A, which is hereby incorporated in its entirety) for production of acetic acid by carbonylation of methanol at low pressure and low-water conditions, using a homogeneous iridium-based catalyst

(iv) The BP SaaBre™ process (EP2085375A1, which is hereby incorporated in its entirety) for production of acetic acid from syngas via carbonylation of dimethyl ether at low pressure and low-water conditions, using a series of heterogeneous zeolite-based catalysts

(v) The SABIC process (US Pat. No. 6,030,920A, which is hereby incorporated in its entirety) for production of acetic acid by one-step, direct oxidation of ethane using a heterogeneous mixed metal oxide catalyst based on molybdenum and vanadium

(vi) The Showa Denko process (EP0620205A1, which is hereby incorporated in its entirety) for production of acetic acid by one-step, direct oxidation of ethylene using a heterogeneous supported palladium-based catalyst.

Other technologies for acetic acid production from waste or underutilized CO_2 are also available. An electrocatalytic CO_2 reduction technique is reported to produce two-carbon ethylene (Prajapati *et al.* 2022, which is hereby incorporated in its entirety), with the moisture-containing CO_2 emissions from biological fermentation being suited to this approach. Ethylene can be used to generate acetic acid as known in the art (US3970697A, EP0620205A1, which are each hereby incorporated in their entirety). An electrochemical CO_2 conversion to CO by a transition metal dichalcogenide (TMDC) catalyst (Asadi *et al.*, 2016, which is hereby incorporated in its entirety) yields substrate for producing acetic acid by the methods list above and in US8394988B2, which is hereby incorporated in its entirety. Zheng *et al.* 2022, which is hereby incorporated in its entirety, employ a catalyst for acetic acid production from CO_2. Beneficially, employing such technology with our instant invention, circular systems also apply to the waste stream of O_2 generated by catalytic processes (or other process oxygen sources as known in the art) to be fed into the algae fermentation process as described in Example 3 (FIG. 1 embodiments 100 and 110), to improve algae growth and reduce energy input for compressed air used to aerate the fermentation tank. Other potential paths to regenerative feedstock include thermocatalytic transformation of syngas/biogas as well as photocatalytic and plasmacatalytic routes (Martín-Espejo *et al.* 2022, which is hereby

incorporated in its entirety, and references therein). The ability to produce the carbon feedstock from fermentation gases generated during the seed train and destination tank production (per Example 3) or other facility CO_2, such as alcohol fermentation sites or cement factories, means a major fermentation cost input (carbon) is not subject to swings in market price or availability for the commodity (acetic acid). If conducted nearby or onsite, shipping cost for feedstock is minimized or negligible, further reducing cost and carbon footprint. Further on cost, the acetic acid generated does not need to be dried to 100% acetic acid. It can be dried to 50% acetic acid for example, reducing the drying cost (see Example 3 on acetic acid feedstock specific to the *Chlamydomonas reinhardtii* fermentation) and thus feedstock cost.

CO_2 emissions at a *Chlamydomonas* fermentation facility are generated by several sources including natural gas burned for the steam generator, dryers like spray dryers, and from algae metabolism. CO_2 generated from steam generation and spray drying can provide about 43% and about 8%, respectively, of the required acetic acid for the *Chlamydomonas reinhardtii* of the instant invention. Specific numbers depend on the facility size, duration of cultivation, and thus number of harvest/sterilization cycles per year of the algae. The biological and food grade CO_2 released during growth of the improved higher purity *C. reinhardtii* lines of the instant invention is modeled to provide 20% to 45% of the required acetic acid or acetate for the algae, if all the recycled biological carbon is utilized in the feedstock. The acetic acid/acetate usage rate for a specific line of the algae and the operational practices being employed (such as draw and fill fermentation, **FIG. 1 embodiment 40**) as process variables can impact the measured value. In certain embodiments, a majority, totaling about 60% to about 88% of the acetic acid required for production of the algae of the instant invention can be 'green' acetic acid from emissions of the facility itself. The remaining amount of acetic acid needed (this will be higher for lower acetic acid metabolizing strains) can be generated from other industrial carbon waste streams, including CO_2 waste streams, or even sourced from direct air capture. Alternatively, the acetic acid can be produced by a microbial fermentation by acetogenic micro-organisms as is known in the art. Anaerobic or oxidative fermentation by bacteria can produce acetic acid, such as vinegars up to 20% acetic acid by fed-batch *Acetobacter* fermentation (**Xu *et al.* 2011**, which is hereby incorporated in its entirety).

Thus, the instant invention discloses a wholemeal ingredient of algae that is improved by being produced more sustainably, more efficiently, and more economically sound through 1) being less wasteful by enabling selective carbon partitioning into protein and pigment rather than into non-nutritive starch; 2) enabling use of recycled carbon, including biological food-grade carbon captured from algae fermentation emissions, that is upcycled into acetic

acid/acetate for use as the carbon nutrient in the fermentation feedstock; or 3) enabling algal protein production by reducing water and land usage over 90% compared to terrestrial plant or animal protein. A more sustainable algal wholemeal is a valued differentiator for the food and beverage industry, and notably for the pet food industry since there are few options among algae raw materials that offer higher quality nutrition of high protein meal with very low starch for use in pet food compositions.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

The above summary is not and should not be seen in any way as an exhaustive recitation of all embodiments of the present invention.

The following examples are provided to describe the invention in further detail. These examples serve as illustrations and are not intended to limit the invention. The composition of foods in the present invention is not intended to be limited by the origin of the improved high protein, very low starch or substantially starchless algae, such as, for example, *Chlamydomonas reinhardtii* component.

EXAMPLE 1— ESTABLISHMENT OF IMPROVED PHENOTYPES, STRAINS AND HETEROTROPHIC CULTURES OF *CHLAMYDOMONAS REINHARDTII*

This example is directed to novel cell types of *C. reinhardtii* that are cultivated under heterotrophic conditions to yield the improved phenotype of higher protein and substantially starchless biomass for use in making edible compositions of the invention. From a commercial standpoint, microalgal cell types that are used in the breeding development of novel phenotypes preferably are already proven in heterotrophic cultivation, with an acceptable growth rate in fermentation and a known composition. Therefore, the yellow botanical sport of production strain KAS1001 from US Pat. No. 11,034,968 B2, which is hereby incorporated by reference in its entirety, designated KAS2004, with 40% protein content and 31% starch content dry weight algae, and possessing a cell wall, is selected as one parent for mating. The color measurement of the yellow dried biomass is described as “yellow, warm red” of value 114 U

in the Pantone Formula Guide color chart. KAS2004 is mated with second mating parent, KAS2006, which is a wall-deficient light green line that carries a chromosomal *sta6* mutation and that we adapted to heterotrophic cultivation. The color measurement of the green dried biomass produced under such cultivation is described as "yellow, green, black" with a value of 2307 U or about 7496 U in the Pantone Formula Guide color chart.

Numerous obstacles had to be overcome to achieve successful hybridization between these parents. A first obstacle that had to be overcome is that the heterotrophically grown *Chlamydomonas* KAS2004 and KAS2006 cells are predominantly non-flagellated, which deters mating. A second obstacle is that about half the cells in a culture are sticky and clumped, rather than individual cells needed for successful mating and for discerning and separating the larger resulting zygotes. Third and fourth obstacles are that one parent lacks a cell wall and is smaller in size, requiring unknown parental cell ratios/volumes during crossings and also risking a result of progeny incompatibility with fermentation production since a cell wall is preferred for tolerance of high agitation/high aeration for reaching high cell densities and productivities. A fifth obstacle is the yellow color of the production parent, resembling zygotes that are also yellow during normal green crosses. Compounding those obstacles is that both lines are cultivated in the dark and also that the production line is yellow and thus one cannot rely on assessment of color for viability during gametogenesis or use of strong light to become motile gametes, as is normal in mating of *C. reinhardtii*.

Nevertheless, one crossing is successful with the novel step of desiccation of both zygotes and non-zygotic cells after promoting agglutination through proximity. This yields dozens of progenies for initial screening for novel cell types. Without attempting to explain the outcome, zygotes are discovered to survive desiccation, while non-zygotic cells die from desiccation. This step is performed in 6-well plates in the dark in a sterile laminar flow cabinet. Liquid is pipetted out of the wells while holding the plate at a slight angle, removing most cells with the liquid but leaving behind zygotes that stick to the plate wells. Zygotes are clearly visible and distinguished from non-zygotes by the zygotes being larger, rounder and less see-through (i.e., are darker). Plates are allowed to dry for several hours. Thereafter, Tris-acetate-phosphate (TAP) medium (or other acetate containing medium such as HSA) is added, and the plate is incubated in light for three days to germinate the zygotes. These cultures are plated on agar plates to generate colonies for screening. The desired trait of substantially starchless is screened by amylase digestion with glucose monitoring (see Example 2). The desired trait of presence of a cell wall is indirectly screened by microscopy based on a larger size of such cells compared to wall-deficient cells, a feature we had observed for the parental lines. Cell wall

screening is optional initially since wall status can be assessed later (such as with exclusion dyes) and since the requirement of a cell wall may not apply if wall-less biomass can be produced economically under fermentation conditions providing sufficient oxygenation. The color of progeny is assessed visually by liquid cultured biomass after picking colonies from a solid plate. Only four of twenty colonies prove to be substantially starchless lines possessing cell walls. These were selected for further propagation and selection. They are subcultured from agar-solidified medium in plates into liquid medium in flasks (see Example 3 for culture media). Morphologically these are similar to the origin parent KAS2004 in cell size and shape during cultivation; and cells once in fermentation production subsequently lose their flagella. Multiwell plate tests using heterotrophic culture show that progeny line KAS2010 is capable of metabolizing nitrogen in the form of urea and ammonium but nitrate only poorly. Parent KAS2004 grows very slowly in heterotrophic cultivation on nitrate while the wall-less parent KAS2006 grows comparatively better in heterotrophic cultivation on nitrate. The other progenies are similar except for KAS2014 which grows well on urea, nitrate, and ammonium. Growth on nitrogen types is optional, used for example for information to confirm hybrid status of the colonies. Surprisingly, a range of colors from olive green to yellow on plates and in flasks is obtained from the substantially starchless progeny lines KAS2010, KAS2014, and KAS2015; progeny KAS2013 is yellow that as a dried powder is described as "yellow, warm red" of value 113 U or 114 U in the Pantone Formula Guide color chart. In *C. reinhardtii*, the chloroplast cpDNA is thought transmitted only from the mating type positive (*mt+*) parent. This follows non-Mendelian transmission of organellar genes, with the rapid loss of the cp nucleoids derived from the *mt⁻* parent within 60 minutes after zygote formation (Nishimura *et al.* 1999). Thus, the yellow phenotype of the production parent KAS2004 (*mt+*) was anticipated among progenies. Surprisingly, olive green progeny line KAS2010, which is closer in hue to the light green of the KAS2006 mating type *mt-* parent in heterotrophic culture, gives spontaneous rise to several yellow colony isolates over time, used to generate line "KAS2010-yellow". These progeny lines yield yellow liquid cultures that unexpectedly produce higher carotenoids during exponential growth (see **FIG. 2** and Example 4) than the yellow parent. The substantially starchless progeny lines also differ from each other by relative growth rate, with KAS2013-yellow growing slower, at about 0.8 to 0.9/day specific growth rate in a 10-L fermentor than KAS2010 with about over 1.3/day under the same fermentor conditions; KAS2014, and KAS2015 also grew relatively slower in flask than KAS2010 based on visual cell densities.

Illustratively, based on the successful matings described above, the improved process of hybridization described herein followed by screening will find broad application in algae strain development programs. Specific to generating robust high protein and starchless progeny, the first parental alga is another member of a group of *C. reinhardtii* algae with robust features for industrial heterotrophic production that is mated with a second parental alga of a group of starchless *C. reinhardtii* of oppositive mating type, such as available from the Chlamydomonas Resource Center. The former group contains a parental cell line with a protein content below 50%. The latter group contains a genetic or epigenetic modification affecting the starch biosynthetic pathway that can occur as a result of coding sequence mutation, gene editing, gene knockout, or control region mutation, or epigenetic modifications. Irrespective of their genetic origin, *Chlamydomonas* phenotypes that display undetectable starch content, i.e., below 2.25%, along with protein content above 50%, above 60%, more preferably above 70%, are suited for use. The improved *C. reinhardtii* and its biomass for food compositions is further characterized in subsequent examples.

EXAMPLE 2—CHEMICAL AND FUNCTIONAL CHARACTERIZATION OF HIGH PROTEIN SUBSTANTIALLY STARCHLESS *CHLAMYDOMONAS REINHARDTII*

The higher protein, negligible starch microalgal materials used in this example are obtained from strains described in Example 1. Chemical characterization of the obtained *C. reinhardtii* material establishes the high protein substantially starchless composition of the microalgal ingredient for food, animal feed, food supplements and beverages along with determining other features of value for function, nutrition or consumer appeal. By means of illustration, this example pertains to biomass, but characterization can equally pertain to biomass component parts that are materials that are extracted, delipidated or otherwise concentrated and is non-limiting. Materials analyzed are described in Example 3 taken from fermentors and include final algal product (**FIG. 1, embodiment 90**). The characterization of the biomass is by established assays. In one embodiment, chemical assays include those shown in **Table 1**.

Table 1. Some methods of compositional analysis used for assessing *Chlamydomonas reinhardtii*. (AOAC, Association of Official Analytical Chemists; AOCS, American Oil Chemist Society).

Composition	Analytical Method
Crude Protein	AOAC 990.3
Amino acids	AOAC 994.12, AOAC 985.28
Crude Fiber	AOCS Ba6a-05; AOAC 978.10
Fat	AOCS Am 5-04; AOAC 954.02
Fatty acids	AOAC 963.22
Starch	AOAC 996.11, AACC 76-13.01
Moisture	AOAC 930.15; Karl Fischer titration
Ash	AOAC 923.03

Proximate analysis without an analytical method specified is determined by calculation. Carbohydrate content is calculated by mass difference, i.e., 100 minus (Protein + Lipid + Ash + Moisture). Determinations are based on dry weight of material. Residual moisture in dried biomass is usually about 5% or less. Drying of the biomass for analysis is done by lyophilization but other methods can be used for drying such as heating. For certain measurements, cells are pulverized by any number of methods such as grinding, milling, pressing, lysing, or sonication followed as needed by separation by filtration, centrifugation or gravity separations as is known in the art. Acid hydrolysis for lipids and for proteins/amino acids under high temperatures facilitates measurements, as a standard method. Components such as pigments can be assayed in pulverized material, notably for carotenoids by chromatography (TLC, HPLC) and for heme by spectroscopy or other components (**Hopp *et al.* 2020**), with appropriate standards and controls as in known in the art. For other measurements whole biomass is used such as for ash content, moisture content and the like. Digestibility is also analyzed at the laboratory level. In one embodiment, after a sample is measured for amino acid composition by method AOAC 994.12, which is hereby incorporated in its entirety, a human digestion simulation is used to break down the proteins into amino acids that are reacted with ninhydrin reagent and quantified. Using the limiting amino acid value, digestibility is corrected to produce a Protein Digestibility Corrected Amino Acid Score (PDCAAS). Caloric content and vitamins are assessed as is known in the art. Minerals and heavy metals are determined by AA (Atomic Absorption Spectroscopy) or by ICP-AES (inductively coupled plasma atomic emission spectroscopy). Solid samples are dry-ashed and dissolved in dilute HCl prior to ICP-AES. For assessment of starch content, samples of lyophilized algal biomass are provided to two independent analytical labs, New Jersey Feed Laboratory, Inc. and Eurofins. Samples are also analyzed in-house using an enzymatic kit for starch following manufacturer's protocol from Megazyme (Neogen) or following AOAC 996.11 specifications. Dried algae biomass from a 10 mL culture in flask is resuspended at 10

g/L (100 mg in 10 mL) in reverse-osmosis (RO) water and autoclaved for 5 minutes to rupture cells. Once sample reaches room temperature a reading for glucose is taken and the value is below detectable (this occurs in both starchless and starch containing biomass, i.e., a below detectable amount of glucose is released from starch through the autoclave process). Then 25 µL of amylase (3000 U/mL) and 25 µL amyloglucosidase (3260 U/mL) are added and incubated at 70°C for 4 hours. Glucose is then read again after sample reaches room temperature. The substantially starchless biomass gives a reading of below detectable. Glucose readings are taken with a CVS ADVANCED glucose meter. A reading that is 'below detectable' means below 2.25% glucose (i.e., the enzymatically digested starch) by algae weight is undetectable; 0.225 g/L glucose is the detection limit of the CVS ADVANCED glucose meter. Using the detection limit of the amount of glucose (from starch) in the reaction of the Megazyme Total Starch Assay Kit (K-TSTA-100A) per AOAC 996.11 assayed spectrophotometrically, namely 18 mg/L, the assay reads below detectable (i.e., is undetectable) for 20 g biomass per liter that generates less than 18 mg/L glucose (from starch), thus subceeding 0.09% (18 mg/20 g). For 10 g biomass per liter, undetectable is below 0.18% (18 mg/10 g) using the kit. All samples tested for starch with readings that are below detectable using the glucose meter are also undetectable using the more sensitive spectrophotometer-based kit. A starch- containing wildtype *Chlamydomonas* biomass assayed gives a reading of 3.4 g/L glucose, indicating it is about 34% starch and the enzymes are effective at digesting starch (i.e., a control to show the enzymes are active when obtaining the starchless biomass reading). Other assays such as iodine staining with microscopy can be utilized for determination for presence or absence of starch in cells, as known in the art. Pigment analyses are described in Example 3. Functional aspects are assessed following protocols of **Bleakley and Hayes**, 2021, which is hereby incorporated by reference in its entirety, for *Chlamydomonas* extracted protein and biomass. This includes water activity, water-holding capacity (WHC), oil-holding capacity (OHC), pH, solubility, emulsifying activity, and stability among other functional features as is known in the art. Once the algae component is part of a food, standard methods of sampling and analyses can be used, for example as described in the food standards of the Codex Alimentarius (Ref.: see worldwide website: fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B234-1999%252FCXS_234e.pdf). For food evaluations, samples are ensured to be free or contain acceptable levels of heavy metals, pesticides, toxins and cyanotoxins, and other as required for food safety as is known in the art.

