

REDUCTION OF ENDOTOXINS IN BACTERIAL PROTEIN PREPARATIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[01] This application claims the benefit of U.S. Provisional Application No. 63/216,744, filed on June 30, 2021, which is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

[02] The present invention relates to microbial protein compositions from which endotoxins have been reduced or removed and that are suitable for incorporation into food products for human and animal consumption.

BACKGROUND

[03] Lipopolysaccharide (LPS) is a polysaccharide complex that is a structural component associated with the outer membrane of a Gram-negative bacterial cell. It consists of long chains of sugar moieties that are covalently bound to lipids. These chains form a network that protects bacteria, forming a gelatinous layer that is attached to the bacterial surface. LPS has amphiphilic characteristics due to its hydrophilic polysaccharide and hydrophobic lipid moieties. Its fundamental structure includes three parts: (i) lipid A, (ii) core sugar, (iii) and O antigen (O-polysaccharide). Lipid A is composed of 4 to 7 fatty acid chains bound to two glucosamines, and a core sugar part that is composed of 8 carbon sugar, keto-deoxyoctonate (KDO), which is highly conserved among bacterial species. The core region is an oligosaccharide containing characteristic sugar residues, KDO and heptose, and its chemical variation is more limited than that of O-antigen. Lipid A acts as a membrane anchor and in gram-negative bacteria, LPS is anchored to the outer membrane via lipid A. The "canonical" LPS structure is represented by *Escherichia coli* LPS. It consists of a 1,4'-biphosphorylated glucosamine disaccharide bearing six fatty acids, which are unbranched chains 12 or 14 carbon units long. Other lipid A species, however, show variability in the number, length, and composition of attached fatty acids, as well as variability in the level of phosphorylation and number and types of substituted groups found attached to the phosphate residues [Erridge, C., Bennett-Guerrero, E., & Poxton, I. R. (2002). Structure and function of lipopolysaccharides. *Microbes and Infection*, 4(8), 837–851. [https://doi.org/10.1016/S1286-4579\(02\)01604-0](https://doi.org/10.1016/S1286-4579(02)01604-0); Dixon, D. R., & Darveau, R. P. (2005). Lipopolysaccharide heterogeneity: Innate host responses to bacterial modification of lipid A structure. *Journal of Dental Research*, 84(7), 584–595. <https://doi.org/10.1177/154405910508400702>].

[04] One function of LPS is to keep the outer surface of bacterial cells moist and slightly negatively charged, and to shield from compounds that may damage the cell, while allowing nutrients to pass through. The physical barrier that separates the inside of a bacterial cell from the outside environment is its membrane, a double lipid layer interspersed with proteins, to which LPS is connected via lipid A, a phosphorylated lipid.

[05] An immunological response to LPS may be triggered by its binding to the receptors on immune cells and some epithelial cells, which causes activation of nuclear transcription factors by intracellular signals. It is believed that CD14 serves as a high-affinity receptor for LPS, forming a CD14–LPS complex after catalytic transfer of LPS monomers by LPS-binding protein (LBP). [Gioannini, T. L., & Weiss, J. P. (2007). Regulation of interactions of Gram-negative bacterial endotoxins with mammalian cells. *Immunologic Research*, 39(1–3), 249–260, <https://doi.org/10.1007/S12026-007-0069-0>, which is incorporated herein by reference in its entirety].

[06] It is believed that LPS first binds to LPS-binding protein in the blood, and that the complex is then transported to the CD14 receptor molecule on the cell surface of macrophages. There, LPS is transferred to the receptor complex containing TLR4 and MD2, triggering intracellular signaling events. [Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., & Miyake, K. (2002). Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nature Immunology* 2002 3:7, 3(7), 667–672. <https://doi.org/10.1038/ni809>, which is incorporated herein by reference in its entirety].

[07] If an animal is injected with LPS, this will often generate a fever, which makes LPS a pyrogenic substance. This immunogenic response to certain types of LPS is connected to LPS generally being considered an endotoxin. The endotoxic effect of LPS is believed to be mainly due to lipid A, while the polysaccharides are less endotoxic or non-toxic. While LPS contains large, variable polysaccharide and oligosaccharide regions, the relatively conserved lipid region (lipid A) is the endotoxic and biologically active moiety responsible for septic shock.

[08] Once LPS is in the bloodstream, it is removed by various means. Studies have found that most LPS measured in plasma a few minutes after intravenous administration is then transported to the liver for metabolic degradation; with smaller amounts of plasma LPS metabolized in the spleen, lungs, kidneys, and adrenal glands, and further excreted into the bile [Kleine, B., Freudenberg, M. A., & Galanos, C. (1985). Excretion of radioactivity in faeces and urine of rats injected with ³H,¹⁴C-lipopolysaccharide. *British Journal of Experimental Pathology*, 66(3), 303. [/pmc/articles/PMC2041064/?report=abstract](https://pubmed.ncbi.nlm.nih.gov/2041064/), which is incorporated herein by reference in its entirety].

[09] LPS is a ubiquitous substrate. For example, almost all foods contain 1 ng to 1 µg of LPS per gram of their weight. Moreover, humans constantly come into contact with huge amounts of bacteria in the oral and intestinal mucosa. The estimated number of human commensal bacteria range from 10³ to 10¹² per gram of tissue [Mitsuoka, T. (2000). Significance of Dietary Modulation of Intestinal Flora and Intestinal Environment. *Bioscience and Microflora*, 19(1), 15–25. <https://doi.org/10.12938/BIFIDUS1996.19.15>]. Thus, humans are constitutively exposed to LPS throughout their lives. Some reports indicate that exposure to LPS in this manner may be important for the maintenance of host immune balance (antiallergic predisposition) [von Mutius (2007) *Europe Proc Am Thorac Soc* 4:212-216; von Mutius (2007) *immunobiology* 212:433-439], and protection from bacterial infections in the intestine [Masuda, et al. (2011) *J Innate Immun* 3:315-326]. Some

epidemiological studies have shown that LPS is inversely correlated with risk of allergic disease (hygiene hypothesis) [Von Mutius, E. (2007). Asthma and allergies in rural areas of Europe. *Proceedings of the American Thoracic Society*, 4(3), 212–216.

<https://doi.org/10.1513/PATS.200701-028AW>]. The LPS found in the environment is abundant and structurally diverse. Although long-studied, because of the complexity of the molecule and of the biological responses to it, LPS is still at the frontier of biological research.

[10] Oral administration of LPS demonstrates completely different results when compared to parenteral administration. Some reports have indicated that oral administration of LPS may not be harmful to animals. [Oketani, K., Inoue, T., & Murakami, M. (2001). Effect of E3040, an inhibitor of 5-lipoxygenase and thromboxane synthase, on rat bowel damage induced by lipopolysaccharide. *European Journal of Pharmacology*, 427(2), 159–166. [https://doi.org/10.1016/S0014-2999\(01\)01234-1](https://doi.org/10.1016/S0014-2999(01)01234-1); Schryvers, A. B., Schollaardt, T., Woods, D. E., Williams, K., & Bryan, L. E. (1987). Efficacy of oral immunization with *Pseudomonas aeruginosa* lipopolysaccharide. *Serodiagnosis and Immunotherapy in Infectious Disease*, 1(5), 379–392. [https://doi.org/10.1016/0888-0786\(87\)90049-7](https://doi.org/10.1016/0888-0786(87)90049-7); Illyés, G., Kovács, K., Kocsis, B., & Baintner, K. (2008). Failure of oral *E. coli* O83 lipopolysaccharide to influence intestinal morphology and cell proliferation in rats: Short communication. *Acta Veterinaria Hungarica*, 56(1), 1–3. <https://doi.org/10.1556/AVET.56.2008.1.1>]. Although LPS has been considered an endotoxin in the field of intravenous pharmaceuticals, oral and dermal routes of LPS administration have been reported to not lead to adverse effects [Inagawa, H., Kohchi, C., & Soma, G.-I. (2011). Oral administration of lipopolysaccharides for the prevention of various diseases: benefit and usefulness. *Anticancer Research*, 31(7), 2431–2436].