After the screening of about 20 progenies is completed under heterotrophic conditions, lines with the desired traits of substantially starchless and having cell walls have a representative proximate composition of about 72.1% protein, 14.92% fat, 11.32% ash, 4.75% moisture, 0.45% fiber, and undetectable starch under nutrient replete conditions. All such lines retain the high protein and starchless phenotype after more than two years maintenance on agar plates in darkness with monthly subculture. For example, the proximate composition of biomass from a cell line designated KAS2010 that is maintained for 27 months and then grown as a 10-L heterotrophic fermentation culture under nutrient replete conditions shows 73.45% protein, 12.61% fat, 12.89% ash, 3.75% moisture, 0.49% fiber, and no starch. Caloric value is 3643 Kcal/kg.

Protein values higher than 73.5% are anticipated by reducing the ash content to below 12.6%, such as to about 5% ash to produce biomass which is 79.5% protein. Ash reduction can occur for example by more precise feeding of the carbon feedstock to reduce residual acetic acid in the medium, minimized use of anti-foam during cultivation, or by washing the biomass to rinse away any residual medium components before drying. Combining ash reduction with lowering of lipid content by 1% or more via carbon (acetic acid) depletion during cultivation produces biomass above 80% protein content (e.g., 80.5%). Remarkably, these protein values are 83% improved over the parental production line, KAS2004 containing 40% protein and 31% starch. Unexpectedly, there is no elevated level of lipids in the novel higher protein starchless *C. reinhardtii* phenotype under nutrient replete conditions of the present invention. Lipid content stays between about 12% to about 15% for these phenotypes, like the wildtype KAS2004 parent (having starch) with about 14% lipids under balanced nutrient replete conditions.

These results demonstrate that the chemical composition of the novel algae phenotype of this invention is simplified by lacking substantive starch content and thus being of higher purity compared to *C. reinhardtii* biomass containing starch above 2.25%. For best protein nutritive value, it is desirable for higher purity algal biomass to have the highest possible protein content. Further, from a nutritional viewpoint, it is desirable to have a *C. reinhardtii* ingredient that provides proteinaceous metabolites without also adding calories in the form of food energy from starch or increasing the glycemic index (GI) of food formulations. Since protein itself does not impact blood sugar levels, it does not have a GI ranking and will not raise blood sugar levels. Subsequently several of these progenies also prove to be superior production lines in fermentation over the industrially suited parent (see Example 4). Taken together these provide an improved ingredient for formulation in food and beverage

compositions as a nutrient, functional ingredient, or with other benefit as described in the examples herein. With the presence of carotenoids in some samples (see Example 4), addition of the biomass to foods and beverages can also deliver antioxidants, such as to protect against oxidative damage.

The percentages of algal biomass and its components in a cooked food are typically assessed from the measured weight of biomass used in the raw product on a dry solids basis of the composition or based on the weight or volume of the finished product. It is understood that during the cooking process there can be a loss of liquids that would effectively increase the percentage of algal biomass or components if assessed in a cooked composition. in the novel higher protein starchless *C. reinhardtii* phenotype

Overfeeding of the above higher protein substantially starchless lines during heterotrophic cultivation with all nutrients (nitrogen, phosphate, other macro and micronutrients) in excess (by at least three-fold the initial concentration in the fermentor medium), except for the acetic acid, is discovered to lead to reduced protein and somewhat elevated fat content compared to when nutrients are not in excess. For example, a composition of 57.7% crude protein with 19.2% fat is obtained, a lower protein and higher fat content than the 73.45% protein and 12.6% fat content obtained in the same line cultivated with replete nutrients that are 'balanced', i.e., less than three times the initial concentration amount in the fermentor medium. The lower protein and higher fat composition has total carbohydrates of 7.63% and is starchless. This lower protein and higher fat material has higher caloric value of 3912 Kcal/kg. The composition of the cultivation medium that led to overfeeding has a relatively lower C:N (carbon to nitrogen) ratio with sufficient carbon being supplied relative to excessive nitrogen along with excessive all other nutrients when compared to cultivation medium with nutrients replete and in balance. Another form of a relatively lower C:N ratio, with excess urea N alone, results in 62% protein and 15% fat. In all cases the baseline protein content exceeds 50% under nutrient replete cultivation including at the outset of the growth phase. Advantageously, these combinations offer a range of compositions for *C. reinhardtii* that contain greater than 50% protein as a baseline and are substantially starchless as may be desirable in certain food applications. Thus, the *C. reinhardtii* of this invention are capable of biomass phenotypes with a range of protein and fat contents in addition to being substantially starchless for inclusion in food, beverage and supplement compositions.

These phenotypes during overfeeding illustrate an unexpected outcome for these *C. reinhardtii* genotypes: that a relatively lower C:N ratio in the context of all nutrients in excess (by at least three-fold the initial concentration in the fermentor medium) including nitrogen

produces the lowest protein content and highest fat content, while a relatively lower C:N ratio with only the urea N in excess also reduces the protein content but less so. Unusually, avoiding a deficiency in N with these substantially starchless genotypes does not inhibit elevated fat levels at the expense of protein levels, as would otherwise be anticipated by a person skilled in the art of algae cultivation. A further unexpected outcome for phenotype is also obtained with these substantially starchless genotypes under different conditions for a lower relative C:N ratio, namely because of acetate depletion without changing the nitrogen. About 3 hours or more of withholding addition of acetic acid to a culture that otherwise was in active growth mode leads to a reduction in biomass and fat within the biomass. Fat content is reduced by about 22% under acetate deplete conditions (lower C:N ratio) while biomass decreases by about 11% compared to nutrient replete cultivation conditions.

TABLE 2. Amino acid profiles of substantially starchless high protein *Chlamydomonas reinhardtii* biomass, represented here by line KAS2010, and starch-containing parent strain KAS2004 expressed as amino acid percentage of total amino acids.

Amino Acid	KAS2010 % of Total	KAS2004 % of Total
Methionine	2.15	2.03
Cystine	1.70	1.10
Lysine	6.16	7.15
Phenylalanine	4.01	4.49
Leucine	7.80	7.53
Isoleucine	2.96	2.79
Threonine	4.70	4.74
Valine	4.70	5.21
Histidine	1.82	2.75
Arginine	14.33	11.21
Glycine	5.45	5.25
Aspartic Acid	9.01	9.48
Serine	4.35	5.08
Glutamic Acid	11.14	11.38
Proline	6.01	5.33
Hydroxyproline	1.44	2.33
Alanine	7.45	7.87
Tyrosine	3.64	3.26
Tryptophan	1.19	1.02
Total	**100%**	**100%**

Analysis of all the progeny lines with protein over 70% and starch being undetectable, including KAS2010 and derivatives by way of illustration, reveal their protein to contain all the essential amino acids and in similar amounts. Results show a complete protein profile that

can contribute to a healthy diet. Values for the nine essential amino acids were compared for histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine as percentage of total amino acids from biomasses of KAS2010 (representing the starchless high protein biomasses) and the starch-containing parent KAS2004 (**Table 2**). For biomass of KAS2010 the sum of essential amino acids is 36% and for the parent KAS2004 is 38%. The minimum percent for essential amino acids is met for the KAS2010 protein to be considered a nutritionally complete protein, namely a minimum of 1.8% histidine, 2.5% isoleucine, 5.5% leucine, 5.1% lysine, 2.5% methionine + cysteine, 4.7% phenylalanine+ tyrosine, 2.7% threonine, 0.7% tryptophan, and 3.2% valine. The values for methione plus cystine are 3.85% for KAS2010 and 3.13% for KAS2004. The ratio of methione plus cystine:lysine is also higher, with 0.625 for KAS2010 compared to 0.438 for the parent. Comparing amino acid profiles across the averages of animal and plant (pulses), the *Chlamydomonas* of the instant invention is more like animal-based protein than plant protein. The *Chlamydomonas* has 43% more methionine (plant average 1.5%, animal average 2.8%) and 47% more lysine (plant average 4.2%, animal average 7.7%) than plant protein. Results from the PDCASS and digestibility of the algal protein (uncooked scores) show a high PDCASS score of 0.846, high protein digestibility of 0.940, and high amino acid score of 0.900. For the key amino acids comprising collagen peptides - namely glycine, proline, hydroxyproline, and alanine - totals are unchanged between biomasses of KAS2010 and the parent at 20.1% of total amino acids. Hydroxyproline, a non-proteinogenic amino acid in the cell wall of *Chlamydomonas reinhardtii*, is 38% lower in the KAS2010 progeny from the cross between a walled and a wall-less phenotype, at 1.44% of total amino acids.

For the fatty acids, a combination of healthy dietary fatty acids is present in all the wholemeals of the higher protein substantially starchless *C. reinhardtii* under nutrient replete conditions. Similar to the other lines with protein exceeding 70% and being substantially starchless, the high protein substantially starchless line KAS2010 has a low omega-6: omega-3 PUFAs (polyunsaturated fatty acids) ratio of 0.7. A lower omega-6: omega-3 PUFAs ratio such as this is better than a higher ratio over 5 for lowering the risk of many chronic diseases and thus can benefit different edible compositions including algal milk (Example 10). The fatty acids profile for KAS2010 is compared to its starch-containing parent, KAS2004 (see **Table 3**). The total saturated fatty acids are almost the same for both lines, 32%, as are the PUFAs of 34% and 31% for KAS2010 and KAS2004, respectively. The parent and progeny samples share the common food fatty acids C16:0 (palmitic) and C18:1ω9&ω8 (oleic) as comprising almost 40% of the combined total fatty acids. Both contain palmitoleic acid (16:1ω7) with known anti-

inflammatory properties and benefits to help inhibit metabolic syndrome associated with diabetes and obesity. The parent and progeny samples both have C:18 unsaturated fatty acids comprising C18:1ω7 (*cis*-vaccenic acid, with KAS2010 at 8% and KAS2004 at 6%) and C18:3ω3 (α-linolenic acid, with KAS2010 at 12% and KAS2004 at 9%). The latter omega-3 PUFA is an essential fatty acid for humans and has recognized anti-inflammatory properties. Further fatty acid profiles shared between KAS2010 and KAS2004 for the include the following: total monounsaturated C18:1ω7+ C18:1ω9&ω8 fatty acids of 20.5% and 15.6%, respectively; C18:1ω7+ C18:2ω6 of 16.3% and 24.6%, respectively; and C18:1ω7+ω9+ C18:2ω6 of 29% and 34%, respectively. KAS2010 shows lower essential fatty acid C18:2ω6 (linoleic, 8.34%) than KAS2004 (18.49%). The high protein starchless KAS2010 shows presence of a fatty acid that is absent in the parent, C18:2ω4.

TABLE 3. Fatty acids profiles of biomasses from improved higher purity *Chlamydomonas reinhardtii,* exemplified here by strain KAS2010, compared to its starch-containing production parent KAS2004 grown in heterotrophic fermentation using acetic acid feedstock.

Fatty Acids	C# : Double Bonds	KAS2010 (% of total)	KAS2004 (% of total)
Myristic	14:0	0.45	0.38
Myristoleic	14:1	0.05	0.18
Pentadecanoic	15:0	0.06	0.06
Palmitic	16:0	27.27	28.01
Palmitoleic	16:1ω7	6.32	3.28
Hexadecadienoic	16:2	0.11	0.00
Hexadecatrienoic	16:3	0.12	0.00
Hexadecatetraenoic	16:4	5.94	3.46
Heptadecanoic	17:0	0.11	0.12
Heptadecanoic	17:1	0.97	0.00
Stearic	18:0	3.52	3.55
Oleic	18:1ω9&ω8	12.47	9.70
Oleic	18:1ω7	7.99	6.12
Linoleic	18:2ω6	8.34	18.49
Linoleic	18:2ω4	7.83	0.00
Linolenic	18:3ω6	0.00	0.00
Linolenic	18:3ω3	12.01	9.03
Octadecatetraenoic	18:4ω3	0.00	0.00
Arachidic	20:0	0.11	0.05
Eicosanoic	20:1ω9	0.09	0.09
Eicosanoic	20:1ω7	0.00	0.00
Eicosadienoic	20:2ω6	0.05	0.00
Eicosatrienoic	20:3ω6	0.00	0.00
Arachidonic	20:4ω6	0.00	0.00

Fatty Acids	C# : Double Bonds	KAS2010 (% of total)	KAS2004 (% of total)
Eicosapentaenoic (EPA)	20:5ω3	0.00	0.00
Behenic	22:0	0.12	0.00
Lignoceric	24:0	0.10	0.00
Others	n/a	5.97	17.48
		100	100

EXAMPLE 3 – CULTURE OF NOVEL IMPROVED PHENOTYPES OF *CHLAMYDOMONAS REINHARDTII* TO OBTAIN HIGH PROTEIN SUBSTANTIALLY STARCHLESS ALGAL PRODUCT

This example employs lines of *C. reinhardtii* that produce biomasses which are high in protein in excess of 50% by weight and are substantially starchless when cultivated under heterotrophic conditions. Among the several lines described in Example 1, there is a noticeable difference in growth rates at both the flask level and the fermentor level. Line KAS2010 and its color variants appear the most robust. These are largely exemplified here as representative and non-limiting for improved higher protein substantially starchless lines that form the starting point (**FIG. 1, embodiment 10**) for the seed train (**FIG. 1, embodiment 20**) as part of the biomass production process (**FIG. 1**). Cultures are generally grown following Example 3 of US Pat. No. 11,034,968 B2 but with modifications in medium components and other details described below.

Using the high protein substantially starchless *C. reinhardtii* lines, 50 mL liquid cultures are maintained weekly and used to seed a total volume of 2.7 L (3x 900 mL cultures in 2 L vented flasks) is grown in batch mode in heterotrophic flask medium. The 2-L flask seed culture is used to generate a fermentation culture of 7.2 L in a 14 L vessel (10 L working volume). The 2-L flasks are incubated at 28°C in the dark with shaking at 115 rpm for sufficient time to be well into the growth phase and before entering stationary phase, as measured by cell densities having an OD750 of 0.15 in early growth phase and increasing to an OD750 of about 0.6 in late growth phase. Flask growth media consist of 0.11 g/L urea, 0.05 g/L magnesium sulfate heptahydrate, 0.05 g/L calcium chloride dihydrate, 0.02 g/L potassium phosphate, 0.01 g/L iron-EDTA, 0.0063 g/L iron chloride hexahydrate, 22 mg/L tetrasodium EDTA, 0.3 mg/L cobalt sulfate, 6 mg/L manganese sulfate, 0.8 mg/L zinc sulfate, 0.2 mg/L copper sulfate, 0.7 mg/L ammonium molybdate, and 0.4 mg/L boric acid. Additionally, the flask medium contains either 2.78 g/L Tris base with 1.15 mL/L acetic acid or it may initially contain 1.6 g/L sodium acetate to allow for maintenance growth of low-density flask cultures. Flask cultures are

transferred to 4.5 L of fermenter heterotrophic growth media (same as flask heterotrophic media supplied with 0.7 g/L Tris base and an initial 0.29 mL/L acetic acid or 0.4 g/L sodium acetate in a 14 L fermentation vessel (Eppendorf BioFlo110) as a continuing part of the seed train scale-up (**FIG. 1, embodiment 20**) with an initial biomass density of 0.2 g/L. Cultivation of the seed train algae preferably occurs in darkness; optionally it can be mixotrophic under ambient or other lighting if desired. The 7.2 L fermentation culture is incubated at 28°C with gas exchange provided by 7.2 L/min sparged air and a Rushton 2-blade impeller at 100 rpm at pH 7.8. Using BioCommand software, peristaltic pumps, and head plate ports the pH is maintained at pH 7.8 to 7.3 throughout the duration of the fed-batch fermentation with pH-triggered additions of 20% acetic acid supplied at 5% pump speed, other nutrients are frequently supplied (every 4 to 0.5 hours) at 10% pump speed throughout the fermentation to keep nutrient levels near starting concentration of fermenter heterotrophic growth media. The concentration of acetic used in the feed bottle can vary as desired from less than 20% to higher than 20% depending on volumes and method of supplying the feedstock (pumps, feed method without burning cells and such). This can range for small tanks using about 3% acetic acid up to 100% using a separate acetic acid feed. As described in **US11034968 B2** this production method covers a range of Chlamydomonadales including *Chlamydomonas* for heterotrophic culture at pH 5.5 to 8.5, and thus the pH set points for triggering acetic acid/acetate feeds can vary accordingly as is known in the art and are not limited to maintaining pH 7.3 to 7.8. As an example, the culture pH set point can be lowered to values below pH 7.0. Accordingly, an increase in culture pH is balanced by addition of organic acid as the carbon source in a fed-batch manner and pH of a culture can be maintained below pH 7.0, such as between 6.5 and 7.0 using the appropriate set points as is known in the art. Dissolved oxygen is maintained above 30% by increasing the amount of sparged air into the vessel up to 10 L/min and increasing agitation up to 600 rpm. Samples (10 mL) are collected every 24 hours for dry weight analysis to determine growth rate. Samples are also collected off the destination production tank (**FIG. 1, embodiment 30).** Seed is used to inoculate the destination bioreactor (**FIG. 1, embodiment 30**), such as a 100 L vessel (Eppendorf BioFlo610) for these examples or the seed train continues into another larger seed vessel as desired prior to producing algal biomass in the destination bioreactor (**FIG. 1, embodiment 30**) under aeration and mixing conditions for providing sufficient dissolved oxygen to support the target cell density.

As seen with nitrogen metabolism among the substantially starchless progenies and parent (see Example 1), different genotypes metabolize specific nutrients differently. A person skilled in the art will recognize that not all strains of the higher protein substantially starchless

C. reinhardtii genotypes will metabolize a particular nutrient similarly, or a particular combination of nutrients similarly, and that nutrient mixtures may need to be modified from one strain to another in order to provide the appropriate nutrient mixture.

An optional added step during the last 3 hours to 24 hours of **FIG. 1, embodiment 30** is the addition of a process aide, vanillin, as a component of the culture medium (see Example 6 for details). Production of biomass in the destination bioreactor can optionally be modified to using a draw and fill or continuous mode of operation (**FIG. 1, embodiment 40**). In this way the production vessel is partially emptied into a harvest vessel and the extant culture continues to grow with refill by fresh culture medium for further production. Prior to harvest of biomass from the destination bioreactor (**FIG. 1, embodiment 30**), heterotrophic conditions can be followed by optional cultivation under illumination around 30 μE or higher (**FIG. 1, embodiment 50**) for a brief period of light treatment such as to increase pigmentation. The feature of protein exceeding 50% by weight and being substantially starchless is unchanged after light exposure. The harvest culture in a draw and fill operation (**FIG. 1, embodiment 40**) can also go through optional light-finishing (**FIG. 1, embodiment 50**) or can proceed to harvest with dewatering (**FIG. 1, embodiment 60**). The algal biomass is preferably harvested in log phase. Harvest can be in a stationary phase if biomass retains the preferred phenotype. Harvest can be at a desired cell density or fermentation time. Harvest proceeds by dewatering the algal culture from the culture medium (**FIG. 1, embodiment 60**). It is concentrated by further removal of moisture (**FIG. 1, embodiment 60**). Biomass used in compositions can be dewatered into a fresh product such as a wet paste or flowable paste for some applications. Dewatering is well known in algae culture and includes centrifugation, filtration, electrostatic aggregation, or other food-grade processes as known in the art. Biomass can also be partially or completely dried such as from 10% moisture content down to bone dry for other applications. In certain embodiments, bone dry can be defined as less than about 10% moisture, about 9% moisture, about 8% moisture, about 7% moisture, about 6% moisture, about 5% moisture, about 4% moisture, about 3% moisture, about 2% moisture, about 1% moisture, or less. Biomass is optionally suspended in distilled water and sterilized with high-temperature, short-time (HTST) or with an autoclave for rinsing or for alteration of composition such as minimization of green color after step **FIG. 1, embodiment 60** prior to drying, mixing for homogeneity or further pulverized for powdering purposes. Methods of drying can be any number as known in the art including spray dried, drum dried, heat dried, vacuum dried, freeze dried or other cold processing, the latter being useful for preservation of some functional properties of the algal biomass. Dry powdering methods are also established in the art. This

yields the novel improved algal product (**FIG. 1, embodiment 70**) to be formulated into the edible compositions in a variety of finished products as exemplified herein (**FIG. 1, embodiment 90**). Optionally the output can be processed with a pre-treatment such as by enzymes or heat, prior to food formulation (**FIG. 1, embodiment 80**). This pre-treatment step may occur after the material is first dewatered to some degree and before the material has been dried. Importantly, the higher protein, very low starch phenotype is produced with no requirement of a stress phase during cultivation. This can shorten a final production cycle, for example to 2 days in the destination bioreactor (**FIG. 1, embodiment 30)** for a harvested nutrient replete fed-batch production. If a nutrient stress is used, with overfeeding or nutrient depletion such as acetate depletion, a final production cycle of longer than 2 days is used. For example, a final production cycle can be 2 days for growth and 6 hours for cultivation under acetate deplete conditions.

The time duration of cultivation in the destination reactor depends on several variables including seeding density, growth rate, and mode of operation (draw and fill, continuous, batch harvest). The fermentation culture in the destination reactor is sustained for a period sufficient to produce growth and desired yields whereby it is longer than about 24 hours, about 48, or about 72 hours or more. In some embodiments the total fermentation cycle time is about 72, about 96, about 120 hours, about 144 hours, or about 168 hours or by any duration falling within the range of 24 hours to 168 hours or a duration that is economically justified. Those numbers apply to cultures that are completely harvested. Draw and fill can be 24 hours or less depending on how much of the culture is drawn (80% for example), also continuous culture will extend beyond 168 hours. The feed rate of acetic acid/nutrients will match the harvest rate to maintain the culture volume and density to allow for continuous culture beyond 168 hours.

Carbon inputs and supplemental oxygen inputs as part of aeration inputs into the seed train bioreactors and into the destination bioreactor (**FIG. 1 embodiment 100**) can include carbon that is recycled and upgraded into acetic acid/acetate feedstock including from the fermentation of the *Chlamydomonas reinhardtii* (**FIG. 1 embodiment 100**) or from other industrial carbon sources including CO_2, carbon monoxide, syngas, methane and such (**FIG. 1 embodiment 110**) as described elsewhere herein. The aeration input (**FIG. 1 embodiment 100)** is ambient air (that contains O_2) that optionally can be enriched with process oxygen that is from a waste stream of O_2 generated by catalytic processes or other process oxygen sources as known in the art to be fed into the algae fermentation process for aeration and mixing.

EXAMPLE 4 – GROWTH RATES AND PRODUCTIVITIES FOR *CHLAMYDOMONAS REINHARDTII* PHENOTYPES THAT PRODUCE IMPROVED HIGHER PURITY WHOLEMEAL

This example characterizes the suitability of the novel *Chlamydomonas reinhardtii* phenotypes to generate biomass and protein in an expeditious manner. Performance of the higher protein substantially starchless strains from the prior examples is compared to performance of the heterotrophic parental production line KAS2004 containing 40% protein and 31% starch in fermentation production. Heterotrophic cultivation conditions to produce biomass and the sampling of cultures is described in Example 3. Samples of 10 mL are immediately centrifuged at 3000 *g* for 5 minutes, supernatant is removed, cell pellet is frozen at -80°C and freeze-dried to determine dry weight. Protein and pigment contents and productivities (rate of product formation or *qp*) are determined using the dried biomass. Protein is quantified per Example 2. Pigments are extracted from ground freeze-dried biomass with 50 µL of ethanol per mg of biomass for 5 minutes at room temperature. Percent pigment is calculated from carotenoid bands excised from a TLC plate that are quantified based on a A476 curve for each pigment calibrated using standards as is known in the art. Pigments can also be extracted from dried biomass (25 mg) in DMSO (2-3 mL) and then acetone (2-3 mL; repeated extractions as needed until the pellet is colorless) for analysis by HPLC as is known in the art, performed at a third-party laboratory.

The biomass densities of the improved high protein substantially starchless lines from KAS2010 in fermentation correspond to a specific growth rate of 1.41/day (0.059/hour) that can be maintained over at least 96 hours with no detectable lag phase, compared to a specific growth rate of 1.34/day for the KAS2004 parent. At industrial production scale this differential in growth rates has a significant beneficial impact on yield. Unexpectedly, the higher protein substantially starchless lines grow faster than the starch-containing production parent. This outcome is unexpected for cells with no measurable reserve energy in the form of stored starch granules. However, another substantially starchless progeny, KAS2013-Y (yellow colony obtained from KAS2013) has a lower growth rate of about 0.8 to 0.9/d in a 10 L vessel and also grows poorly on plates. This results in lower protein productivity for KAS2013-Y compared to other starchless progenies.

A fermentation culture from KAS2010 and derivatives started with 0.2 g/L of biomass of *C. reinhardtii* with a logarithmic phase of at least 48 hours with a specific growth rate of 1.41/day yields 13.8 g/L biomass in 72 hours. A fermentation culture started with 1 g/L of biomass of *C. reinhardtii* with a specific growth rate of 1.41/day yields 68.9 g/L biomass in 72

hours. This yields a *qp* of protein at 702 mg/L-hour calculated for 73.45% protein content. In comparison, the yellow parent KAS2004 started with 1 g/L biomass produces 54.9 g/L biomass in 72 hours with a *qp* of protein at 305 mg/L-hour as calculated for 40% protein. For starchless lines grown for lower protein but still above 50% protein with similar growth rates, the protein productivity will be less than 702 mg/L-hour but still exceed that of the unimproved parent that contains starch.

Protein content for substantially starchless lines grown as described in Example 3 for an ash content of 5% will exceed 73.45% protein and exceed 80% protein by weight. The protein productivity is 760 mg/L-hour for 79.5% protein, starchless lines. The protein productivity is 770 mg/L-hour for 80.5% protein, starchless lines. For draw and fill mode of fermentation production, the protein productivity rates are 1766 mg/L-hour and 1788 mg/L-hour for 79.5% and 80.5% protein contents by weight, respectively.

The substantially starchless lines cultivated with overfed nutrients that are three times or more in concentration compared to the initial concentration in growth medium used at the start of a fermentation will result in lower protein content and lower productivities. For example, referencing Example 3, urea at 0.11 g/L would be overfed if at 0.33 g/L. Another example is magnesium sulfate heptahydrate at 0.05 g/L would be overfed at 0.15 g/L. In one case, an overfed biomass contains about 58% crude protein with about 19% fat. In another case, an overfed biomass contains about 62% protein and about 15% fat. In some cases, the medium comprising an over-supply of nutrients that results in biomass with lower protein content, at about 62% protein or at about 58% protein, has a relatively lower C:N (carbon to nitrogen) ratio compared to medium yielding about 73% protein and about 12% fat.

The improved higher protein substantially starchless *C. reinhardtii* phenotype has a better acetic acid/acetate (carbon feedstock) use rate, based on conversion into biomass, than the starch-containing parent with lower protein. Further, it has an intermediate acetic acid use rate between the two parents, as substantially starchless zygotes, such as, for example, KAS2010 uses about 2.7 g acetic acid/g biomass; KAS2006 uses about 2.4 g acetic acid /g biomass; and KAS2004 uses about 3 g acetic acid /g biomass. On a unit protein basis, the improved phenotype has a much more efficient carbon usage (i.e., acetic acid/acetate usage) as seen by an acetic acid/acetate conversion ratio of 3.4 to 3.75 for 80% and 72% protein content in biomass, respectively, based on 2.7 g acetic acid per g biomass expressed on protein basis (e.g., 2.7 g acetic acid /0.72 g protein = 3.75 ratio) compared to a ratio of 7.5 to 10 for a starch-containing lower protein phenotype of 40% to 30% protein, respectively, based on 3 g acetic acid per g biomass expressed on protein basis (e.g., 3 g acetic acid /0.3 g protein = 10 ratio).

The wildtype phenotype with 31.3% starch content in effect uses about one-third of the fed acetic acid to produce the non-nutritive starch component of the biomass. This is a wasteful, unsustainable metabolism that does not occur with the substantially starchless high-protein phenotypes. The improved phenotype of 72% to 80% protein and substantially starchless uses about one-half (7.5/3.75) to one-third (3.4/10) the acetic acid/acetate for protein production than a wildtype under dark fermentation cultivation. This shows that algal lines that produce the higher purity biomass of 72% to 80% protein and are substantially starchless use 2- to 3-times less acetic acid/acetate per unit protein (calculated as 7.5/3.75 and 10/3.4, rounded up) than unimproved biomass with 30% to 40% protein containing starch above 2.25%. This demonstrates much more efficient and sustainable metabolism of added carbon feedstock (in the form of acetic acid/acetate) into nutritionally valuable compounds, namely protein. For reference, acetic acid is 40% carbon by weight.

Using line KAS2010-Yellow, a fermentation culture yields a *qp* of pigments at 5.74 mg/L-hour (per 0.6% total carotenoids in biomass). In comparison, the yellow parent KAS2004 at 54.9 g/L in 72 hours with 0.3% carotenoids yields a *qp* of pigments at 2.29 mg/L-hour. The pigment content of the improved *C. reinhardtii* is sufficient for functional food compositions when used at about 1% or higher wholemeal inclusion based on lutein alone, with foods containing about 30 µg/g lutein considered functional foods (**Diprat *et al.* 2020**; for example, a 200 g serving provides 6 mg lutein as daily recommendation for decreasing risk of developing macular degeneration). In some embodiments, the antioxidant features imparted by the antioxidant-containing composition are imparted to an uncooked product. In some embodiments, the antioxidant functionalities imparted by the antioxidant-containing composition are imparted to the cooked product.

Progenies KAS2010, KAS2014 and KAS2015 overall have more pigments per dry weight than the yellow KAS2004 parent. This is visualized by broader/darker bands in comparative thin layer chromatographs loaded on an equivalent weight basis. The vast improvement in pigment content was evident by eye such that TLC bands do not need to be excised and loaded into a spectrophotometer to obtain numerical values. Such pigment production under nutrient replete heterotrophic cultivation (e.g., no depletion of nitrogen or other nutrients that cause stress to trigger carotenogenesis or chlorophyll biogenesis as is known in the art) is unexpected in the high protein substantially starchless *C. reinhardtii* of the instant invention. The chemical composition of the improved higher purity *C. reinhardtii* biomass (**Example 2**) show that novel strains do not partition carbon from acetic acid/acetate into accumulated starch. Thus, they lack access to a key energy and carbon storage compound

(starch) for secondary metabolism and carbon partitioning. The strains also lack the requirement for carotenoids and other pigments needed for active photosystems since they are under dark cultivation. Thus, it is surprising that carbon partitioning occurs into both proteins and pigments during the rapid growth phase of these improved *C. reinhardtii* cultures to produce the improved wholemeal. The white version of the improved *C. reinhardtii*, which lacks substantial pigments, selectively partitions carbon into the higher nutritional value protein rather than into lower nutritional value starch like the pigmented strain versions. Under mixotrophy these lines will also demonstrate more efficient production of nutritionally valuable compounds by selective partitioning of feedstock carbon into protein and/or pigment over starch. It is known in the art that for *C. reinhardtii* the specific growth rate under mixotrophic conditions can be higher than the specific growth rate under heterotrophic conditions, and exposure to light increases pigmentation.

Taken together, the wholemeal of the improved higher purity *Chlamydomonas reinhardtii* phenotype is more efficiently produced in terms of utilizing carbon, provided in the form of acetic acid in the fermentation feedstock, by its selective partitioning it into higher nutritional value protein or pigment rather than into lower nutritional value starch compared to the wholemeal of non-improved *Chlamydomonas reinhardtii* phenotype with protein subceeding 50% and containing starch.

Accordingly, the results of this example demonstrate that the improvement of the *Chlamydomonas reinhardtii* strains refers not only for their improved chemical composition but also to their added improved performance in cultivation by fast growth, high protein productivity, dark heterotrophic growth, ability to accumulate pigments by carotenogenesis in the dark, and efficient, selective carbon metabolism into nutritionally valuable compounds. Illustratively, productivity and efficiency can be further increased through optimization of fermenter seeding density (that can directly affect final density for a given time period) and of operations, as is known in the art. This includes optimizing temperatures, aeration, pressure, nutrients and nutrient ratios, inoculation ratio, through cell type selection, or adding optional exposure to light. Notably, this includes operations using draw and fill (see **FIG. 1, embodiment 40**) wherein a vessel used for draw and fill reaches 80 g/L, 90% culture is harvested every 36 hours when 80 g/L is again reached. This calculates to 1622 mg/L-hour protein productivity for biomass with 73% protein (80 g/L / 36 hours x 73% protein). Unexpectedly, it is discovered that KAS2010 grown at 32°C has a faster growth rate than 30°C, 28°C, and room temperature (19°C-23°C); 28°C is the default fermentation temperature. Using this value, productivity can increase to at least 1.8/day biomass based on temperature elevated

by 2°C. Substantially improved productivity in fermentation is important for food applications. Heterotrophic production of *Chlamydomonas* with precisely controlled feeding and culture conditions allows for unprecedented nutritional consistency and quality of an agricultural nutritional whole food protein.