[11] It has been reported that LPS can be inactivated by low pH such as occurs in the abomasum or monogastric stomachs and that it can bind to numerous substances, including proteins and divalent cations such as Ca^{2+} and Mg^{2+} , reducing its bioavailability [Khiaosa-ard, R., & Zebeli, Q. (2018). Diet-induced inflammation: From gut to metabolic organs and the consequences for the health and longevity of ruminants. *Research in Veterinary Science*, 120, 17–27. <https://doi.org/10.1016/J.RVSC.2018.08.005>]. An intact gut appears to allow only a tiny fraction of the LPS produced by gram-negative bacteria residing within the gut to pass the mucous barrier and enter deeper tissue and the blood stream, Any LPS that does leave the gut appears to be redirected to the liver, where it is degraded and rapidly cleared from the bloodstream [Guerville, M., & Boudry, G. (2016). Gastrointestinal and hepatic mechanisms limiting entry and dissemination of lipopolysaccharide into the systemic circulation. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, 311(1), G1–G15.

<https://doi.org/10.1152/AJPGI.00098.2016/ASSET/IMAGES/LARGE/ZH30111671300003.JPG>].

Both chylomicrons and bile salts are reported to reversibly inhibit LPS pyrogenic activity, suggesting that there are multiple protective mechanisms that diminish LPS toxicity.

[12] According to the European Pharmacopoeia [*European Directorate for the Quality of Medicines and Healthcare - European Directorate for the Quality of Medicines & HealthCare*. (n.d.). Retrieved June 27, 2022, from <https://www.edqm.eu/en/home>], the limit of endotoxin that can be tolerated in parenterally administered substances is five endotoxin units (EU) per kg body mass. This specification leads to a maximum tolerable endotoxin amount of 350 EU per individual (based on a body mass of 70 kg), or a tolerance of 350 EU/mL for a parenteral product where a single dose would be 1 mL. However, there is no limit available for endotoxin present in orally administered products.

[13] Increasingly feed additives for livestock, such as amino acids and vitamins, are produced by Gram-negative bacteria, such as *Escherichia coli*. Farm livestock are exposed continuously to endotoxins in their environment, including in feed, and to large quantities of LPS present in Gram-negative bacteria in the gastrointestinal tract. Wallace, et al. (2016), <https://doi.org/10.1186/s12940-016-0087-2>, proposed that feed additives produced by Gram-negative bacteria should not add greater than 1,000 IU/mg endotoxin concentration to the feed.

[14] Despite some uncertainty as to the appropriate level of LPS in orally and dermally administered products, for bacterial preparations that are incorporated into food, feed, or skin products, there is an interest in practical approaches to reducing the level of LPS in such products.

BRIEF SUMMARY OF THE INVENTION

[15] In accordance with the present invention, biomass (e.g., microbial biomass, produced by growth of a microorganism), or a protein product derived therefrom (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), is treated to reduce or eliminate endotoxins (e.g., lipopolysaccharide (LPS)), and is optionally also treated to reduce or remove (e.g., eliminate) nucleic acids. Certain embodiments further entail a microorganism, such as a chemoautotrophic microorganism, e.g., an oxyhydrogen microorganism, that is grown in a bioreactor, e.g., in a culture growth medium in a bioreactor, thereby producing microbial biomass. In some embodiments, a gas composition is introduced into the bioreactor that contains a carbon and/or energy source for growth of a chemoautotrophic microorganism. In certain embodiments, the gas composition comprises hydrogen, carbon dioxide, and oxygen for growth of an oxyhydrogen microorganism. Alternatively, the biomass may be grown heterotrophically using an organic carbon source, such as a sugar molecule, instead of gas feedstock. In certain embodiments, the microorganism comprises *Cupriavidus necator*, such as, but not limited to, DSM 531 or DSM 541. The biomass, or a protein product derived therefrom, is treated to reduce or eliminate endotoxins (e.g., lipopolysaccharide (LPS)), and is optionally also treated to reduce or remove nucleic acids.

[16] After treatment to reduce or eliminate endotoxins, the biomass or protein product derived therefrom may be processed into or incorporated as an ingredient in a food or animal feed product.

[17] Microbial biomass grown as described herein may be harvested and processed into a meat analogue product, e.g., a meat analogue product, or another food product, or an animal feed product. The biomass may be initially processed into one or more protein products, including single cell protein (e.g., whole cell biomass), cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof. Such initial processing may comprise (1) freeing organic molecules (including at least a portion of proteins of the microorganism) from the microorganism cells through cellular excretion, secretion, or cell lysis; and (2) treating the freed organic molecules to break down the bonds between at least some of the proteins' amino acids to create peptides with a desired chain length (e.g., polypeptides, oligopeptides (peptides having 2-20 amino acids), or free amino acids). These protein products may then be treated to remove (reduce or eliminate) endotoxins (e.g., LPS), and optionally also treated to remove (reduce or eliminate) nucleic acids, and then processed into a food product, such as a meat analogue product or other food product, or an animal feed product.

[18] In accordance with an embodiment, the protein product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% free amino acids. In accordance with an embodiment, the protein product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% oligopeptides. In accordance with an embodiment, the protein product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising 20 to 50 amino acids, or 21 to 50 amino acids. In accordance with an embodiment, the protein product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising 50 to 200 amino acids, or 51 to 200 amino acids. In accordance with an embodiment, the protein

product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising 200 to 500 amino acids, or 201 to 500 amino acids. In accordance with an embodiment, the protein product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising less than 1000, 900, 800, 700, 600, 500, 400, 300, 200, or 100 amino acids. In accordance with an embodiment, the protein product comprises any of about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% of a combination of free amino acids, oligopeptides, and polypeptides having 20 to 50 amino acids, or 21 to 50 amino acids, wherein the ratio of free amino acids to oligopeptides to polypeptides having 20 to 50 amino acids, or 21 to 50 amino acids, is about 1:1:1, or about 0 to about 3:about 0 to about 3:about 0 to about 3, or about 3 to about 6:about 0 to about 3:about 0 to about 3, or about 0 to about 3:about 3 to about 6:about 0 to about 3, or about 0 to about 3:about 0 to about 3:about 3 to about 6, or about 3 to about 6:about 3 to about 6:about 0 to about 3, or about 0 to about 3:about 3 to about 6:about 3 to about 6, or about 3 to about 6:about 0 to about 3:about 3 to about 6, or about 6 to about 9:about 0 to about 3:about 0 to about 3, or about 0 to about 3:about 6 to about 9, or about 6 to about 9:about 0 to about 3, or about 0 to about 3:about 0 to about 3:about 6 to about 9, or about 6 to about 9:about 6 to about 9:about 0 to about 3, or about 0 to about 3:about 6 to about 9:about 6 to about 9, or about 6 to about 9:about 0 to about 3:about 6 to about 9. In accordance with an embodiment, the protein product is free or essentially free of whole cells.