EXAMPLE 5 – COMPOSITION FOR FOOD SUPPLEMENTS USING IMPROVED HIGHER PURITY *CHLAMYDOMONAS* INGREDIENT

The physical form of a food supplement comprising the improved *Chlamydomonas* material when as a dried algal material is a solid agent such as a tablet, capsule, granule, or loose powder. The form of the food supplement when using a paste of the improved *Chlamydomonas* material is as solution, a suspension, or an emulsion. One preferred embodiment is to use a dried powdered or flaked *Chlamydomonas* material as described in **Example 3**. This provides for ease of handling and storage. Before drying, biomass is optionally suspended in distilled water and sterilized with HTST or with an autoclave for rinsing or for alteration of composition such as minimization of green color. The material is then dried, formed or compressed into small bits, or pulverized for homogeneity or for powdering purposes as is known in the art. The high protein substantially starchless *Chlamydomonas* material can be formulated into a food supplement by commonly used methods using the dry homogeneous or powdered *Chlamydomonas* biomass as the main component in combination with a vegetable capsule for one form of an edible composition. Additionally, the *Chlamydomonas* biomass as main ingredient can be mixed with a known carrier such as carboxymethylcellulose or its preferred plant-based substitutes, inorganic salt, corn starch or other as known in the art; or other component as an excipient, a binder, a disintegrant, a flavoring, a lubricant, a stabilizer or preservative. The dosage can be in any number of quantities and is not particularly limited; a serving size will be recommended. An effective dosage using the *Chlamydomonas* material will deliver a similar or superior nutrition as existing commercial supplement products. For protein nutrition, a serving size of 8 g (2 tablespoon) of a composition comprised of dried algae (72% protein content) blended with 0.03 g to 0.06 g stevia powder, protein will be 5.7 g or about 10.1% DV (percent Daily Value). For the same serving size using dried algae of about 60% protein (produced as described elsewhere in the examples), the finished product will be 4.8 g protein or about 8.5% DV (percent Daily Value). This composition provides protein content like this supplement product (see link) but without the carbohydrates (12.5%) and is used similarly by sprinkling into a smoothie or sports

drink, used as a salad topper or on soup, see worldwide website: sunfood.com/spirulina-crunchies-4-oz-raw-natural-low-temp-dried.html.

Consumers will compare the improved *Chlamydomonas* material with other algae for its benefits over the market leaders in algae-based nutritional supplements compositions. Historically *Spirulina* (*Arthrospira platensis*) is the first "superfood" introduced into the market. Protein quality can be assessed by protein digestibility as is known in the art. Protein digestibility for the improved higher purity *C. reinhardtii* of the instant invention is 94% and with precision fermentation production compared to field cultivation shows consistency in composition. The improved higher purity *C. reinhardtii* profile indicates it is a complete protein with desirable amino acid profile (see **Example 2, Table 2**). When provided with samples of loose powder to taste and informed of nutritional differences and industrial production practices/sustainability between the facultative heterotrophic improved *Chlamydomonas* and photoautotrophic *Spirulina* produced in outdoor raceways, a group of six volunteers queried for specific customer values (nutritional profile, taste and texture, serving size, cost, sustainable, safe/traceable) indicated a unanimity for a better end user experience (for better health, tastes better, quicker and easier to eat, saves money, better for aiding the planet health, and confidence in product.)

EXAMPLE 6 – PREPARATION OF POWDER MIX, BATTER, AND DOUGH WITH PROTEIN FORTIFICATION, SENSORY IMPROVEMENT, AND FLOUR REPLACEMENT USING CHLAMYDOMONAS OF IMPROVED COMPOSITION

In some embodiments, the improved higher purity compositions of *Chlamydomonas reinhardtii* biomass are used in edible compositions that combine algae with additional dry ingredients that, upon addition of water, produce a liquid batter. The added algal component provides protein fortification- for an added 3% or higher Daily Value protein- at rates of inclusion lower than other algae comprised of less than 50% protein. This proceeds without adding extra starch to affect the batter cooking or off-flavors to affect the consumer appeal. In one example, dry ingredients are added to a bowl followed by addition of hot water. The batter is mixed by hand using a spoon. The improved higher protein starchless *C. reinhardtii* ingredient blends in easily. The batter retains small bubbles similar to batter with no added algae. The final dry matter mixture (i.e., not including water weight) comprises 8% weight dried algae. The formula for pancake batter sufficient for 12 pancakes is 10 g improved *C. reinhardtii* KAS2010, 124 g (1 cup) Krusteaz Buttermilk Protein Pancake Mix (an egg-free product though this can readily be substituted with powdered egg-containing mix), and 200 mL

water. Pancakes are cooked using a non-stick skillet over medium heat for 2 minutes per side using ½ tablespoon of Earth Balance Organic Whipped Buttery Spread for every 3 pancakes. **FIG. 3** shows a cooked, airy, protein-fortified pancake with a pleasing chartreuse interior comprising high-protein, substantially starchless *C. reinhardtii.* The process of cooking and the resulting product appearance and 'spring' are identical (aside from the color) to batter with no added algae. Taste-testing of the algae pancakes reveals descriptions ranging from "algae not noticeable" to "having a hint of green tea", such as can be balanced for example with maple or fruit syrup, or fruit sauce as natural sweeteners commonly used with pancakes. After 24 hours of refrigerated storage, there is no change in appearance and spring of the pancakes. Analysis of the composition of the algal component from this batch shows 69% protein, undetectable starch, 16% lipid, 10% ash, 4% moisture. Beneficially, as evidence of protein fortification, the consumption of one serving size of 6 pancakes provides about 20.5 grams protein or about 13% Daily Value (DV), an increase over the about 15 grams protein or about 10% DV without the added algae. Protein is increased by 36% without adding starch-derived sugars or calories (such as would occur using unimproved algae containing starch) and without compromising the batter's rheological properties or the cooked pancakes physical properties (other than color) compared to no-added algae composition. Inclusion of less than 8% (dry matter basis) yields the same 20.5 g total protein in a serving size for algal wholemeal with higher protein content such as 73% or 80%, namely inclusion of 7.5% or 6.9% dry matter, respectively.

Advantageously, in further examples, a dry mix or a batter composition comprises green or yellow wholemeal obtained from algal cultivation in vanillin-containing culture medium or from soaking dried biomass in a vanillin solution. Evaluation of the pancakes with 8% inclusion dry matter yields similar results as described above in terms of pancake form and texture, with the sensory quality of taste being "tasty" and "typical pancake". The algal biomass is produced following **Example 3**, with the added step of vanillin addition during the last 3 to 24 hours as a finishing step. Vanillin is employed in the instant invention as a novel process aide in the culture medium of the *Chlamydomonas* to reduce oxygen consumption. Vanillin is a known tryptophan quencher for taste improvement and an inhibitor of cell proliferation, with concentrations as low as 50 mg/L demonstrated effective in various cell lines (**Durant and Karran 2003**). Surprisingly, use of 20 mg/L vanillin (Sigma V1104) in flask culture of *C. reinhardtii* already produces a notably altered biomass with vanilla scent from very small - just 7 mg - algae samples. This is based on testing vanillin concentrations ranging from 0 mg/L to 40 mg/L assessed over three days at 28°C heterotrophic culture in the dark. This indicates

vanillin lower than 20 mg/L is also anticipated to be effective. Vanillin inhibited cell proliferation in a dose dependent manner and thus should be used at the lowest concentrations as a process aide for reduced oxygen consumption with effective flavor enhancement.

Alternatively, the dried algae are treated by soaking overnight or stored as a paste or slurry with preservatives in an aqueous vanillin solution of at least about 1.9 mg/mL for aroma modification. While vanillin is appropriate for an application such as sweet pancakes, pre-treatment in other appealing aroma/flavor solutions such as poultry broth, tuna juice, filet mignon flavor and such can be considered for other food compositions.

In a further example, the wholemeal can benefit a savory pancake composition such as Asian-style scallion pancakes. The wholemeal is utilized as an ingredient for protein fortification added to the dry matter of a thick batter (i.e., dough) with partial flour replacement, or added to the filling. The scallion pancake dough comprises 4.8% dry matter algae or 3% algae for total wet weight. To a mixture of 285.5 g all-purpose flour (containing 28.55 g protein) with 3.5 g salt in a stand mixer equipped with a dough hook, 118 g hot water pre-blended with 14.5 g algae (containing 10 g protein) is added slowly, followed by 59 g cold water. It is kneaded for 3-5 minutes and then allowed to rest before rolling out. Importantly, the addition of the algal wholemeal retains the elastic nature of the dough when compared to the flour-only control, with 300 g flour and 0 g algae (containing 30 g total protein), despite the lack of algal starch and the decrease in the flour component. Beneficially, replacement of flour with algal wholemeal improves protein digestibility due to higher digestibility of the *Chlamydomonas* protein (94%) compared to wheat protein (87%). Replacement of flour with algal wholemeal also reduces the gluten content of the composition. All-purpose flour has about 8-11% gluten content; gluten is the protein. Replacing half the savory pancakes protein content from flour with algal wholemeal results in 50% reduction in gluten content. For a basic filling, ingredients are flour, salt, and vegetable oil (peanut oil, light olive or canola) with a dash of sesame oil plus the chopped green onion (scallion) mixed into a smooth paste. The algae-containing dough rolls out with a rolling pin with the expected extensibility and stickiness to form the thin rectangle sections for each pancake. The flour-salt-oil paste is spread on top and then chopped green onion (and optional algae if not used in the dough) is added on top of that. These are shaped, rolled out, and cooked in hot oil, being covered for one minute, then continued cooking on the other side without a cover until done. Protein is increased by 22% without adding starch-derived sugars or calories (such as would occur using unimproved algae containing starch) and without compromising the dough's rheological properties apparent during handling compared to the flour-only control pancake.

Taken together, the effective amount of ingredient for the liquid batter or dough is that which adds protein value to a desired content without compromising the physical properties of the uncooked dough or batter or the physical and flavor properties of the cooked final food product. Amounts may vary depending on being sweet or savory compositions and on the fillings or saucing used.

EXAMPLE 7 – PREPARATION OF PROTEIN FORTIFIED PLANT-BASED OR OTHER NON-ANIMAL DERIVED MEAT-LIKE PRODUCTS AND PASTA FILLINGS CONTAINING IMPROVED COMPOSITIONS OF *CHLAMYDOMONAS REINHARDTII*

In some embodiments, the *Chlamydomonas*-containing compositions combine algae with additional ingredients such as plant-derived or cell culture-derived or other non-animal sourced or non-animal meat components to produce a meat-like algae-containing product. A first example is algae-containing vegan or vegetarian meatballs without use of pulse (legume) proteins or texturized wheat proteins as common sources of protein for meat replacement in recipes. Food allergies to pulses such as peas, soy, chickpeas are not uncommon and may lead to life-threatening reactions. Algae protein in the form of wholemeal from the high protein, substantially starchless *Chlamydomonas reinhardtii* component is a much more water-conservative and land-conservative protein than soy-derived or wheat-derived protein. To form the meatballs, 200 g fresh shitake mushrooms, 8.6 g dried algae from **Example 1**, 8 pitted green olives, 3 large cloves garlic, 12 basil leaves, and 1/2 tsp fennel seed are minced in a food processor. Thereafter the mixture is combined with 1/2 cup quick cook oats, 1 egg (or 15 g chia seed soaked in 2.5 TBSP water for 5 minutes to replace egg), 1/4 tsp paprika, 1/8 tsp red pepper flakes, salt and pepper. This is formed into 12 1-inch meatballs, placed on a lined baking sheet, brushed with oil, then baked at 350°F for 35 minutes, turning them over at the halfway point. Alternatively, the meatballs can be browned in a skillet in neutral oil, 4-5 minutes. The resulting protein fortified food product holds its form like the meat-like meatballs without added algae. The cooked mushroom-algae vegetarian meatball color is a pleasing brown using green algae powder. Advantageously, inclusion of the algae increases the protein content by almost 70%, from 3% to 5%. This recipe provides an uncooked composition with 37% calculated total protein value from the added improved *Chlamydomonas* component based on 72% crude protein and 3% algal inclusion. Due to the high protein content of the algal ingredient, inclusion can be reduced to 2% and still provide protein enrichment by over 50% compared to the control vegetarian meatballs without algae. The effective dosage of algae provides protein enrichment with acceptable flavor to this vegan food while preserving physical properties expected for its

meat-containing counterpart. The meatballs lend themselves to red/orange sauces such as tomato-based and/or red pepper-based sauces which can include additional protein sources such as ground cashews. **FIG. 4** shows a food composition of baked algae-mushroom meatballs with creamy red pepper sauce produced by incorporation of the improved algae component of the present invention. Advantageously, the use of higher protein substantially starchless algae in food compositions enabled by the instant invention facilitates a much lower algal inclusion rate than using *C. reinhardtii* of the highest measured protein value in prior art. For the same protein value, 25% less wholemeal is required at 3% inclusion of the instant invention versus 4% inclusion assuming an about 50% protein content biomass for the latter. Advantageously, this provides flexibility in food compositions and ingredient proportions to attain flavor, texture or other desirable attributes in this or other meat analogue products containing *C. reinhardtii*. such as meat-like patties. A second example is a food composition that is an algae-containing pasta filling that is a ravioli or dumpling type food. In some embodiments, the high protein substantially starchless C. *reinhardtii*-containing compositions employ algae with additional ingredients to produce a filling intended for ravioli and dumpling as an alternative to meat-containing fillings. The fillings contain algal protein with healthy polyunsaturated fatty acids and are free of cholesterol. Algae wholemeal containing heme or mixed with red *Haematococcus pluvialis* wholemeal delivers a red color to the meat analogue food compositions.

EXAMPLE 8 – PREPARATION OF MEAT-CONTAINING COMPOSITIONS WITH IMPROVED HIGHER PURITY *CHLAMYDOMONAS REINHARDTII* MATERIAL FOR PET FOOD OR PET TREATS

Chlamydomonas reinhardtii-containing compositions can be produced that combine the algae with meat ingredients to produce a meat-containing product. Such meat may be derived from livestock, poultry, open nature-sourced or aquaculture-sourced fish, shellfish and crustaceans, or cell-cultured meat and cell-cultured seafood. Algal biomass containing additional pigment such as chlorophyll imparts a deeper pink or reddish or brownish color to the meat product. This can be for cooked or uncooked or raw product. Once heated the green chlorophyll material to some extent loses color to appear gray, cream or whitish, with light green biomass more effectively losing its green hues compared to dark green biomass (**FIG. 5**). In some embodiments, the color of the meat product is unaltered by the addition of the algae in cooked product. The addition of the improved higher purity microalgal material in an effective amount may serve functional roles such as benefitting the palatability (aroma/taste)

of a composition, water holding capacity of the composition, or serve as a binding agent and without added starch, which is shunned in pet foods.

In one example, the high protein substantially starchless algae is incorporated into a pet food for its palatability value, specifically into a cat food. As cats are hypercarnivores, it is recognized that cat food should avoid carbohydrates and have 40% or more protein and lower than 50% fat. Thus, the higher purity algal ingredient of the instant invention with high protein and being substantially starchless is a good match for cat food formulations for a range of ages and diets. Two-bowl choice tests for feeding can indicate preferred aroma or palatability, which is essential for eliciting the feeding response for a diet formulated to support a cat's health, vitality and longevity. Often this is with no wheat, soy, dairy or eggs and may also include no poultry to avoid supply issues seen from disease threats such as avian flu. Ingredients can be dosed in a composition as is known in the art and can include salmon, menhaden fish meal, carrots, blueberries, taurine, and flaxseed. The dry algal ingredient is suited to be made into snacks, kibble or into moist pet foods or even pet beverages as can be prepared by a person skilled in the art. The algal wholemeal may benefit from a pre-treatment of digestion to free amino acids such as through addition of a commercial protease added to liquid mixture to hydrolyze proteins followed by a step of inactivation as is known in the art such as heat. It is known in the art that supplementation with glycine and other amino acids especially as free amino acids, are of benefit for pet foods. The amino acid profile including glycine that is revealed in **Example 2** of the improved higher purity biomass rich in protein and being substantially starchless is promising for enhancing palatability, especially for food compositions with raw material of plant (non-animal) origin. As a source of aroma (flavor) or other palatability for cat food, the algal wholemeal ingredient of the instant invention used in small quantities about 1% or less, to about 0.1%, is sufficient; optimal levels can be determined empirically for different formulations as is known in the art. When not mixed into the food product by the manufacturer, the powdered biomass of the instant invention can be deployed by the pet owner by blending in a little and then sprinkling a bit on top, for dry or wet preparations. While the wholemeal itself is appealing to the animal, a pre-treatment in other appealing aroma/flavor solutions such as poultry broth, tuna juice, filet mignon flavor and such can be considered for marketing purposes.

In a second example, the high protein substantially starchless algae is incorporated into a pet food for its protein value. The effective dosage of algae provides a similar or superior amount of protein for replacing an ingredient without also adding starch that should be avoided in pet food (discussed elsewhere herein). Algae in quantities of 3% or 4% or more by weight

are sufficient for chicken meal replacement (of 65% protein) used in the same quantity for a source of protein in dog food and cat food. Chicken meal has a similar form to the dried algae ingredient, being dehydrated and a powder. The substituting or adding 3% (in USA) or 4% (in Europe) algal wholemeal to the food allows reference to the algae in the product name, which can be an important marketing tool. For some animals such as canines the algal ingredient can score neutral in appeal in two-bowl choice tests at the inclusion rates tested. The improved higher purity microalgal ingredient has excellent storage of at least two years, a preferred feature for pet food manufacturers.

EXAMPLE 9 – FLAVOR MODIFICATION IN FOOD AND BEVERAGE COMPOSITIONS USING THE IMPROVED *CHLAMYDOMONAS REINHARDTII* WHOLEMEAL

A food or beverage finished product can include use of flavor or taste blockers, modifiers or maskers, such as for higher dosing of the algae or specific applications or market geographies, used in liquids and in solids. This is in addition to that described for vanillin treatment in Example 6. Taste modifiers can include without limitation, those that act on taste receptor families such as the plant-derived bitter blocker from Natural Taste Consulting applied at 0.1%. Example of maskers or modifiers (which may also be sweeteners) include stevia, thaumatin protein, brazzein protein or monellin protein suitable for external addition or internal delivery with a genetically modified *Chlamydomonas*. **US11034968 B2** describes expression of an introduced thaumatin protein in *Chlamydomonas* used for flavoring and flavor masking. Other flavors are dry mustard and siracha. Dosages will vary per food type and can be determined empirically.

EXAMPLE 10 – IMPROVED ALGAE MILK AND CREAM COMPOSITIONS USING IMPROVED HIGHER PURITY *CHLAMYDOMONAS REINHARDTII* WHOLEMEAL

Plant-based milk is a multibillion global market, benefitting those consumers who are lactose intolerant, vegan, avoid excess saturated fats, or want a milk more sustainable than from dairy with livestock's high use of natural resources (water and land) and the associated methane emissions. Availability of options with algae-based milk and cream product offerings will appeal to those avoiding nut-based milk from an allergy basis, and in particular avoiding almond milk for sustainability reasons since the almond crop requires very high amounts of irrigation water. In this example, an algae milk beverage is produced using the high protein, substantially starchless, yellow or cream *Chlamydomonas reinhardtii* wholemeal of the present invention. Optionally the algal biomass used in the composition is produced in fermentation

cultivation with a vanillin process aide as described in Examples 3 and 6. The algae milk consists of algal wholemeal combined with a thickener, vitamins A and D, and water in appropriate proportions, along with optional sweetener or other additions. The wholemeal is predominantly intact and is homogenized or micronized for a uniform fine consistency. Thickener and the algae are first slowly dispersed in water followed by optional addition of vitamins before high pressure homogenization and pasteurization as known in the art. Common thickeners are xanthan gum or xanthan gum and guar gum agar used in known ratios.

An improved composition of algae milk is enabled by the instant invention compared to other algae milk. The inclusion rate of the higher purity *C. reinhardtii* algal wholemeal can be guided by protein contents considered to be effective for healthy vegan milk compositions. One effective amount is based on soy milk, which can have 1.44% protein (3.5 g protein in 243 grams; see worldwide website: nutritionix.com/i/nutritionix/unsweetened-soy-milk-1-cup/56ba04d150ef45d146b213af). A 2% inclusion of improved *C. reinhardtii* wholemeal with 72% crude protein of the instant invention provides a similar protein value (1.48%). Another dairy analogue composition is Silk brand Oat milk that contains 1 g protein in 240 mL product or about 0.4% protein. Illustratively, this corresponds to about 0.7% inclusion of algal wholemeal of the instant invention (1.7 g algae in 240 g product for a 60% protein *C. reinhardtii* plus water and thickener in above proportions). Based on meeting the same protein nutrient targets, these inclusion rates will be lower compared to using other algae with less than 50% protein and may benefit comparative tastes.

Different algae milks are made to compare the use of substantially starchless algae of the instant invention with starch-containing *C. reinhardtii* wholemeal. For test purposes these are unsweetened with no added vitamins. The protein target is 1.48% of the milk composition on wet weight basis. The recipe comprises 5 g algae (2.06%) of starchless 72% protein *C. reinhardtii,* 0.3 g xanthan gum (0.13%); and 237.7 g water, for 243 g product. The recipe for the other test milks is 9 g algae (3.7%) of starch-containing algae with 40% protein and 19% or 31% starch**,** 0.3 g xanthan gum (0.13%) and 233.7 g water, for 243 g product. The freeze-dried algae are non-homogeneous flaky powders in these tests; homogeneous, finely powdered preparations are preferred. The solids are added to water at room temperature along with the xanthan gum and blended for one minute with a hand-held immersion blender. Thereafter the mixture is boiled in a small, uncovered saucepan for one minute (temperature reads at least 180°F) and then transferred to a glass beaker in an ice bath for chilling.

The improvements in an algal milk using the higher purity *C. reinhardtii* are numerous compared to using other algae for algae milk. One improvement to the composition is by virtue

of no starch being contributed from the algal wholemeal and thus no associated starch-based sugars and calories. A second improvement is by virtue of the higher digestibility of the *C. reinhardtii* protein compared to other sources of vegan protein, described elsewhere herein. A third improvement is reduced fats compared to other vegan dairy analogues, with healthy fatty acids unique to the *C. reinhardtii* algal wholemeal. The algae milk has 0.3% total fat (0.65 g in 243 g milk) compared to 1% in soymilk (2.5 g in 249 g). About 62% of the total algae milk fatty acids comprise unsaturated fatty acids. Humans can produce all but two of the fatty acids they require for good health. These two essential fatty acids, linoleic acid C18:2ω6 and α-linolenic acid C18:3ω3, are present among the *Chlamydomonas* fatty acids in the improved algal milk product (about 20% of total fatty acids). These combined with the C18:1ω7 and C18:1ω9&ω8 fatty acids that are also present enables a unique fatty acid profile that comprise over 40% of total fatty acids; see Example 2 for fatty acid profiles. A fourth improvement is that the wholemeal, and notably the yellow wholemeal, of the improved *C. reinhardtii*, provides an improved nutritive value of the algal milk due to the algal carotenoids in combination with the effective protein and lipid values. Advantageously, taken together the overall compositional profile of milk analogue using the improved higher purity *C. reinhardtii* wholemeal is unavailable in other algae milk.

For algal vegan cream, xanthan gum content in the milk is increased to obtain the desired consistency. For the starchless algal milk above is added xanthan gum for a total in the composition between about 0.3% to 0.79%. The latter is very thick but still flowable. Other thickeners can be used as is known in the art. Beneficially, the result is a vegan cream that can also be considered reduced fat. For comparison a light cream or reduced fat cream comprises about 20% milk fat. The algae cream made with the starchless *C. reinhardtii* is about 0.3% fat.

This example of improved algae milk typifies some general advantages of the new *C. reinhardtii* ingredient offering used in edible compositions over the prior art, namely:

the flexibility in choice of ingredients that is valued in the trade for multiple food and beverage compositions. Innovative ingredients make foods and beverages healthier; and

the criticality of the *C. reinhardtii* wholemeal of the instant invention comprising a combination of being substantially starchless with a high protein content exceeding 50%, along with other cell constituents and other features described herein for making an improved food composition.

As described in the examples herein of food, food supplement and beverage compositions for people and pets, including this algae milk, numerous benefits are enabled by the novel higher purity *C. reinhardtii* wholemeal that are not possible when using starch-

containing wildtype *C. reinhardtii* or other starch-containing microalgae wholemeal in conventional compositions including in vegetarian/vegan compositions including the following:

Compositions with one or more of protein supplementation (enrichment, fortification), improved protein quality (in digestibility, protein content, and/or amino acid profile), antioxidant or pigment/color supplementation, animal-based protein replacement, egg and or flour replacement, sensory improvement, higher water absorbance for rehydration or shelf-life improvement, enhanced palatability, all while including healthy fatty acids concurrent with healthy nutritive protein produced more sustainably (more efficient, less wasteful) while excluding non-nutritive starch;

food compositions benefit from no added algal starch-based sugars and empty calories;

food compositions benefit from no added algal starch with effects on food formulation, cooking and food properties such as occurs with starch retrogradation;

food compositions benefit from certain nutritional targets (i.e., complete protein levels, all essential amino acids, better protein quality including better digestibility) now being attainable without increased algal inclusion rate compared to formulations using other microalgae or other *Chlamydomonas* biomass with lower protein;

food compositions benefit from certain nutritional targets now being attainable at lower algal inclusion rates than previously possible;

the technical process in formulation of an edible composition remains unaltered;

a food product's textural properties remain unaltered or indeed are even improved (such as preferred textural properties of the extruded cooked algae noodle in Example 15);

food compositions benefit from the unique fatty acid combinations of the improved *C. reinhardtii*;

food compositions benefit with improved nutritive value from antioxidant carotenoids in certain colored wholemeal of the improved *C. reinhardtii* in combination with effective protein value;

food compositions benefit from comprising an ingredient that is produced more sustainably by being less wasteful of nutrients provided during algal growth compared to ingredients from other *C. reinhardtii* that accumulate starch. This more economically sound approach is via selective partitioning of carbon nutrient into the preferred production of protein or pigments rather than into non-nutritive starch; and lastly

food compositions benefit from comprising an ingredient that is produced more sustainably by using carbon nutrient supplied during algal growth that is recycled from CO_2

emissions in the form of acetic acid/acetate feedstock. Aerobic fermentations with *C. reinhardtii* utilizing acetic acid/acetate as the primary carbon feedstock provides a distinct advantage over fermentations using glucose, such as practiced with microalgae outside of the Chlamydomonadales taxon. This is because converting CO_2 into monosaccharide feedstocks is uncommon whereas converting CO_2 into acetic acid feedstock is common and helps close the carbon cycle for this ingredient especially when sourced from the algae's own fermentation emissions.

EXAMPLE 11 – PROTEIN FORTIFICATION USING IMPROVED HIGHER PURITY *CHLAMYDOMONAS REINHARDTII* WHOLEMEAL FOR AN IMPROVED OIL-IN-WATER EMULSION

This example pertains to use of algal wholemeal with an improved composition of high protein over 50% and being substantially starchless to boost the protein value of a food composition that is an oil-in-water emulsion containing egg, namely a mayonnaise or dressing. There is a clear benefit for consumers and manufacturers in having options for increased nutritive value of commonly used dressings and spreads including for mayonnaise. The improved stable oil-in-water emulsion described here is a 3% w/w 'algae mayonnaise' with over double the protein content (115% increase) of the unfortified control mayonnaise enabled by successful incorporation of our novel algal substance. The control formulation is calculated to have a 5.5 g protein value (from the egg yolks) in a composition of 295.4 grams total, while incorporation of the algae biomass at 73% protein weight provides an additional 6.7 g protein beyond the egg yolk protein in a composition of 304.6 grams total. For a serving size of 13 g (1 tablespoon), this is 0.24 g protein for the unfortified control mayonnaise and 0.53 g protein in the protein-fortified 'algae mayonnaise'. Beneficially, there is no addition of starch-derived sugars in the food composition by inclusion of the algae.

The *Chlamydomonas* algae ingredient used in the mayonnaise is a lyophilized flaky powder. It results from heterotrophically-produced *Chlamydomonas* as described in Example 3. The ingredients shown in **Table 4** produce a 72% oil mayonnaise for the control with no added algae and a 70% oil 'mayonnaise' for the algae-fortified product.

Table 4. Recipes for mayonnaise without algae (the control) and for a 3% w/w 'algae mayonnaise' with added wholemeal of improved higher purity *Chlamydomonas* material to fortify protein content without adding algal starch.

Ingredients	Control (grams)	Algae-fortified (grams)
Egg yolk	36.7	36.7
Salt	6.4	6.4
Dry mustard	0.8	0.8
Lemon juice	9.5	9.5
White distilled vinegar	30	30
Extra light olive oil (Bertolli)	212	212
Improved *C. reinhardtii* wholemeal	0	9.2
Total	295.4	304.6

The algal wholemeal is mixed in 50 g vegetable oil with the food processor and set aside for 30 minutes while the other ingredients are prepared. Egg yolks and acids are creamed for 10 seconds with the mustard and salt. The algae-oil mixture is then slowly spooned in over a few minutes. The remaining 162 g vegetable oil is then slowly added for the final mayonnaise. A control mayonnaise without algae is made along with the algae mayonnaise formulation. Emulsification is carried out using a single-speed Cuisinart Prep 7, 7-cup food processor in a 26.7°C (80°F) room. The run time for the control is 13-14 minutes to allow for slow addition of oil to ensure obtaining the desired thickening. Unexpectedly, the preparation using algal wholemeal thickened noticeably faster than the egg-alone control and was finished within 9-10 minutes. This was particularly surprising given the warm room and the heating of the base motor during the food processor operation, with the base becoming hot to the touch; mayonnaise thickens best in a cool environment. Additional mixing for another 2 minutes had no further visible effect on thickness.

Both fresh preparations of the control and 'algae-fortified mayonnaises' appear identical in gloss and texture (**FIG. 6**). The one-day-old preparations after overnight chilling appear identical in gloss, texture and stiffness. The slight 'green' odor detected in the freshly made warm room temperature (80°F) 'algae mayonnaise' is almost completely dissipated after overnight refrigeration and is unnoticeable after five weeks refrigerated storage. The 'algae mayonnaise' made using green algae produces an avocado green condiment similar to the appearance of guacamole spreads. This differs from the creamy white sauce typical of commercial mayonnaises but nevertheless has consumer appeal especially for the health-conscious consumer. Use of a cream-colored high protein algal ingredient of the instant invention in place of the green ingredient is expected to provide the typical creamy color. The algae-containing and the control finished products each are pH 4, meeting the desired specification for mayonnaise of pH between 3.6 and 4. After using the food processor

described above, both products show small air bubbles less than 1.5 mm size dispersed throughout the emulsions.

Remarkably the fresh algae-fortified emulsion is homogenous with excellent thickening. This is unexpected considering the fortification ingredient is not an extracted protein isolate or protein hydrolysate– preferred for their ability to be present at the oil-water interface during emulsification – but instead is an un-fractionated, un-milled wholemeal. No coalesced curdling is evident during or after the formulation process. There are no lumps or pockets of unmixed algae visible. Thus, there is no negative emulsification impact of the high protein substantially starchless wholemeal.

At the outset, the fresh 'algae mayonnaise' in the warm room temperature visually appears slightly less firm than the control mayonnaise based on its ability to hold a peak at the warm room temperature. However, the one-day-old mayonnaises after overnight chilling appear identical in stiffness in terms of ability to hold a peak. The products are stored at 4°C. Viscosity measurements, recorded in centipoise (cP), are taken at 4°C for chilled samples with a Brookfield RV Series viscometer using spindle HB-5 at 50 rpm with the following results shown in **Table 5.**

Table 5. Viscosity measurements for various dressings and mayonnaises, in centipoise (cP).

Storage time	Control (no algae) Mayonnaise	Best Foods Real Mayonnaise	Algae-fortified Mayonnaise	Follow Your Heart Vegenaise (no egg)
1 week	13,000-14,000 cP	10,000-11,000 cP	7,000-8,000 cP	4,000-5,000 cP
9 weeks	13,000-14,000 cP	--	7,000-8,000 cP	--

Results indicate a viscosity value for the 'algae-fortified mayonnaise' midway between an egg-less plant-based dressing, Follow Your Heart Vegenaise, and a commercial Best Foods Real Mayonnaise. This indicates that this specific composition already fits into a range of commercial product offerings. The laboratory-made egg control (no algae) is higher than all other samples. After nine weeks refrigerated storage there is no coagulation and viscosities remain unchanged (**Table 5**). It is anticipated that emulsions of the improved algae biomass solids, with inclusions ranging from less than 3% to higher than 3%, can be combined with a range of natural edible oils and produced in industrial food processing equipment for an improved food-grade oil-in-water product suited for commercial use.

The effective amount of algal wholemeal inclusion for the oil-in-water emulsion in this example pertains to the protein content as one nutrient of interest. Since it is for protein fortification, any amount would serve. For demonstration purposes the choice of doubling the

protein content was made based on published works using protein hydrolysate as fortifying agent that doubled the protein (Parvathy *et al.*, 2020, which is hereby incorporated by reference in its entirety). Total protein in the 'algae-fortified egg mayonnaise' is calculated at 4% (dry weight protein on wet weight of the finished product) using the improved algae ingredient with 73% protein content with 3% wholemeal inclusion. There is a much lower 1.9% total protein calculated for the egg mayonnaise control. The algae protein constitutes 2.2% in the product and over half, 55%, of the total product protein. A second nutrient of interest would be the pigment antioxidants of lutein and beta-carotene. A composition that is a 3% w/w 'algae mayonnaise' comprising yellow wholemeal with 0.6% carotenoids contains about 0.02% carotenoids.

EXAMPLE 12 – IMPROVED PASTA COMPOSITIONS USING IMPROVED PURITY *CHLAMYDOMONAS REINHARDTII* WHOLEMEAL IN EXTRUDED PASTA

Compositions of pasta can be improved nutritionally using algal biomass for protein enrichment and also improved by removal of animal products. The latter has the benefit of reducing cholesterol such as from eggs. However, textures of pastas are critically affected by proteins and starch as functional ingredients. This example describes an extruded food product using for the first time a dried improved *C. reinhardtii* wholemeal that is very high in protein and substantially starchless. It further describes compositions of pasta that are egg-less with addition of the improved *C. reinhardtii* algae component to produce an improved pasta. Being egg-less results in elimination of cholesterol in the food product. The experiment is designed with the intent for the added algae component to replace the egg component in its protein value, recipe volume, and functional properties to produce an acceptable extruded pasta. The pasta is exemplified by Spaetzle, an extruded egg noodle found in European cuisines comprised of flour, eggs, salt, water. Dough in this example is prepared using all-purpose flour (Gold Medal), tap water, salt, and egg or egg replaced completely with improved algal wholemeal of *C. reinhardtii* of the present invention. Equal amounts of dough are pressed by hand through an Spaetzle extruder into clean cooking water for cooking at a rolling boil for 8 minutes in 5 cups of tap water in a 2-quart saucepan. The cooked pasta is drained and allowed to sit for 10 minutes prior to evaluation. Samples were packaged and stored as precooked, frozen, or dehydrated/ dried.