[19] In accordance with an embodiment, the protein product is combined with other edible ingredients to form a food product, including a meat analogue product which mimics one or more physical characteristics and/or functional properties of meat, such as texture, flavor, aroma, and/or appearance. Such other ingredients may be selected from apple cider, apple cider vinegar, baking powder, baking soda, beans, beef, beet juice, beet powder, black pepper, brown sugar, butter, canola oil, caramel, carrot fiber, carrots, cashews, cheese, chicken, chocolate, citrus, citrus extract, coconut oil, condensed milk, dairy, egg, egg substitute, fish, flour, garbanzo bean, garlic powder, honey, liquid smoke, maple syrup, margarine, monosodium glutamate, mustard powder, oil, olive oil, onion powder, paprika, pork, potato, potato starch, rice flour, salt, sodium benzoate, soy (protein

and/or oil), soy sauce, spices, spirulina, sugar, sunflower oil, tomato juice, tomato powder, tomato sauce, tomatoes, turmeric, vanilla, vinegar, vitamins and minerals, walnuts, water, wheat, wheat flour, wheat gluten, xanthan gum, yeast, and/or yeast extract.

[20] In accordance with an embodiment, the protein product comprises at least about 5%, at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, or at least about 85% of the meat analogue product on a dry basis, *e.g.*, by weight on a dry basis.

[21] In one aspect, methods are provided for producing a food product. The methods include: (a) processing microorganism cells that are harvested from a culture medium to produce a protein product, *e.g.*, a microbial protein product, wherein the protein product includes one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, and oligopeptides, and wherein the protein product includes endotoxins from the microorganism cells; (b) processing the protein product to reduce the concentration of the endotoxins; and (c) processing the protein product to produce a food product.

[22] In some embodiments, the concentration of endotoxin in the protein product is reduced by at least about 2-fold, or by about 2-fold to about 6000-fold, or by any of at least about 10-fold 100-fold, 1,000-fold, 10,000-fold, or 100,000-fold, or by about 2-fold to about 100,000-fold, or by about 10-fold to about 100,000-fold, or by about 10-fold to 100-fold, about 100-fold to about 1,000-fold, about 1,000-fold to about 10,000-fold, or about 10,000-fold to about 100,000-fold.

[23] In some embodiments, the protein product includes nucleic acids, and the protein product is processed to reduce the concentration of said nucleic acids prior to or in conjunction with step (b) or prior to step (c). For example, the nucleic acid content of the protein product is less than about 9% (w/w).

[24] In some embodiments, the food product includes at least about 10% of the protein product by weight.

[25] In some embodiments, the food product is a food item, a food ingredient, a nutritional product, an animal feed product, or a pet food product. In one embodiment, the food product is a meat analogue product. In other embodiments, the food product is a dairy product, a dairy replacement product, a bakery product, a confection, a health or protein bar, a protein powder, a sports and/or energy drink, a protein shake, or a smoothie.

[26] In some embodiments, the food product is a vegetarian or vegan food product. In some embodiments, the food product is an organic food product, a pesticide-free food product, an herbicide-free food product, a fungicide-free food product, an antibiotic-free food product, or a non-genetically-modified (non-GMO) food product. In some embodiments, the food product is a probiotic food product or a prebiotic food product. In some embodiments, the food product contains no animal protein or fat. In some embodiments, the food product includes one or more plant protein

source, in addition to the microbial protein product. In some embodiments, the food product includes an insect or algae protein source, in addition to the microbial protein product.

[27] In some embodiments, the microorganism from which the protein product is derived is a chemoautotrophic microorganism, such as an oxyhydrogen microorganism. In some embodiments, the oxyhydrogen microorganism is a *Cupriavidus* microorganism, such as, but not limited to, *Cupriavidus necator* DSM 531 and/or DSM 541.

[28] In some embodiments, step (a) includes: (i) freeing organic molecules from the microorganism cells, via excretion, secretion, or cell lysis, or a combination thereof, wherein the organic molecules include proteins; and (ii) treating the freed organic molecules to hydrolyze peptide bonds between at least a portion of amino acids in at least a portion of the proteins, thereby producing a hydrolysis protein product that includes polypeptides comprising 20 to 50 amino acids, oligopeptides comprising 2 to 20 amino acids, and/or free amino acids.

[29] In some embodiments, step (c) includes combining the protein product with other edible ingredients to form the food product.

[30] In another aspect, a food product is provided, which is prepared in accordance with any of the methods disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[31] Fig. 1 shows a diagram of an embodiment of a single cell protein production process whereby CO₂ is converted into a high protein powder having low LPS and/or low EU and whereby the protein powder is further used as an ingredient to produce a meat analogue product.

[32] Fig. 2 schematically shows an embodiment of production of nutrients from CO₂, H₂, and other inorganic inputs. Removal of endotoxin content and extraction of high protein and low EU nutrients. **410**: inoculation of culture medium with microorganism cells; **420**: growth of microorganism cells for production of microbial biomass; **430**: harvest of microorganism cells from culture medium; **440**: removal or reduction of endotoxin content in microbial biomass; **450**: drying to produce of a low EU dry composition; **460**: grinding of the low EU composition, producing **470**: a low EU protein powder.

[33] Fig. 3 schematically shows an embodiment of production of nutrients from CO₂, H₂, and other inorganic inputs. Removal of endotoxin and lipid content and extraction of high protein and low EU and low lipid nutrients. **510**: inoculation of culture medium with microorganism cells; **520**: growth of microorganism cells for production of microbial biomass; **530**: harvest of microorganism cells from culture medium; **540**: removal or reduction of endotoxin content in microbial biomass; **550**: defatting of the composition, producing a low EU defatted composition; **560**: removal of odor and/or other undesirable characteristics; **570**: drying to produce a low EU dry composition; **580**: grinding of the low EU composition, producing **590**: a low EU defatted protein powder.

[34] Fig. 4 shows a block diagram of an embodiment a method described herein, whereby a high protein, low nucleic acid isolate is derived from the cells as described herein.

[35] Fig. 5 shows runtime data from a continuous run of *Cupriavidus necator* on H₂ and CO₂ substrates that maintained an axenic culture for over 1,000 hours.

DETAILED DESCRIPTION

[36] The invention provides food products, such as meat analogue products, and animal feed products that include protein products derived from microorganisms. Prior to incorporation into the food or feed product, the protein product is treated to remove endotoxins, and optionally also treated to remove nucleic acids, as described herein.

[37] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, et al., *Dictionary of Microbiology and Molecular Biology*, second ed., John Wiley and Sons, New York (1994), and Hale & Markham, *The Harper Collins Dictionary of Biology*, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the methods, systems, and compositions described herein.

[38] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, and biochemistry, which are within the skill of the art. Such techniques are explained fully in the literature, for example, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook et al., 1989); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984; *Current Protocols in Molecular Biology* (F. M. Ausubel et al., eds., 1994); *PCR: The Polymerase Chain Reaction* (Mullis et al., eds., 1994); and *Gene Transfer and Expression: A Laboratory Manual* (Kriegler, 1990).

[39] Numeric ranges provided herein are inclusive of the numbers defining the range.

[40] Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

Definitions

[41] "A," "an" and "the" include plural references unless the context clearly dictates, thus the indefinite articles "a", "an", and "the" as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

[42] The term "about" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of $\pm 5\%$, $\pm 1\%$, or $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the disclosed methods or in connection with a disclosed composition.

[43] "Acetogen" refers to a microorganism that generates acetate and/or other short chain organic acids up to C4 chain length as a product of anaerobic respiration.

[44] "Acidophile" refers to a type of extremophile that thrives under highly acidic conditions (usually at pH 2.0 or below).

[45] The term “amino acid” refers to a molecule containing both an amine group and a carboxyl group that are bound to a carbon, which is designated the alpha-carbon. Suitable amino acids include, without limitation, both the D- and L-isomers of the naturally occurring amino acids, as well as non-naturally occurring amino acids prepared by organic synthesis or other metabolic routes. In some embodiments, a single “amino acid” might have multiple sidechain moieties, as available per an extended aliphatic or aromatic backbone scaffold. Unless the context specifically indicates otherwise, the term amino acid, as used herein, is intended to include amino acid analogs.