For the egg replacement recipe, whole egg mass of 50 g is replaced with 50 g of algal wholemeal plus water, with the algae amount calculated to replace the protein value of the egg assumed to be 6-7 g. Algal wholemeal (10 g, 60-70% protein content, substantially starchless)

is prepared by mixing with the recipe's total water volume (40+49.3 =89.3 g) in a glass beaker held in a water bath at 82°C to 88°C for 5 minutes to form a thick slurry. This is allowed to cool for some minutes then is mixed by hand with the flour and salt dry matter to form a sticky dough that adheres to the mixing utensil and does not drip out of the extruder bowl holes by gravity when allowed to sit before extrusion. This raw dough resembles the desired consistency of the control egg noodle dough. The recipe shown below exemplifies an algal wholemeal inclusion at 13% of the total dry matter (flour, algae, salt). According to the flour label, the flour contains 0.77 g carbohydrates per gram. The same grams of flour (66.7 g) are used in the egg-less dough as in the control dough with egg so there is no flour substitution by algae.

Table 6. Recipe for traditional Spaetzle pasta dough with egg (the control) and for an egg-less dough with added wholemeal of improved *C. reinhardtii* containing high protein and no starch.

Component in Raw Dough	Control Dough with Egg (g)	Egg-less Dough with Algae (g)	% of Total Mass in Control with Egg	% of Total Mass in Egg-less Dough with Algae
All-Purpose Flour	66.7	66.7	47.7%	39.7%
Improved Algae	0	10	0	6.0%
Water for egg replacement	0	40	0	23.8%
Egg, beaten	50	0	35.8%	0
Salt	2	2	1.4%	1.2%
Water	21	49.3	15%	29.3%
Total	139.7	168	100%	100%

The cooked pasta made from the algae-containing egg-less dough forms a firm (but not rubbery or chewy), full length noodle consistent with the egg control and of similar sheen (**FIG. 7**). It is surprisingly homogeneous in shape with a more uniform diameter of 5 mm rather than ranging from 4 to 6 mm for the control noodles. Foaming and cooking loss of matter (an indicator of cooking quality) are desirably low and appear similar compared to the egg control. Typically, egg is required as a binder to hold the flour and water dough together. Overall, the cooked algae pasta aroma and flavor at 6% algae per total mass of wet dough that is not sauced scores only slightly lower (at -1, slightly disliked) compared to the control which is ranked as neutral/indifferent (0) on a 7-point scale of -3 (strongly dislike) to 3 (strongly like) for the green version of the improved *C. reinhardtii* ingredient. This can be managed through using chartreuse, yellow or cream/white wholemeal with their milder overall flavor. Optionally it can also be managed by lower inclusion to taste without compromising the noodle firmness, appearance, and texture, such as about 2%-3% of 73%-80% protein substantially starchless

wholemeal. It may also be altered by some use of semolina with its more earthy aroma. This pasta is traditionally served with a sauce or gravy which benefits the overall flavor profile.

This is the first report of substantially starchless algae being used in a typical egg noodle composition with the egg ingredient removed. The same grams of flour (66.7 g) and thus same grams of starch (49.3 g) are used in the egg-less dough with algae as in the control dough with egg; there is no flour substitution by algae in this example and egg contains only a trace amount of carbohydrate. Most of the carbohydrate in flour is starch (96%), and carbohydrates comprise about 77% of the all-purpose flour mass, making it a high-glycemic food ingredient. Calculated on a mass basis using the mass totals in the recipes above, the percent of total mass that is starch in the control dough is 35%; that in the egg-less algae dough is 29%. See **Table** 7. Beneficially, there is no algal carbohydrate (starch/sugar) component contributed by use of the improved algae of the present invention. Lack of algal starch removes its contribution to sugars and impact on food properties such as occurs with starch retrogradation.

Table 7. Amount of starch calculated to be in raw pasta doughs containing egg (the control) or egg-less and containing improved *Chlamydomonas reinhardtii* algal wholemeal. Percent of total mass that is starch is calculated based on the mass totals shown in **Table 6**.

Component in Raw Dough	Starch in Control Dough with Egg (g)	Starch in Egg-less Dough with Algae (g)	% of Total Mass that is Starch in Control	% of Total Mass that is Starch in Algae Dough
All-Purpose Flour	49.3	49.3	35%	29%
Improved Algae	0	0	0	0
Egg	0	0	0	0

The percent dry matter in the recipes is about 49% in the control egg dough and about 47% in the egg-less algae dough. This is due to the extra water added into the algae dough to obtain the desired dough consistency for extrusion; a one-for-one replacement of the lost egg mass (50 g) with water (40 g) and algae (10 g) mass is insufficient to produce a good dough. Unexpectedly, the algae test dough forms a desirable cooked noodle consistent with the cooked egg control despite the algae dough's lower starch content and lower percent dry matter. Lower carbohydrate diets are healthful along with healthy sources of protein and fats in the diet, thus the algae composition offers an improvement over the traditional control pasta. Starch plays a key role in gelatinization whereby fresh or dried pasta swells as it absorbs water during cooking, resulting in the preferred texture and form of the finished cooked product. Beneficially, by virtue of the improved substantially starchless algal wholemeal's apparent flour and water binding function in the absence of egg, the pasta's overall glycemic index can

be lowered compared to the egg control without compromising noodle structure. Unexpectedly, the lower starch algae noodle is more homogeneous in form. This can benefit the product quality control process and consumer appeal. Homogeneity using the improved *C. reinhardtii* ingredient may rely primarily on apparent strengthening of the gluten network by added algal proteins.

Calculated on a wet mass basis using the mass totals in the recipes of **Table 6**, the percent of total wet mass that is protein in the uncooked control dough is 9% and that in the egg-less algae dough is 8%; see **Table 8**. Using wholemeal comprised of 80% protein, the total protein in the egg-less dough is 14.67 g, or 8.7% of the total mass that is protein in the algae-containing dough. Since the mass totals include the extra water added to the algae pasta dough, the overall protein value goes down compared to the control. The calculations do not account for any mass losses that may occur during the cooking process. This is remedied by final processing into the dehydrated/dried noodle product (the common form for product sales), which label will reflect equivalent protein values as the egg-containing version. The food formulation was based on an algal inclusion amount that replaces the protein value of the egg.

Table 8. Amount of protein calculated to be in uncooked pasta doughs containing egg (the control) or egg-less and containing improved *Chlamydomonas reinhardtii* algal wholemeal. Percent of total wet mass that is protein is calculated based on the mass totals shown in **Table** 6. The algae noodle dough has higher water content and thus lowers the % protein per wet mass.

Component in Raw Dough	Protein in Control Dough with Egg (g)	Protein in Egg-less Dough with 60%-Protein Algae (g)	Protein in Egg-less Dough with 70%-Protein Algae (g)	% of Total Mass that is Protein in Control	% of Total Mass that is Protein in Algae Dough (60%-protein algae)	% of Total Mass that is Protein in Algae Dough (70%-protein algae)
All-Purpose Flour	6.67	6.67	6.67	5%	4%	4%
Improved Algae	0	6	7	0%	4%	4%
Egg	6	0	0	4%	0%	0%
Total	12.67	12.67	13.67	9%	8%	8%

Unexpectedly, the algae-containing dough forms a good noodle despite overall lower protein in the context of lower starch in the uncooked dough composition. Thus, it is discovered that the algal protein itself - a non-gluten protein- in the improved high protein *Chlamydomonas reinhardtii* ingredient is an important contributor to the cooked Spaetzle structural quality (diameter, firmness/strength, length, and homogeneity) and cooking behavior. Importantly the algal wholemeal also provides much higher (10-times more) protein nutritive value in the absence of egg in the pasta over using a common egg replacement for a vegan food composition with olive oil and aquafaba (replacement for 1 whole egg is only 0.6 g protein from aquafaba

vs 6 g protein for the improved algae). The selection of ingredients having proteins with appropriate water-holding capacity (WHC) is also important in food compositions. This applies to pasta and plant-based meat products (see Example 7) and to their performance such as under the force of extrusion and during rehydration. This preferred property of high WHC is demonstrated by the improved *Chlamydomonas reinhardtii* ingredient of the instant invention (see also Example 17). In addition, the brief period of heat-induced protein denaturation during the algal wholemeal pre-treatment step may further improve this essential functionality of the protein. Yet another improvement is better nutrition through improved protein digestibility. Beneficially, egg-less pasta can be produced as a food composition by using the improved algae of the instant invention with greater than 50% protein, substantially starchless and with a desirable protein digestibility of 94% that contributes higher protein digestibility value than cooked egg. The added improved *C. reinhardtii* wholemeal contributes antioxidants in the form of carotenoids and phenolic/flavonoid compounds naturally present, chlorophylls as optional natural color when using green algal biomass, nutritional lipids, and can also contribute essential micronutrients that address deficiencies when used in appropriate amounts. Additionally, pasta as a popular food has appeal to deliver health and functional benefits unique to *C. reinhardtii* as described in the Background and elsewhere. Taken together, the novel algae component can replace egg in an extruded pasta composition and produce a superior pasta.

In a second example for improved food compositions, the inclusion rate using improved *Chlamydomonas reinhardtii* wholemeal can be as little as about 0.1% by dry weight or can be 1% or 2% dry weight basis of the composition as an effective amount to deliver algal pigment for functionality. This can be to confer a color or taste (as discussed elsewhere in the instant invention) to a pasta or other food or feed, or for a practical function to include "algae" among ingredients listed on a food label for marketing purposes or to convey a marketing message of appearing "healthy". This lower amount (0.1%) of dried green higher protein substantially starchless algal wholemeal confers a pale green hue to a dehydrated pasta noodle.

EXAMPLE 13 – GENERATING COLOR OPTIONS USING *CHLAMYDOMONAS REINHARDTII* IN FOOD AND BEVERAGES

Color options for natural ingredients have a place in a variety of applications for food and beverages. Colors include desirable neutral hues such as cream, yellows and whites. Enabling algae-based protein fortification with various color options also ties in with concepts for food and beverage concepts and varies geographically. Yellow hues for example have a positive association with turmeric as tonic and "health food" ingredient. Green hues for

example lead to positive associations with green tea or spinach as "health food" ingredients. In beverages including fruit juices, vegetable juices, and smoothies, green hues are linked with kale, avocado, kiwi fruit as desirable healthy ingredients. **FIG. 2** shows some examples of applications representing a wide variety of colors of the improved *Chlamydomonas reinhardtii* of the present invention. In some instances, color variants occur as sports in a propagated line of the improved high protein substantially starchless phenotype. In some instances, color variants are generated in a parent line and a progeny line by mutagenesis. An olive-green liquid culture of KAS2010 from Example 1 and KAS2011 (derived from KAS2004) are treated with ultraviolet C (UVC) light for 18 seconds and then plated to isolate two yellow colonies for KAS2010 and one yellow and one white/cream color variant among thousands of green colonies from KAS2011. A single cell isolate from the latter further yields a chartreuse (light green) line. In some instances, heat treatment of the ingredient, intermediates, or the finished product itself can alter the original color of an ingredient or a composition. Heating can occur during pasteurization or HTST (flash pasteurization) prior to **FIG. 1, embodiment 70** such as during step **FIG. 1, embodiment 60**; or during pre-treatment, formulation, texturing or extrusion as part of **FIG. 1, embodiments 80** or **90**. By way of illustration, biomass of the chartreuse (light green) version of *C. reinhardtii* KAS2011 was heated for 5 minutes at 80°C at 10 g/L or higher to produce a cream color as shown in **FIG. 5.** Such treated material can also be frozen and used as a wet paste in formulations for food or beverage compositions. For beverages, microalgal powder from *Spirulina* as an example is typically used in quite small amounts less than 0.5% weight in smoothies or juice drinks. With the color options of the improved algae of the instant invention, inclusion rates can be at or higher than 0.5% weight without 'muddying' or dominating the desired colors from other natural ingredients like berry red or banana cream. And with the high protein content of the improved algae, even 0.5% inclusion provides higher protein than other microalgae options.

EXAMPLE 14 – PACKAGED BIOMASS OF IMPROVED HIGHER PURITY *CHLAMYDOMONAS REINHARDTII* FOR FOOD SUPPLEMENTATION

Capsules and tablets are commercially acceptable forms for personal administration of nutritional supplements. The algae biomass dosage contained therein depends on the targeted outcome, be it supplementation for protein, antioxidants, or other features or combination of features that are desired. Capsules and tablets can contain over 90% *Chlamydomonas reinhardtii* and optionally include an excipient to facilitate tablet compaction or an antioxidant or de-humectant for shelf stability. Particle size of the C. *reinhardtii* material can be adjusted

to enable material flowability, efficient compaction, and end-product uniformity, homogeneity, and other quality features. This concerns downstream processing and handling of the biomass, including heat treatment, pasteurization, drying methods and any added comminution or pulverization of powders, flakes, granules, or aggregates, and/or sieving if needed. Tablets can be formulated, produced and evaluated for example following **Osorio-Fierros *et al.*, (2017)**, which is hereby incorporated by reference in its entirety, including for acceptable measures of post-processing handling, packaging and end-product storage. Compositions for capsules contain the ingredients of at least the algae material and the vegetable cellulose capsule. Additional compositions for tablets or capsules can include an inert bulking agent such as microcrystalline cellulose; an antioxidant such as tocopherols (Vitamin E) or sodium ascorbate (Vitamin C); anti-caking, flow, or binder agent such as vegetable stearate; preservative as known in the art for shelf stability; blending agent (emulsifier) such as soy lecithin for good mixing or inhibiting separation of any added sweeteners or flavoring agents and the like. One non-limiting example is a 250 mg tablet comprised of 96.3% microalgae and 3.7% excipient. Unlike using other algae, this food supplement composition has no algal starch and has very high protein digestibility. Another non-limiting example includes 0.5 g dry weight compacted high protein substantially starchless *Chlamydomonas reinhardtii* biomass combined with one food-grade #0 gelcap for an edible composition. Water activity is at or below that required for microbial stability and quality assurance. This example also includes the high protein low/no starch *Chlamydomonas reinhardtii* combined with other natural botanical materials in the form of tablets or capsules to produce a composition of the microalgae with at least one other powdered or granulated botanical material. The proportion of algae to other botanical can be 30%, 40%, 50%, 60% 70%, 80% 90% or about 99% algae to the corresponding proportion of botanical to reach 100%, with optional other excipient or shelf life extender included as well, as in known in the art. The other botanicals can include mango, fig, date, pure stevia powder, glucose sweetener, or other sweeteners or flavor modifiers as known in the art and listed elsewhere herein. These are useful for achieving mechanical properties for improved compaction or for consumer appeal, modified flavor or flavor masking, sweetening, expanded nutritional or fiber supplementation and the like.

EXAMPLE 15 – COMPOSITIONS USING AN IMPROVED HIGHER PURITY *CHLAMYDOMONAS REINHARDTII* SUBSTANCE IN SAVORY BROTHS AND SOUP STOCKS

The improved *Chlamydomonas reinhardtii* ingredient that is high protein and substantially starchless lends itself to use in broths and soup stocks for flavoring, accentuating the umami flavor, and for protein enrichment. The novel ingredient provides the presence of high glutamic acid- a natural compound for savory taste as a component of the higher protein content- along with no algal starch that would otherwise add texture changes, thickening, or cloudiness to the broth if using other *C. reinhardtii* algae with starch and lower protein content. The effective dosage of microalgae will be what gives the desired flavor and protein enrichment for a broth or soup stock composition. Also, there is a strong positive association of "algae" with Asian cuisine. Dashi broth is one example since it uses kelp seaweed in its formulation. For protein-fortified basic Dashi broth, 1 TBSP of yellow or orange/brown improved *C. reinhardtii* is added to 4 US cups (1 quart) of water- that has been pre-boiled with 1 strip 2- x 4-inch piece of kombu dried kelp and then removed- along with 3 TBSP dried bonito flakes (the microalgae algae can also substitute for 1 TBSP of bonito). The water is brought to a boil and then strained. For a darker broth that is better suited to use the green microalgae, to 4 US cups water use 1 strip kombu, 1 cup dried shiitake mushrooms, 1 TBSP improved *C. reinhardtii*, ¼ cup mirin, 1/8 cup shoyu, 1 tsp grated ginger root, and optional niboshi (up to ½ cup, omit if vegan). After pre-boiling the kombu and removing it, add the mushrooms to simmer for 30 minutes, adding the improved *C. reinhardtii* in the final 5 minutes. Then add the remaining ingredients and strain before serving. This will provide about 2 grams of protein from the microalgae and 3 grams protein from the bonito flakes, increasing the protein value by over 50%. It can serve as a base for miso soup or as a stand-alone broth for rice, noodles and the like. The improved *C. reinhardtii* in flaked form lends itself to sprinkling on the same.