[46] The phrase “and/or,” as used herein in the specification and in the claims, should be understood to mean “either or both” of the elements so conjoined, *i.e.*, elements that are conjunctively present in some cases and disjunctively present in other cases. Other elements may optionally be present other than the elements specifically identified by the “and/or” clause, whether related or unrelated to those elements specifically identified unless clearly indicated to the contrary. Thus, as a non-limiting example, a reference to “A and/or B,” when used in conjunction with open-ended language such as “comprising” can refer, in one embodiment, to A without B (optionally including elements other than B); in another embodiment, to B without A (optionally including elements other than A); in yet another embodiment, to both A and B (optionally including other elements); etc.

[47] The term “artificial meat” or “meat substitute” or “imitation meat” or “meat analogue” or “meat-like food product” as used herein refers to a food product that is not derived from an animal, or that contains a substantial amount of non-animal protein source, but has structure, texture, aesthetic qualities, and/or other properties comparable or similar to those of animal meat, including livestock (*e.g.*, beef, pork), game (*e.g.*, venison), poultry (*e.g.*, chicken, turkey, duck), and/or fish or seafood substitutes/analogues. The term refers to uncooked, cooking, and cooked meat-like food product. These terms also encompass cultured meat products.

[48] The term “biomass” refers to a material produced by growth and/or propagation of cells, such as microorganism cells. Biomass may contain cells and/or intracellular contents as well as extracellular material, including, but not limited to, compounds secreted by a cell.

[49] The term “bioreactor” or “fermenter” refers to a closed or partially closed vessel in which cells are grown and maintained. The cells may be, but are not necessarily, held in liquid suspension. In some embodiments, rather than being held in liquid suspension, cells may alternatively be grown and/or maintained in contact with, on, or within another non-liquid substrate including but not limited to a solid growth support material.

[50] The term “carbon fixing” process, reaction or pathway refers to enzymatic reactions or metabolic pathways that convert forms of carbon that are gaseous under ambient conditions, including but not limited to CO₂, CO, and CH₄, into carbon-based biochemicals that are liquid or solid under ambient conditions, or which are dissolved into, or held in suspension in, aqueous solution.

- [51] "Carbon source" refers to the types of molecules from which a microorganism derives the carbon needed for organic biosynthesis.
- [52] "Carboxydophilic" refers to microorganisms that can tolerate or oxidize carbon monoxide. In preferred embodiments a carboxydophilic microorganism can utilize CO as a carbon source and/or as a source of reducing electrons for biosynthesis and/or respiration.
- [53] "Chemoautotrophic" refers to organisms that obtain energy by the oxidation of chemical electron donors by chemical electron acceptors and synthesize all the organic compounds needed by the organism to live and grow from carbon dioxide.
- [54] In the claims, as well as in the specification, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively.
- [55] A "consortium" refers herein to two or more different species or strains of microorganisms and/or multi-cellular organisms, which are grown together, for example, grown in co-culture in the same growth medium.
- [56] The term "culturing" refers to growing a population of cells, *e.g.*, microbial cells, under suitable conditions for growth, in a liquid or solid medium.
- [57] The term "derived from" encompasses the terms "originated from," "obtained from," "obtainable from," "isolated from," and "created from," and generally indicates that one specified material finds its origin in another specified material or has features that can be described with reference to another specified material.
- [58] "Energy source" refers to either the electron donor that is oxidized by oxygen in aerobic respiration or the combination of electron donor that is oxidized and electron acceptor that is reduced in anaerobic respiration.
- [59] "Extremophile" refers to a microorganism that thrives in physically or geochemically extreme conditions (*e.g.*, high or low temperature, pH, or high salinity) compared to conditions on the surface of the Earth or the ocean that are typically tolerated by most life forms found on or near the earth's surface.
- [60] The term "gasification" refers to a generally high temperature process that converts carbon-based materials into a mixture of gases including hydrogen, carbon monoxide, and carbon dioxide called synthesis gas, syngas or producer gas. The process generally involves partial combustion and/or the application of externally generated heat along with the controlled addition of oxygen and/or steam such that insufficient oxygen is present for complete combustion of the carbon-based material.
- [61] "Halophile" refers to a type of extremophile that thrives in environments with very high concentrations of salt.
- [62] "Heterotrophic" refers to organisms that cannot synthesize all the organic compounds needed by the organism to live and grow from carbon dioxide, and which must utilize organic

compounds for growth. Heterotrophic organisms cannot produce their own food and instead obtain food and energy by taking in and metabolizing organic substances, such as plant or animal matter, *i.e.*, rather than fixing carbon from inorganic sources such as carbon dioxide.

[63] "Hydrogen-oxidizer" refers to a microorganism that utilizes reduced H₂ as an electron donor for the production of intracellular reducing equivalents and/or in respiration.

[64] "Hyperthermophile" refers to a type of extremophile that thrives in extremely hot environments for life, typically about 60 °C (140 °F) or higher.

[65] The term "knallgas" refers to the mixture of molecular hydrogen and oxygen gas. A "knallgas microorganism" is a microbe that can use hydrogen as an electron donor and oxygen as an electron acceptor in respiration for the generation of intracellular energy carriers such as Adenosine-5'-triphosphate (ATP). The terms "oxyhydrogen" and "oxyhydrogen microorganism" can be used synonymously with "knallgas" and "knallgas microorganism," respectively. Knallgas microorganisms generally use molecular hydrogen by means of hydrogenases, with some of the electrons donated from H₂ that is utilized for the reduction of NAD⁺ (and/or other intracellular reducing equivalents) and some of the electrons from H₂ that is used for aerobic respiration. Knallgas microorganisms generally fix CO₂ autotrophically, through pathways including but not limited to the Calvin Cycle or the reverse citric acid cycle ["Thermophilic bacteria", Jakob Kristjansson, Chapter 5, Section III, CRC Press, (1992)].

[66] The term "lipid" herein refers to one or more molecules (*e.g.*, biomolecules) that include a fatty acyl group (*e.g.*, saturated or unsaturated acyl chains). For example, the term lipids includes oils, phospholipids, free fatty acids, monoglycerides, diglycerides, and triglycerides.

[67] The term "lysate" refers to the liquid containing a mixture and/or a solution of cell contents that result from cell lysis. In some embodiments, the methods described herein comprise a purification of chemicals or mixture of chemicals in a cellular lysate. In some embodiments, the methods comprise a purification of amino acids and/or protein in a cellular lysate.

[68] The term "lysis" refers to the rupture of the plasma membrane and if present, the cell wall of a cell such that a significant amount of intracellular material escapes to the extracellular space. Lysis can be performed using electrochemical, mechanical, osmotic, thermal, or viral means. In some embodiments, the methods described herein comprise performing a lysis of cells or microorganisms as described herein in order to separate a chemical or mixture of chemicals from the contents of a bioreactor. In some embodiments, the methods comprise performing a lysis of cells or microorganisms described herein in order to separate an amino acid or mixture of amino acids and/or proteins from the contents of a bioreactor or cellular growth medium.

[69] "Methanogen" refers to a microorganism that generates methane as a product of anaerobic respiration.

[70] "Methylophile" refers to a microorganism that can use reduced one-carbon compounds, such as but not limited to methanol or methane, as a carbon source and/or as an electron donor for their growth.

[71] The terms "microorganism" and "microbe" mean microscopic single celled life forms, including bacteria, fungi, and algae.

[72] The term "molecule" means any distinct or distinguishable structural unit of matter comprising one or more atoms, and includes for example hydrocarbons, lipids, polypeptides and polynucleotides.

[73] "Oligopeptide" refers to a peptide that contains a relatively small number of amino-acid residues, for example, about 2 to about 20 amino acids.