EXAMPLE 16 – MISCELLANEOUS COMPOSITIONS CONTAINING HIGHER PURITY *CHLAMYDOMONAS REINHARDTII* IN PET AND HUMAN FOODS AND BEVERAGES

Innovative ingredients make snacks healthier. This includes muesli bars, protein bars, or savory snacks such as jerky or crisps. The wholemeal algae ingredient of the instant invention can offer an alternative to using allergenic wheat and soy ingredients. It is anticipated to be well-suited to high-moisture extrusion cooking as is currently done with pea and other pulse proteins as part of the healthy foods trend. Having flexibility in food components is also valued for nutritional reasons. For example, pea protein contains all nine essential amino acids but lacks sufficient methionine plus cysteine to be considered a complete protein as is typical of pulses. Substituting that plant-based protein powder with substantially starchless *Chlamydomonas* powder containing 70% to 80% protein (see Example 2) would provide a food component containing all nine essential amino acids but with sufficient methionine plus

cystine. Thus, an effective amount of the *C. reinhardtii* algae is that which replaces and improves the nutrients of interest in the snack that derived from allergenic pulses, in this case amino acids. As one example, algae containing compositions using the improved higher purity *Chlamydomonas* ingredient can be produced that combine the algae with additional ingredients for a vegetarian/vegan savory dried meat-like snack product such as beef jerky that is convenient and proteinaceous. It is generally contemplated that dry ingredients including the algae are weighed and combined in accordance with the desired formulation. Alternatively, a food-grade algal paste may be used. In one iteration, the green version of 72% protein substantially starchless *C. reinhardtii* is employed to mimic the dark color of dried meat-based jerky and is combined with smoke flavor, preferably natural spices such as smoked paprika, chili powder, cayenne pepper and salt in a dry powder pre-mix. A method of high moisture extrusion cooking is used for texturizing the algal protein into a product with fibrous texture resembling animal meat. The algal wholemeal may be combined with other sources of vegetable proteins, such as for cost purposes and/or label purposes and/or specific nutrition targets such as to provide all nine essential amino acids plus sufficient levels of methionine and cysteine. For a 100 g protein powder mixture to have 2% methionine as percentage of total amino acids, 8 g pea protein can be combined with 92 g of wholemeal algae. The product simulates meat in appearance, flavor, and color, has a chewy or leathery texture also expected of meat, and has an extended shelf-life by virtue of control of free water content and activity of the product.

Recreational beverages containing algae can appeal to consumers due to the nutritional composition of algae combined with appealing colors. Cocktail compositions using the 72% protein substantially starchless *C. reinhardtii* is one such beverage category. For example, green for St. Patrick's Day or mint flavored drinks (Mojito) or yellow for lemon flavored drinks (Lemon drop martini). The effective amount of the *C. reinhardtii* wholemeal inclusion is determined by the desired color and the desired flavor. The white improved *C. reinhardtii* can go into a White Russian-type drink using the algal cream of Example 10. The algae cream enables a vegan product over using dairy-based cream.

EXAMPLE 17 – WATER ABSORPTION CAPACITY OF IMPROVED *CHLAMYDOMONAS REINHARDTII* SUBSTANCE

The ability of an algal ingredient and a food composition to absorb water benefits numerous properties, such as, for example, improved rehydration of dried food preparations; improved sensory properties, such as juiciness, texture (firmness, appearance and mouthfeel,

see previous examples), and increased satiety; improved product shelf life by improving water retention, such as in baked or cooked goods; and improving health and nutrition by helping control nutritional value of food preparations that undergo thermal processing. Therefore, it is of value to assess the ability of the improved high protein substantially starchless *Chlamydomonas* wholemeal to hold water. Water absorption ratios are determined for different lyophilized samples produced under the same fermentation conditions. One is wholemeal with 72.1% protein and is substantially starchless. The other is the unimproved wholemeal with 39.9% protein and 31.2% starch content. Samples (1 g) are mixed at ambient temperature with measured volumes of ambient temperature tap water in pre-weighed glass beakers or tubes and allowed to sit for 60 minutes. Any clear supernatant is removed, and the pastes are weighed. Water absorption ratios are expressed as grams water to gram algal biomass or as a percentage. Results for the about 72.1% protein substantially starchless biomass is 7.5 g/g (750%), and for the wildtype unimproved biomass with 39.9% protein is 4.4 g/g (400%). Thus, the improved higher protein substantially starchless algal material has almost double, 1.7-times, the water absorption capacity of wildtype *Chlamydomonas* material with lower protein and containing starch. With this, inclusion of the improved high protein substantially starchless algal wholemeal in the subject compositions can improve water retention and the myriad of benefits associated therewith, as listed above. When compared on the same caloric value or on the same mass value, inclusion of the algae wholemeal with about 72% protein and substantially starchless in a food composition will aide rehydration due to higher water absorption if compared to the wildtype with lower protein algae of about 40% and containing starch. Weighed dehydrated noodles (dehydrated at 110-115°F for several hours), 15 count, each approximately 3 inches long before dehydration, prepared per Example 12 and Table 6 on mass basis using either test wholemeal of *C. reinhardtii* that is 72.1% protein and substantially starchless or control wholemeal from wildtype *C. reinhardtii* with 39.9% protein and 31.2% starch are placed into gently boiling water for 5 min. Noodles are then removed from the pot by a skimmer spider strain, briefly submerged in cold rinse water, then patted dry using paper towel before re-weighing. The rehydration ratio is calculated by dividing the combined final weight by the combined original weight. Results indicate improved rehydration for the test noodles by at least 7% based on that time period.

EXEMPLARY EMBODIMENTS

Embodiments of the subject invention include, but are not limited to, the following exemplified embodiments:

Embodiment 1. A composition comprising a *Chlamydomonas reinhardtii* biomass and at least one additional edible component,

wherein the *C. reinhardtii* biomass is present in a concentration of about 0.1% to about 99% by weight,

wherein the *C. reinhardtii* biomass has a protein content exceeding about 50% by weight and a starch content less than about 2.25% by weight, and

wherein the *C. reinhardtii* biomass is produced in a heterotrophic, mixotrophic, or autotrophic production process, or any combination of such production processes.

Embodiment 2. The composition of Embodiment 1, wherein the composition is a food, food supplement, or beverage.

Embodiment 3. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass is produced heterotrophically.

Embodiment 4. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass is wholemeal.

Embodiment 5. The composition of Embodiment 1, wherein the heterotrophic or mixotrophic production process comprises providing a carbon nutrient to the *C. reinhardtii*,

wherein the carbon nutrient is acetic acid or an acetate, and

wherein the acetic acid or acetate is supplied to the *C. reinhardtii* during heterotrophic or mixotrophic cultivation.

Embodiment 6. The composition of Embodiment 5, wherein the heterotrophic production process is followed by the mixotrophic production process and the acetic acid or acetate is supplied to the *C. reinhardtii* during heterotrophic or mixotrophic cultivation.

Embodiment 7. The composition of Embodiment 5, wherein the carbon nutrient is derived from a recycled carbon from biological emissions, non-biological carbon from a fermentation systems emissions, industrial sources, or a mixture of biologically- and non-biologically-sourced CO_2; wherein the carbon nutrient is supplied to the *C. reinhardtii* in the form of acetic acid, acetate, or a combination thereof.

Embodiment 8. The composition of Embodiment 7, wherein the recycled carbon is methane, methanol, syngas, carboxylic acid, carbon dioxide, carbon monoxide, ethylene, ethane, ethanol, or acetaldehyde, or any combination thereof.

Embodiment 9. The composition of Embodiment 1, wherein the *C. reinhardtii* comprises a mutation in a starch biosynthetic pathway.

Embodiment 10. The composition of Embodiment 1, wherein the protein productivity of the *C. reinhardtii* is about 305 mg/L-hour.

Embodiment 11. The composition of Embodiment 1, wherein the composition is a mixture, oil-in-water emulsion, batter, dough, pancake, pasta, pasta filling, mayonnaise, dressing, meat-substitute meatball, textured meat, pet food, pet food supplement, pet beverage, pet treat, cat kibble, soup, broth, algae milk, algae cream, muesli bar, protein bar, jerky, crisp, smoothie, sports drink, or cocktail.

Embodiment 12. The composition of Embodiment 11, wherein the pet food, pet food supplement, or pet beverage is for a cat or a dog.

Embodiment 13. The composition of Embodiment 1, wherein, the composition is frozen, dehydrated, or dried.

Embodiment 14. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass further comprises glutamic acid at a concentration of at least about 11% of the total amino acids of the *C. reinhardtii* biomass or glycine at a concentration of at least 5% of the total amino acids of the *C. reinhardtii* biomass.

Embodiment 15. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass is treated by micronization, heat, and/or enzymatic digestion followed by a step of inactivation or hydration in a liquid prior to use in the composition.

Embodiment 16. The composition of Embodiment 15, wherein the liquid is water, milk, egg, vanillin or any combination thereof.

Embodiment 17. The composition of Embodiment 1, further comprising plant-based or cell culture-based protein.

Embodiment 18. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass is present in a concentration of about 0.1% to about 4% by weight of pet food or pet treat.

Embodiment 19. The composition of Embodiment 1, further comprising a carotenoid.

Embodiment 20. The composition of Embodiment 1, further comprising a meat.

Embodiment 21. The composition of Embodiment 20, wherein the meat is a livestock, poultry, fish, shellfish, crustacean, cell-cultured meat, or cell-cultured seafood.

Embodiment 22. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass is green, yellow, chartreuse, gray, cream, whitish, pink, red, or brown in color.

Embodiment 23. The composition of Embodiment 1, wherein the composition is packaged in a capsule or a tablet.

Embodiment 24. The composition of Embodiment 1, further comprising at least one excipient; food-grade vegetable cellulose gel cap; inert bulking agent; antioxidant; anti-caking,

flow, or binder agent; preservative; emulsifier; sweetener, flavoring agent, or flavor masker; or botanical material.

Embodiment 25. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass has a protein content exceeding about 60% by weight and a starch content less than about 2.25% by weight.

Embodiment 26. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass has a protein content exceeding about 70% by weight and a starch content less than about 2.25% by weight.

Embodiment 27. The composition of Embodiment 1, wherein the *C. reinhardtii* biomass has a protein content exceeding about 80% by weight and a starch content less than about 2.25% by weight.

Embodiment 28. A method of growing a *C. reinhardtii* cell, the method comprising:

i) mating a first *C. reinhardtii* strain that has a starch content of at least about 2.25% by weight and a second *C. reinhardtii* strain that contains a mutation or epigenetic modification inhibiting the starch biosynthetic pathway;

ii) desiccating the mated first *C. reinhardtii* strain and second *C. reinhardtii* strain;

iii) removing non-zygotic cells resulting from the mated first *C. reinhardtii* strain and second *C. reinhardtii* strain; and

iv) optionally, germinating a zygote resulting from the mated first *C. reinhardtii* strain and second *C. reinhardtii* strain.

Embodiment 29. The method of Embodiment 28, further comprising:

v) cultivating the zygote in a cultivation medium, wherein a biomass resulting from the cultivation has a protein content of at least 50% by weight and a starch content of less than 2.25% by weight.

Embodiment 30. The method of Embodiment 29, further comprising adding vanillin to the cultivation medium.

Embodiment 31. The method of Embodiment 30, wherein the vanillin is at a concentration of about 1 mg/L to about 500 mg/L.

Embodiment 32. The method of Embodiment 28, wherein the mutation or epigenetic modification in the starch biosynthetic pathway is a mutation of *sta6*, *sta1*, *sta7*, *sta11*, or any combination thereof or epigenetic modification of expression of *sta6*, *sta1*, *sta7*, *sta11*, or any combination thereof.

Embodiment 33. The composition of Embodiment 26, wherein the *Chlamydomonas* biomass further has a fat content of about 12-15% when produced under nutrient replete heterotrophic conditions.

Embodiment 34. The composition of Embodiment 26, wherein the *C. reinhardtii* biomass further comprises starch subceeding 0.09% by weight.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

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CLAIMS

We claim:

1. A composition comprising a *Chlamydomonas reinhardtii* biomass and at least one additional edible component,

wherein the *C. reinhardtii* biomass is present in a concentration of about 0.1% to about 99% by weight,

wherein the *C. reinhardtii* biomass has a protein content exceeding about 50% by weight and a starch content less than about 2.25% by weight, and

wherein the *C. reinhardtii* biomass is produced in a heterotrophic, mixotrophic, or autotrophic production process, or any combination of such production processes.

2. The composition of claim 1, wherein the composition is a food, food supplement, or beverage.

3. The composition of claim 1, wherein the *C. reinhardtii* biomass is produced heterotrophically.

4. The composition of claim 1, wherein the *C. reinhardtii* biomass is wholemeal.

5. The composition of claim 1, wherein the heterotrophic or mixotrophic production process comprises providing a carbon nutrient to the *C. reinhardtii*,

wherein the carbon nutrient is acetic acid or an acetate, and

wherein the acetic acid or acetate is supplied to the *C. reinhardtii* during heterotrophic or mixotrophic cultivation.

6. The composition of claim 5, wherein the heterotrophic production process is followed by the mixotrophic production process and the acetic acid or acetate is supplied to the *C. reinhardtii* during heterotrophic or mixotrophic cultivation.

7. The composition of claim 5, wherein the carbon nutrient is derived from a recycled carbon from biological emissions, non-biological carbon from a fermentation systems emissions, industrial sources, or a mixture of biologically- and non-biologically-sourced CO_2;

wherein the carbon nutrient is supplied to the *C. reinhardtii* in the form of acetic acid, acetate, or a combination thereof.

8. The composition of claim 7, wherein the recycled carbon is methane, methanol, syngas, carboxylic acid, carbon dioxide, carbon monoxide, ethylene, ethane, ethanol, or acetaldehyde, or any combination thereof.

9. The composition of claim 1, wherein the *C. reinhardtii* comprises a mutation in a starch biosynthetic pathway.

10. The composition of claim 1, wherein the protein productivity of the *C. reinhardtii* is about 305 mg/L-hour.

11. The composition of claim 1, wherein the composition is a mixture, oil-in-water emulsion, batter, dough, pancake, pasta, pasta filling, mayonnaise, dressing, meat-substitute meatball, textured meat, pet food, pet food supplement, pet beverage, pet treat, cat kibble, soup, broth, algae milk, algae cream, muesli bar, protein bar, jerky, crisp, smoothie, sports drink, or cocktail.

12. The composition of claim 11, wherein the pet food, pet food supplement, or pet beverage is for a cat or a dog.

13. The composition of claim 1, wherein, the composition is frozen, dehydrated, or dried.

14. The composition of claim 1, wherein the *C. reinhardtii* biomass further comprises glutamic acid at a concentration of at least about 11% of the total amino acids of the *C. reinhardtii* biomass or glycine at a concentration of at least 5% of the total amino acids of the *C. reinhardtii* biomass.

15. The composition of claim 1, wherein the *C. reinhardtii* biomass is treated by micronization, heat, and/or enzymatic digestion followed by a step of inactivation or hydration in a liquid prior to use in the composition.

16. The composition of claim 15, wherein the liquid is water, milk, egg, vanillin or any combination thereof.

17. The composition of claim 1, further comprising plant-based or cell culture-based protein.

18. The composition of claim 1, wherein the *C. reinhardtii* biomass is present in a concentration of about 0.1% to about 4% by weight of pet food or pet treat.

19. The composition of claim 1, further comprising a carotenoid.

20. The composition of claim 1, further comprising a meat.

21. The composition of claim 20, wherein the meat is a livestock, poultry, fish, shellfish, crustacean, cell-cultured meat, or cell-cultured seafood.

22. The composition of claim 1, wherein the *C. reinhardtii* biomass is green, yellow, chartreuse, gray, cream, whitish, pink, red, or brown in color.

23. The composition of claim 1, wherein the composition is packaged in a capsule or a tablet.

24. The composition of claim 1, further comprising at least one excipient; food-grade vegetable cellulose gel cap; inert bulking agent; antioxidant; anti-caking, flow, or binder agent; preservative; emulsifier; sweetener, flavoring agent, or flavor masker; or botanical material.

25. The composition of claim 1, wherein the *C. reinhardtii* biomass has a protein content exceeding about 60% by weight and a starch content less than about 2.25% by weight.

26. The composition of claim 1, wherein the *C. reinhardtii* biomass has a protein content exceeding about 70% by weight and a starch content less than about 2.25% by weight.

27. The composition of claim 1, wherein the *C. reinhardtii* biomass has a protein content exceeding about 80% by weight and a starch content less than about 2.25% by weight.

28. A method of growing a *C. reinhardtii* cell, the method comprising:

i) mating a first *C. reinhardtii* strain that has a starch content of at least about 2.25% by weight and a second *C. reinhardtii* strain that contains a mutation or epigenetic modification inhibiting the starch biosynthetic pathway;

ii) desiccating the mated first *C. reinhardtii* strain and second *C. reinhardtii* strain;

iii) removing non-zygotic cells resulting from the mated first *C. reinhardtii* strain and second *C. reinhardtii* strain; and

iv) optionally, germinating a zygote resulting from the mated first *C. reinhardtii* strain and second *C. reinhardtii* strain.