[74] As used herein in the specification and in the claims, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of," or, when used in the claims, "consisting of," will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (*i.e.*, "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of." "Consisting essentially of," when used in the claims, shall have its ordinary meaning as used in the field of patent law.

[75] The term "organic compound" refers to any gaseous, liquid, or solid chemical compound that contains carbon atoms, with the following exceptions that are considered inorganic: carbides, carbonates, simple oxides of carbon, cyanides, and allotropes of pure carbon such as diamond and graphite.

[76] "Peptide" refers to a compound (a polypeptide) consisting of two or more amino acids linked in a chain, the carboxyl group of each acid being joined to the amino group of the next by a bond of the type R-OC-NH-R', for example, about 2 amino acids to about 50 amino acids, or 21 amino acids to about 50 amino acids.

[77] As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length and any three-dimensional structure and single- or multi-stranded (e.g., single-stranded, double-stranded, triple-helical, etc.), which contain deoxyribonucleotides, ribonucleotides, and/or analogs or modified forms of deoxyribonucleotides or ribonucleotides, including modified nucleotides or bases or their analogs. Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present invention encompasses polynucleotides which encode a particular amino acid sequence. Any type of modified nucleotide or nucleotide analog may be used, so long as the polynucleotide retains the desired functionality under conditions of use, including modifications that increase nuclease resistance (e.g., deoxy, 2'-O-Me, phosphorothioates, etc.). Labels may also be incorporated for purposes of detection or capture, for example, radioactive or nonradioactive labels or anchors, e.g., biotin. The term polynucleotide also includes peptide nucleic acids (PNA). Polynucleotides may be naturally occurring or non-naturally occurring. The terms "polynucleotide," "nucleic acid," and "oligonucleotide" are used herein

interchangeably. Polynucleotides may contain RNA, DNA, or both, and/or modified forms and/or analogs thereof. A sequence of nucleotides may be interrupted by non-nucleotide components. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S ("thioate"), P(S)S ("dithioate"), (O)NR.sub.2 ("amidate"), P(O)R, P(O)OR', CO or CH.sub.2 ("formacetal"), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (--O--) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. Polynucleotides may be linear or circular or comprise a combination of linear and circular portions.

[78] As used herein, "polypeptide" refers to a composition comprised of amino acids and recognized as a protein by those of skill in the art. The conventional one-letter or three-letter code for amino acid residues is used herein. The terms "polypeptide" and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also, included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art.

[79] The term "precursor to" or "precursor of" is an intermediate towards the production of one or more of the components of a finished product.

[80] "Producer gas" refers to a gas mixture containing various proportions of H₂, CO, and CO₂, and having heat value typically ranging between one half and one tenth that of natural gas per unit volume under standard conditions. Producer gas can be generated various ways from a variety of feedstocks, including gasification, steam reforming, or autoreforming of carbon-based feedstocks. In addition to H₂, CO, and CO₂, producer gases can contain other constituents including but not limited to methane, hydrogen sulfide, condensable gases, tars, and ash depending upon the generation process and feedstock. The proportion of N₂ in the mixture can be high or low depending whether air is used as an oxidant in the reactor or not and if the heat for the reaction is provided by direct combustion or through indirect heat exchange.

[81] The term "producing" includes both the production of compounds intracellularly and extracellularly, including the secretion of compounds from the cell.

[82] "Protein digestibility-corrected amino acid score" (PDCAAS) is a method of evaluating the quality of a protein based on both the amino acid nutritional requirements for humans compared to the amino acid content of the protein and the ability of humans to digest the protein.

[83] "Psychrophile" refers to a type of extremophile capable of growth and reproduction in cold temperatures, typically about 10°C and lower.

[84] The terms “recovered,” “isolated,” “purified,” and “separated” as used herein refer to a material (e.g., a protein, nucleic acid, or cell) that is removed from at least one component with which it is naturally associated. For example, these terms may refer to a material that is substantially or essentially free from components which normally accompany it as found in its native state, such as, for example, an intact biological system.

[85] The phrase “substantially free” or “essentially free” as to any given component means that such component is only present, if at all, in an amount that is a functionally insignificant amount, *i.e.*, it does not significantly negatively impact the intended performance or function of any process or product. Typically, substantially free means less than about 1%, including less than about 0.5%, including less than about 0.1%, and also including zero percent, by weight of such component. The terms “substantially free” or “essentially free” shall mean less than 1% of a component.

[86] “Sulfur-oxidizer” refers to microorganisms that utilize reduced sulfur containing compounds including but not limited to H₂S as electron donors for the production of intracellular reducing equivalents and/or in respiration.

[87] “Syngas” or “Synthesis gas” refers to a type of gas mixture, which like producer gas contains H₂ and CO, but which has been more specifically tailored in terms of H₂ and CO content and ratio and levels of impurities for the synthesis of a particular type of chemical product, such as but not limited to methanol or fischer-tropsch diesel. Syngas generally contains H₂, CO, and CO₂ as major components, and it can be generated through established methods including: steam reforming of methane; or through gasification of any organic, flammable, carbon-based material, including but not limited to biomass, organic matter, or peat. The hydrogen component of syngas can be increased through the reaction of CO with steam in the water gas shift reaction, with a concomitant increase in CO₂ in the syngas mixture.

[88] “Thermophile” refers to a type of extremophile that thrives at relatively high temperatures for life, typically about 45 °C to about 122 °C.

[89] “Wild-type” refers to a microorganism as it occurs in nature.

[90] “Yield” refers to amount of a product produced from a feed material relative to the total amount of the substance that would be produced if all of the feed substance were converted to product. For example, yield of the product may be expressed as % of the product produced relative to a theoretical yield if 100% of the feed substance were converted to the product.

Endotoxin removal

[91] Concentration of endotoxin (e.g., LPS) is reduced in microbial protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), prior to incorporation into a food or animal feed product. For example, endotoxin concentration is reduced any of at least about 2, 10, 20, 50, 100, 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, or 3000-fold, relative to an equivalent protein product that has not been treated as described

herein for reduction of endotoxin content. In various embodiments, endotoxin concentration is reduced by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, relative to an equivalent protein product that has not been treated as described herein for reduction of endotoxin content.

[92] In some embodiments, endotoxin concentration is reduced by a treatment that denatures protein to produce a protein concentrate with reduced endotoxin content.

[93] In some embodiments, endotoxin concentration is reduced by a treatment that results in a non-denatured protein. In some embodiments, endotoxin removal may be achieved via lysis of cells, followed by solubilization and separation of protein.

[94] In some embodiments, endotoxin concentration is reduced by acid hydrolysis, for example, with an acid such as, but not limited, to an organic acid (e.g., acetic, formic, carbonic, and/or citric acid), and/or sulfuric, phosphoric, hydrochloric, and/or nitric acid, optionally followed by enzymatic digestion, for example, with an enzyme such as, but not limited to, protease, alkaline phosphatase, phospholipase, or lysozyme.

[95] In some embodiments, endotoxin concentration is reduced by base hydrolysis, for example, with a base such as, but not limited to ammonium hydroxide, potassium hydroxide, sodium hydroxide, and/or calcium hydroxide, optionally followed by enzymatic digestion, for example, with an enzyme such as, but not limited to, alkaline phosphatase, phospholipase, or lysozyme.

[96] In some embodiments, endotoxin concentrations in a non-denatured protein preparation can be reduced via an activated carbon treatment. In some embodiments, gel filtration chromatography, or ion exchange, and/or size exclusion chromatography can be used, instead of or in addition to the activated carbon treatment

[97] In some embodiments, treatment with a detergent, such as Triton X-114 phase separation, can be deployed for endotoxin removal.

[98] In some embodiments, endotoxin removal from non-denatured and solubilized proteins can be achieved via filtration (e.g., ultrafiltration, microfiltration).

[99] LPS endotoxin was originally detected and measured using a pyrogen test based on injection into rabbits [Hort, E. C., & Penfold, W. J. (1912). Microorganisms and their Relation to Fever: Preliminary Communication. *The Journal of Hygiene*, 12(3), 361. <https://doi.org/10.1017/S0022172400005052>]. The Limulus Amoebocyte Lysate (LAL) test (also known as bacterial endotoxin test [BET]) [Rojas-Corona, R. R., Skarnes, R., Tamakuma, S., & Fine, J. (1969). The Limulus coagulation test for endotoxin. A comparison with other assay methods. *Proceedings of the Society for Experimental Biology and Medicine*, 132(2), 599–601], was developed and introduced to clinical medicine in 1970 [Levin, J., Poore, T. E., Zauber, N. P., & Oser, R. S. (1970). Detection of endotoxin in the blood of patients with sepsis due to gram-negative bacteria. *New England Journal of Medicine*, 283(24), 1313–1316]. It is based on lysed amoebocytes obtained from the hemolymph of horseshoe crabs. These cells mostly respond to the lipid A moiety of LPS. LAL technology eventually replaced the test using live rabbits and it is estimated that over

90% of pyrogenic testing is performed by LAL [Hartung, T. (2015). The human whole blood pyrogen test – lessons learned in twenty years. *ALTEX - Alternatives to Animal Experimentation*, 32(2), 79–100. <https://doi.org/10.14573/ALTEX.1503241>]. The Monocyte Activation Test (MAT) is an alternative to the LAL test, which uses human blood cells. It has the advantage that it responds to all pyrogens that potentially induce fever in humans [*Detection of pyrogens using human whole blood* [Article in German] | *ALTEX - Alternatives to animal experimentation*. (n.d.). Retrieved June 27, 2022, from <https://www.altex.org/index.php/altex/article/view/1671>]. MAT was included as a standard method for pyrogen detection in the European Pharmacopoeia in 2010 [European Directorate for the Quality of Medicines and Healthcare - European Directorate for the Quality of Medicines & HealthCare. (n.d.). Retrieved June 27, 2022, from <https://www.edqm.eu/en/home>].

[100] In certain embodiments of the present invention, a protein concentrate (PC), protein isolate (PI), or protein hydrolysate (PH) composition is produced with at least about 10-fold, or at least about 100-fold, or at least about 1,000-fold, or at least about 10,000-fold, or at least about 100,000-fold reduction in endotoxin (EU) content per mass, compared to the starting biomass, from which the said PC, PI, or PH was derived. In certain such embodiments the said reduction in EU per mass is determined by comparing the starting biomass from which the PC, PI, or PH is derived with the PC, PI, or PH product, using one or more of the following tests: pyrogen test, LAL assay, and/or MAT assay. In certain non-limiting embodiments, a PC, PI, or PH composition having reduced EU per mass, compared with the biomass from which it was derived, also has one or more of the following additional features: a nucleic acid content of $\leq$ about 1% or $\leq$ about 2% or $\leq$ about 3% or $\leq$ about 4% or $\leq$ about 5% or $\leq$ about 6% or $\leq$ about 7% or $\leq$ about 8% or $\leq$ about 9% (wt %; nucleic acids/dry wt of the composition); a total amino acids content of at least about 70% or at least about 75% or at least about 80% or at least about 85% or at least about 90% or at least about 95% (wt %; amino acids (e.g., proteogenic amino acids)/dry wt of the composition); an ash content of $\leq$ about 1% or $\leq$ about 2% or $\leq$ about 5% or $\leq$ about 10% or $\leq$ about 15% (wt %; ash/dry wt of the composition); a PDCAAS of at least about 0.7 or at least about 0.8 or at least about 0.9 or at least about 0.92 or at least about 0.95 or at least about 0.97 or at least about 0.99 or about 1.

Nucleic acid removal

[101] In some embodiments, concentration of nucleic acids in the protein product is reduced, prior to incorporation into a food or animal feed product. Removal of nucleic acids may occur prior to , after, or concurrently with removal of endotoxins.

[102] One possible area of concern with respect to direct human consumption of microbial protein product is the nucleic acid content. For human nutrition, the nucleic acid content of single-cell protein (SCP) may need to be reduced to a lower level, if the SCP comprises a significant portion of the diet. The Recommended Daily Allowance of the Food and Nutrition Board, National Research Council for protein is 65 grams per day for a 70 kilogram adult male, and the Protein Advisory Group of the United Nations System recommends that the amount of nucleic acid ingested per day from

microbial protein should be less than two grams per day. Therefore, according to these criteria the ratio of nucleic acid content to protein should be less than three percent, if SCP is the only source of dietary protein.

[103] Certain embodiments of this disclosure relate to a microbial protein product with lowered nucleic acid content. In certain embodiments, the nucleic acid content of the protein product is less than about 9%, less than about 5%, or less than about 3% by weight. In certain embodiments, the ratio of nucleic acid content to protein is reduced to less than about 3%. In certain embodiments, the ratio of nucleic acid to protein is reduced to a level considered safe for providing a substantial proportion, or all of the protein requirement in a human diet, by one of average skill in the art and knowledge in the field. In certain embodiments, the nucleic acid of cells is primarily RNA as well, and the terms RNA and nucleic acid will sometimes be used interchangeably herein. .

[104] In another aspect, a method as described herein for the biological conversion of inorganic and/or organic molecules containing one or more carbon atoms, into organic molecules comprising amino acids, proteins, and/or vitamins produced through a carbon fixing reaction or anabolic biosynthesis, further comprises producing a concentrated protein product, comprising the steps of: a. rupturing said microorganism cells, wherein said cells comprise one or more nuclease enzyme, thereby producing a mixture comprising soluble nucleic acid, nuclease, and protein and comprising insoluble cell wall debris; b. separating the soluble nucleic acid, nuclease, and protein from the insoluble cell wall debris under conditions in which the nucleic acid is hydrolyzed with the nuclease, thereby producing hydrolyzed nucleic acid; d. rendering the protein insoluble; and e. separating the insoluble protein from the remaining soluble materials that comprise the hydrolyzed nucleic acid. In some embodiments, the cells are ruptured by homogenization. In one embodiment, the insoluble protein comprises less than about 5% nucleic acid. In one embodiment, the insoluble protein comprises less than about 3% nucleic acid. **Fig. 1** shows a block diagram of an embodiment a method described herein, whereby a high protein, low nucleic acid isolate is derived from the cells as described herein.

[105] When cells are ruptured by any method, a cellular debris fraction and a soluble cytoplasmic constituent fraction are generally obtained. These fractions may be separated by methods such as, but not limited to, centrifugation or filtration. Among the soluble cytoplasmic constituents are the nucleic acid and the protein, either individually or in conjugation. Substantial amounts of DNA may be liberated by cell lysis, increasing viscosity. In certain non-limiting embodiments DNase (*e.g.*, ~1 mg/ml) is added to reduce the viscosity of the lysed preparation. Following cell lysis, recovery of the protein by isoelectric precipitation may result in a proteinaceous product having a content of nucleic acids that is undesirable in certain embodiments. In certain embodiments, measures well known in the art to reduce or eliminate the nucleic acid content of proteinaceous material are undertaken.