29. The method of claim 28, further comprising:

v) cultivating the zygote in a cultivation medium, wherein a biomass resulting from the cultivation has a protein content of at least 50% by weight and a starch content of less than 2.25% by weight.

30. The method of claim 29, further comprising adding vanillin to the cultivation medium.

31. The method of claim 30, wherein the vanillin is at a concentration of about 1 mg/L to about 500 mg/L.

32. The method of claim 28, wherein the mutation or epigenetic modification in the starch biosynthetic pathway is a mutation of *sta6*, *sta1*, *sta7*, *sta11*, or any combination thereof or epigenetic modification of expression of *sta6, sta1*, *sta7*, *sta11*, or any combination thereof.

33. The composition of claim 26, wherein the *Chlamydomonas* biomass further has a fat content of about 12-15% when produced under nutrient replete heterotrophic conditions.

34. The composition of claim 26, wherein the *C. reinhardtii* biomass further comprises starch subceeding 0.09% by weight.

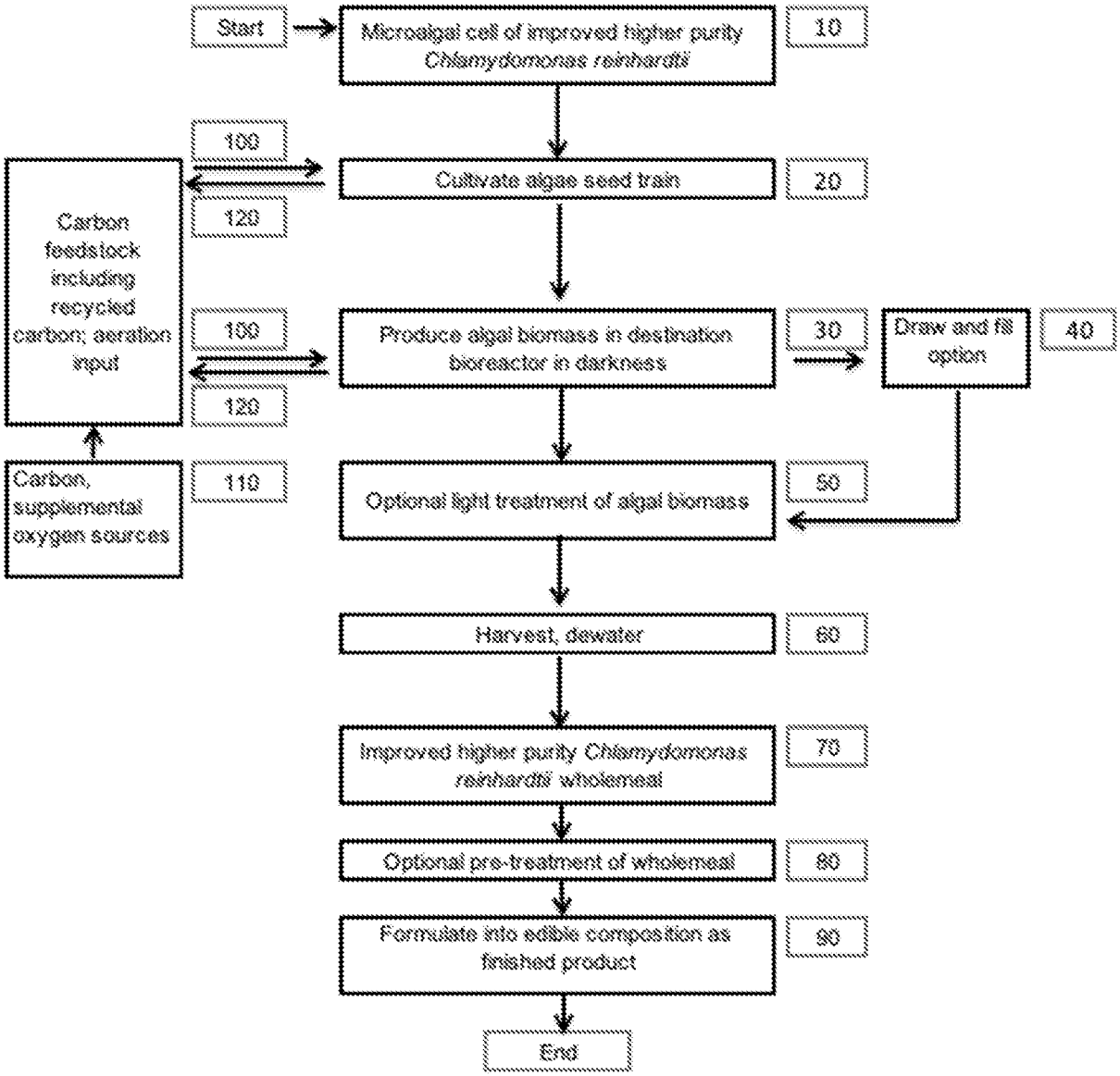

Start
Microalgal cell of improved higher purity *Chlamydomonas reinhardtii*
10
100
120
Cultivate algae seed train
20
Carbon feedstock including recycled carbon; aeration input
100
120
Produce algal biomass in destination bioreactor in darkness
30
Draw and fill option
40
Carbon, supplemental oxygen sources
110
Optional light treatment of algal biomass
50
Harvest, dewater
60
Improved higher purity *Chlamydomonas reinhardtii* wholemeal
70
Optional pre-treatment of wholemeal
80
Formulate into edible composition as finished product
90
End

FIG. 1

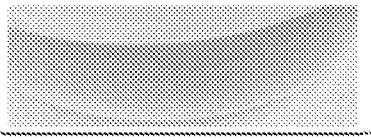
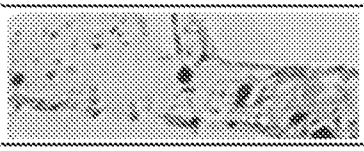
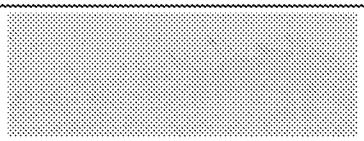
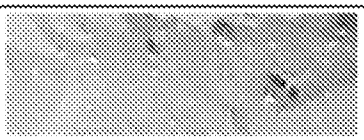
Images of Cultures or Product	Colors & Hues	Applications
A 	Emerald and chartreuse greens	Vegan patties, pancakes, tortilla, soup stocks, salad toppers, juices, smoothies, cocktails
B 	Other greens, including forest and olive	Beef (patties, meatballs, scramble), pasta, soup stock, novelties and special occasions/holidays
C 	Yellows or heat-treated chartreuse	Pasta, tortilla, bakery, beverages, eggs/scramble, mayonnaise, sauces
D 	Creams, white	Same as yellow applications, sustainable algae flour, color-neutral protein fortification
E 	Orange/brown or reddish	Plant-based seafoods and meats; cultured seafood; sausages

FIG. 2

FIG. 3

FIG. 4

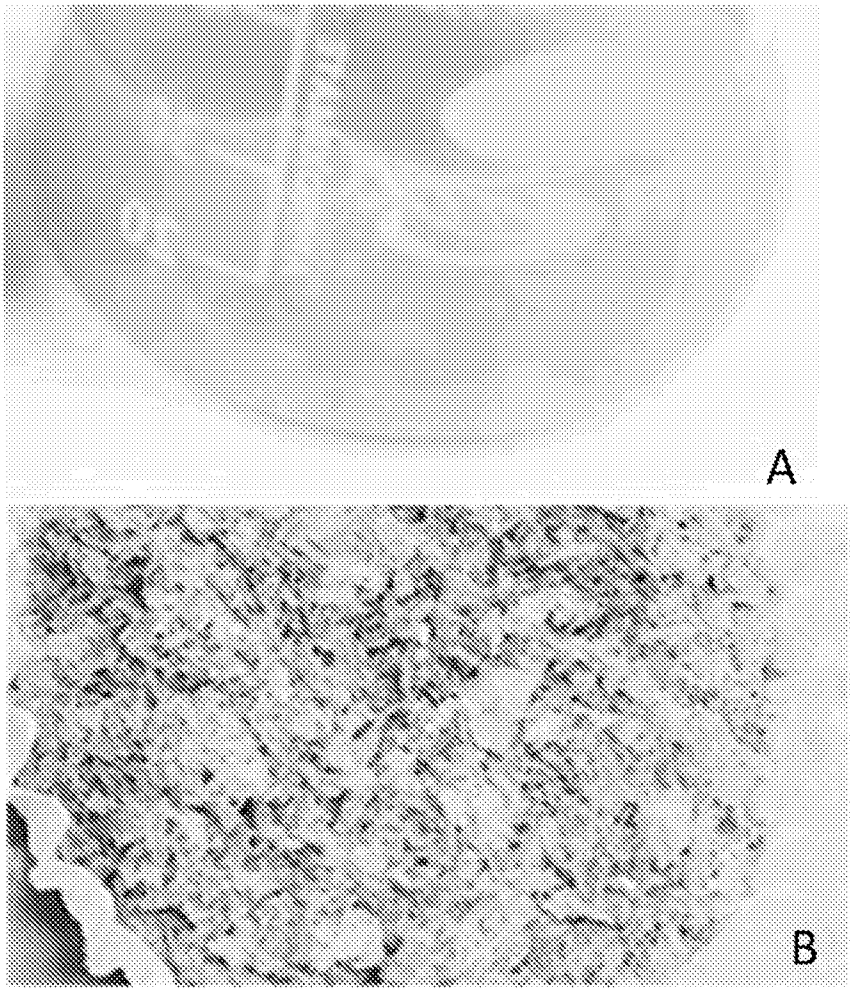


FIG. 5

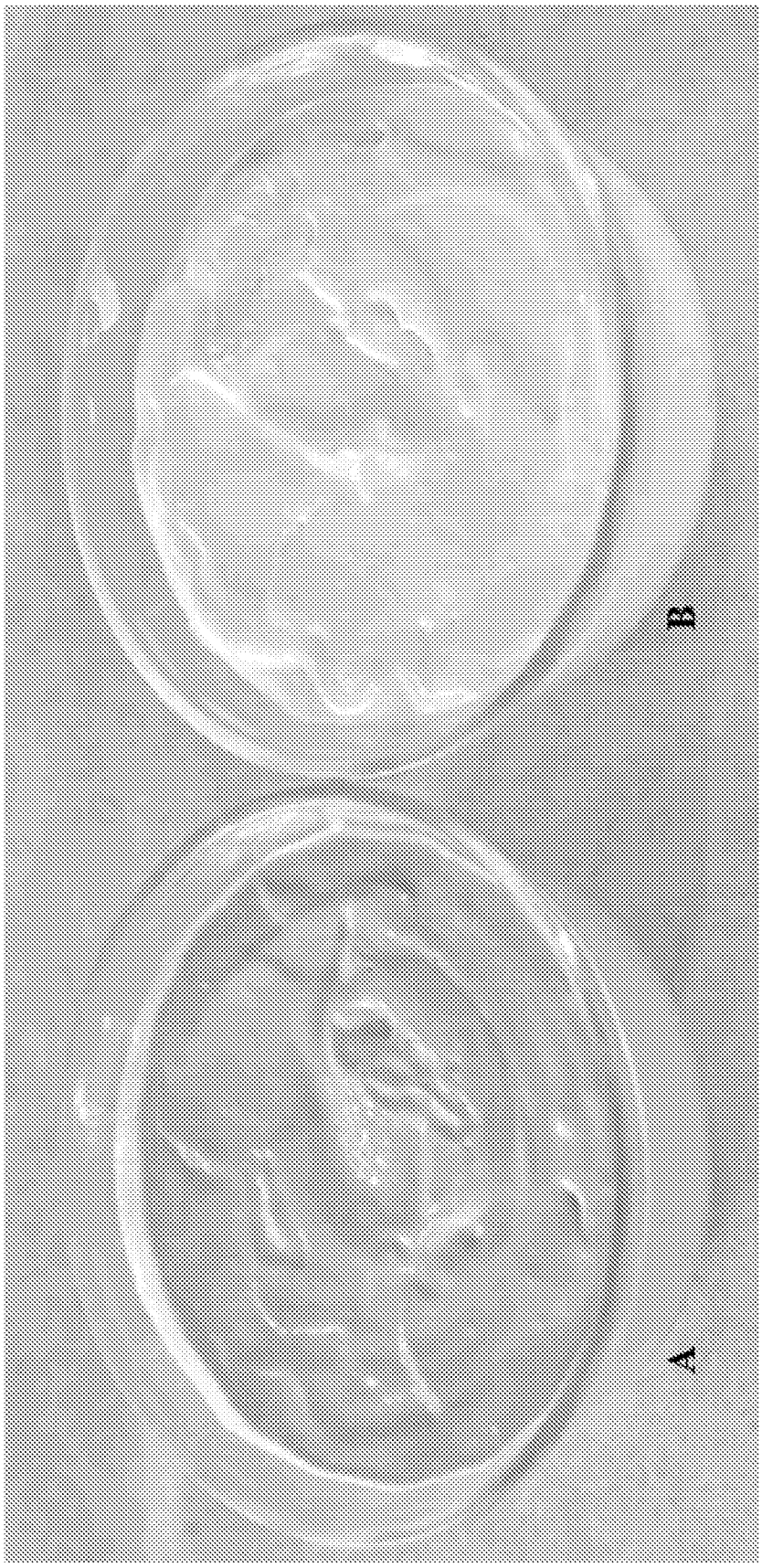


FIG. 6

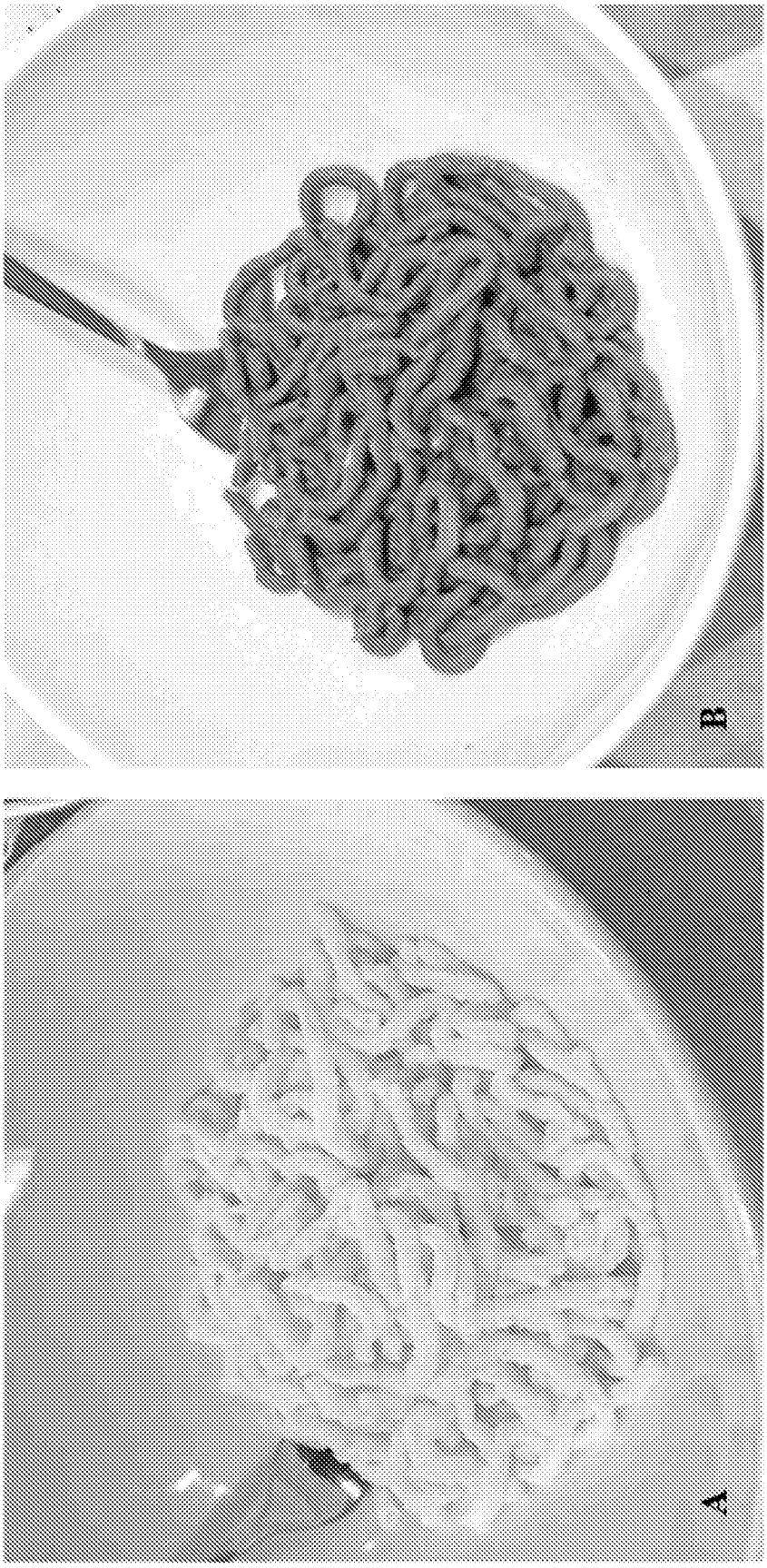


FIG. 7