[106] Certain embodiments of this disclosure relate to a microbial protein product (*e.g.*, one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof) with lowered nucleic

acid content. In some embodiments, a protein product is provided that is relatively free of nucleic acid, but that still provides good nutritional value and acceptable eating (*i.e.*, consumption) quality. In certain embodiments, the nucleic acid content is reduced to less than about 9%, less than about 7%, less than about 5%, or less than about 3% by weight. In certain embodiments, the RNA content is reduced to meet World Health Organization [WHO] guidelines for human consumption. In certain embodiments, the RNA content of the protein product is below 2% dry weight; in some embodiments it is below 1% dry weight. In certain embodiments, a nucleic acid reduction process is included which produces cell material containing two to three grams of nucleic acid, or less, per 100 grams of protein. In certain non-limiting embodiments, the Protein Equivalence Ratio (PER) of the cell isolate is greater than 1. In certain non-limiting embodiments, the composition of the cell isolate in terms of weight percentage is about 65% to about 85% protein, or higher; about 0.5% to any of about 3%, 5%, 7%, or 9% nucleic acid; about 7% to about 15% lipid; about 1% to about 5% ash; and about 5% to about 20% carbohydrate, and/or other N-free, non-lipid organic matter.

[107] In certain embodiments, the cells are subjected to a temperature which inactivates proteases and stops them from breaking down proteins, but which still allows RNase (RNA-digesting enzymes) to break down ribosomal RNA. In certain such embodiments, this temperature is at least about 64°C. In certain such embodiments the small products of this RNA digestion diffuse through the cell walls into the culture broth and so are removed from the final microbial biomass (SCP) product. In certain such embodiments, this RNA removal step occurs in a RNA reduction vessel in fluid connection with the bioreactor. In certain such embodiments, the culture is harvested continuously from the bioreactor. In certain such embodiments, after the RNA content has been reduced, the cell mass is spread over a large moving filter through which most of the liquid is drawn off by vacuum. In certain such embodiments, this leaves behind a thin sheet of high protein material, for example, a thin sheet of high protein material that is low in nucleic acid content.

[108] The cell isolate of certain embodiments herein may be made by a process which involves rupturing the cells and removing the cell wall residue. In certain such embodiments, this cell rupturing is performed in an alkaline medium. In certain embodiments, the reduction of the nucleic acid content can be accomplished by the hydrolysis of the nucleic acid within the cell to fragments of such size that the fragments can be diffused from the cell away from the protein.

[109] It is known that nuclease enzyme, which is present in a number of different microorganisms, hydrolyzes or breaks up nucleic acid molecules to smaller fragments. Hydrolysis of the nucleic acids by enzymatic methods allows the use of much milder conditions in terms of pH and temperature than those generally needed for chemical methods of hydrolysis, where milder is taken to be pH closer to 7, and temperature closer to ambient, respectively. In certain embodiments, the mild conditions obviate the need for acid or alkali resistant equipment. In certain embodiments, an enzymatic process and mild conditions are utilized which better preserve the nutritional quality of proteins than comparable chemical methods of hydrolysis. Several sources of nuclease have been described in the literature. Certain embodiments herein utilize nuclease(s) for the hydrolysis of

nucleic acid polymers that are well known to one of average skill in the art. In certain embodiments, such nuclease(s) are free of secondary enzyme systems such as protease, which could cause a decrease in protein recovery by the co-diffusion of amino acids and/or small peptides out of the cell along with the hydrolyzed nucleic acids. In certain embodiments, the nuclease preparation does not contribute an undesirable flavor to products derived from the nucleic acid reduction process. In certain embodiments, the nuclease preparation used is commercially available. In certain embodiments, the nuclease is food grade. In certain embodiments, a nuclease from yeast is used to break up nucleic acid molecules to small fragments. In certain embodiments, an endogenous and/or exogenous nuclease is utilized to break up nucleic acid molecules to small fragments.

[110] In some embodiments, a process is provided for making a protein isolate in which endogenous nuclease is used to hydrolyze the nucleic acid so that the nucleic acid fragments can be separated from the protein, *e.g.*, by precipitation of the protein. It also is known in the art that the hydrolysis of nucleic acids within a cell can be accomplished by a multi-step heating process to activate a self-contained and/or endogenous nuclease to convert insoluble nucleic acid polymer to soluble nucleic acids. In certain embodiments, a multi-step heating process is used to activate a self-contained and/or endogenous nuclease to convert insoluble nucleic acid polymer to soluble nucleic acids. In certain embodiments, the cell lysate is incubated in such a manner that an endogenous nuclease contained in the soluble portion degrades the nucleic acid present in the cell to a soluble form.

[111] Nucleic acid also can be hydrolyzed by exposing the cell to an external nuclease. In certain embodiments, the cell and/or protein product thereof, such as a cell lysate, is exposed to an external (exogenous) nuclease.

[112] Nucleases are known to be extractable into the soluble fraction above a certain pH. In certain embodiments, the nuclease is soluble at a pH greater than 4. In certain embodiments, the nuclease is soluble at a pH greater than 5.5. In certain embodiments, the pH for nuclease extraction is optimized for minimum alkali addition and/or salt content at adequate soluble nuclease yield. In certain embodiments, the nuclease is extracted at a pH where the nuclease is soluble but inactive, and is maintained in an inactive state until its activity is required, at which point the pH is lowered to regain nuclease activity.

[113] In certain embodiments, a cell strain is improved and/or engineered for increased nuclease activity. In certain embodiments, the nuclease content of the cells is increased by genetic and/or environmental manipulation. In certain embodiments, strains require additional nuclease beyond the endogenous nuclease to adequately reduce the RNA. In certain embodiments, this additional nuclease is provided either from an outside source and/or by selective development of strains for increased internal nuclease.

[114] Nucleases are known to generally have an optimum pH and temperature for maximum aggregate activity (*i.e.*, conversion rate per individual active enzyme multiplied by the number of active enzymes). The reaction rate increases with temperature; however, the fraction of enzymes

that are inactivated also generally increases with increasing temperature. These two counteracting effects generally result in the enzyme exhibiting maximum activity within a certain temperature range. In certain embodiments, this optimum is identified and used following standard experimental methods known to one skilled in the art.

[115] In certain embodiments, the incubation time for RNA breakdown by nucleases is optimized.

[116] Nucleic acid polymers and proteins are known to co-precipitate below a certain pH, producing a nucleoprotein mixture. In certain embodiments, the pH to which the lysate is exposed is kept above this pH so as to avoid the formation of a nucleoprotein mixture.

[117] Chargaff, in Vol. I, *The Nucleic Acids*, states that ribonucleic acid (RNA) can be hydrolyzed by the action of 1N HCl for one hour at 100°C, or by the action of 0.1N NaOH at 100°C. In certain embodiments, the application of acidic or alkaline conditions to the microbial cytoplasmic constituents released by cell rupture results in the hydrolysis of the nucleic acid. In certain embodiments, HCl or NaOH is utilized for the hydrolysis of RNA. In certain non-limiting embodiments RNA is hydrolyzed using about 1N HCl or about 0.1N NaOH at about 100°C for around 1 hour.

[118] In certain embodiments, following hydrolysis of nucleic acid polymers, two fractions are obtained: one fraction comprises solids containing a reduced content of nucleic acid; and the other fraction is the surrounding medium containing dissolved nucleic acid fragments and other diffusible material. In certain embodiments, the protein is made insoluble, so as to separate it from the hydrolyzed and dissolved nucleic acid. In certain embodiments, an insoluble protein fraction is separated from a fraction containing soluble nucleic acid. In certain embodiments reaction conditions, including but not limited to, pH, time, temperature, and/or concentration, are optimized to recover a protein product having a low content of nucleic acid and an acceptable protein yield, and/or are optimized to maximize protein yield at an acceptable nucleic acid content. Certain embodiments also comprise a method of making protein of low nucleic acid content and free of cell wall debris. In certain such embodiments, a nuclease is used to solubilize nucleic acid including but not limited to an endogenous nuclease. The drawing provided in **Fig. 1** is a block diagram of an embodiment of the process of this particular aspect.

[119] Nonlimiting examples of methods for removal of nucleic acids from microbial protein preparations are described in PCT Application No. WO2018/144965, which is incorporated herein by reference in its entirety.

High protein food products

[120] In some embodiments, high protein food compositions are provided as well as methods of making the same. Food compositions herein include a microbial protein product from which endotoxins, and optionally nucleic acids, have been reduced or eliminated, prior to processing or incorporation into the food product. A "protein product" or "microbial protein product" (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein

hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), derived from one or more microorganisms described herein, may be processed or incorporated into a high-protein edible food composition for human and/or animal consumption. A food composition (food product) may be, for example, a food item, and/or a food ingredient, and/or a nutritional product, and/or an animal feed, and/or a pet food product. In some embodiments, the food composition may contain any of at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, or at least about 90% microbial protein product, as described herein, by weight, *e.g.*, by weight on a dry weight basis.

[121] In certain embodiments, the protein products as described herein are utilized in the production of a vegetarian or vegan food product. In certain embodiments, they are utilized in the production of an organic food product and/or pesticide-free and/or herbicide-free and/or fungicide-free and/or antibiotic-free and/or non-genetically modified (non-GMO) food product. In certain embodiments, they are utilized in a locally produced food product. In certain embodiments, they are utilized in a probiotic food product or in a prebiotic food product (*e.g.*, prebiotic nutritional product).

[122] Processes are provided for making a high protein food product, which includes one or more protein product from one or more microorganism as described herein. In certain embodiments, the food product has no animal protein or fats. In certain embodiments, the protein product(s) are incorporated into food products including, but not limited to, dairy products, dairy replacement products, meat products (including livestock, game, poultry, fish, or seafood products), meat replacement and/or imitation meat products (including imitation livestock, game, poultry, fish, or seafood products), bakery products, confections, health and protein bars, protein powders, sports and/or energy drinks, and/or protein shakes and/or smoothies. In certain embodiments, protein products are textured for incorporation into meat products and/or imitation meat products. In certain embodiments, the high protein ingredient can be used as a meat extender in beef patties.

[123] In certain embodiments, a high protein food product as described herein does not include animal fats. In certain embodiments, it has a relatively high ratio of polyunsaturated fats to saturated fats. In certain embodiments, it has a high-quality protein content, roughly comparable to milk protein. In certain non-limiting embodiments, its amino acid content is substantially similar, very close, or identical to that recommended by the United Nations Food and Agriculture Organization as ideal. In certain embodiments, food products made using the protein products of the present invention represent healthy and/or low-calorie foods. In certain embodiments, the protein product has a bland flavor and/or a light cream color and/or easy dispersibility and/or a relatively high water absorption and/or relatively high fat adsorption. In certain embodiments, the protein product can be formed into fibers and/or thermally extruded and/or coagulated into a gel. In certain such non-limiting embodiments, gel coagulation occurs at pH falling in a range of about 3 to about 6 upon heating. In certain embodiments, one or more properties of the protein product makes it well suited for incorporation into food products, including but not limited to dairy products, dairy replacement

products, meat products, meat replacement and/or imitation meat products, bakery products, confections, health and protein bars, protein powders, sports and/or energy drinks, and/or protein shakes and/or smoothies. In certain embodiments, the protein product is textured for incorporation into meat products and/or imitation meat products. In certain embodiments, the protein product can be used as a meat extender, for example, as a meat extender in beef patties. In certain non-limiting embodiments, roughly 30 parts of the protein product can be combined with 70 parts of meat, *e.g.*, ground beef, and in other embodiments, roughly 10 parts of protein product per 90 parts meat, *e.g.*, ground beef. In certain non-limiting embodiments, the protein product is combined with beef and/or other meat products in a ratio that conforms to the requirements set forth by the USDA and/or in accordance with regulations governing the National School Lunch Program (Type A School Lunch). In certain non-limiting embodiments, the protein product is included in a formulation having a combined protein equivalence ratio (PER) of around 2.6. In certain non-limiting embodiments, the water absorption and/or fat binding properties of the protein product aids in reducing shrinkage (fat and water loss) on cooking and/or enhances the moisture and texture of the cooked patty or other meat or food item.

[124] In certain embodiments, protein product produced as described herein is included in a recipe and/or formulation along with one or more of the following ingredients: water; tomatoes; tomato juice; tomato sauce; beans; spices including but not limited to chili spice; seasoning; animal protein; beef; poultry (*e.g.*, chicken, turkey, duck, goose); pork; fish; seafood; soy; wheat; flour; yeast; yeast extract; spirulina; margarine; butter; dairy; cheese; sugar; brown sugar; honey; egg; salt; vanilla; chocolate; baking soda; baking powder; condensed milk; and/or caramel. In certain embodiments said combined ingredients are subjected to one or more of hydrating; blending; mixing; beating; sifting; sprinkling; heating; cooking; frying; deep frying; baking; simmering; browning; boiling. In certain embodiments the said ingredients are fried at around 350°F and/or are baked or cooked at around 375°F to 450°F. In certain embodiments, the protein product is used as a meat extender or meat substitute in one or more of the following: patties; chili con carne; pizza toppings; ground beef; chicken nuggets; fish sticks. In certain embodiments as a meat extender and/or substitute, the protein product replaces about 50% or more than about 50% (*e.g.*, any of at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, or at least about 80%) of the meat ingredient in the food item, in other embodiments they replace less than 50% (*e.g.*, any of less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, or less than about 10%).

[125] In certain embodiments, the protein product ingredient imparts improved nutrition, water absorption, fat binding properties, texture, and/or eating qualities to a food product, such as a cereal based product. In certain embodiments, said protein product ingredient is used to fortify or is otherwise incorporated into a cereal based product. In certain said embodiments, said cereal based product is a breakfast cereal, cookie, cake, pie, brownie, muffin, or bread. In certain embodiments,

the protein product is used as a replacement for milk proteins (e.g., sodium caseinate) and/or as a vitamin and/or mineral supplement in milk or dairy products. In certain such embodiments, the protein product ingredient is used in one or more of non-fat dried milk, powdered milk, or dairy type drinks, such as, but not limited to, instant breakfast mixes, or imitation dairy type drinks including but not limited to soy milk, rice milk, and almond milk. In certain embodiments, the protein product ingredient is used in nutritionally fortified (e.g., protein, vitamin, and/or mineral fortified) candies, deserts, or treats.

[126] In some embodiments, protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), derived from one or more microorganisms described herein, is processed to produce a food product or ingredient thereof, in a process that includes heating the protein product, optionally in combination with other ingredients such as, for example, plant-derived protein, under shearing agitation, followed by extrusion to produce a product of desired texture (e.g., chewy, crunchy, crispy, resists dispersion in water, etc.). In exemplary, non-limiting embodiments, an aqueous paste of protein product (for example, containing about 20 % (w/w) to about 50 % (w/w), about 20% (w/w) to about 40% (w/w), about 30% (w/w) to about 50% (w/w), about 20% (w/w) to about 35% (w/w) or about 35% (w/w) to about 50% (w/w) water), optionally in combination with plant-based materials such as vegetable protein (such as, for example, soybean meal, sesame meal, cottonseed meal, corn meal, wheat meal, and/or peanut meal), is heated to a temperature of about 150° F. to about 400° F, or about 225 ° F to about 275 ° F., for about 10 seconds to about 300 seconds, applying a shearing force, optionally simultaneously with the heating, for example, with a shear rate of about 10 rpm to about 60 rpm and torque of about 200 to about 2,000 meter-grams, and extruding the heated and sheared protein product through a die to provide a shaped extrudate. Optionally, the extrudate is exposed to an oxygen-containing gas stream. In an embodiment, the oxygen-containing gas stream is an air stream (e.g., a dry air stream), for example, at a temperature of about 80° ° F. to about 212° F. for about 0.5 minutes to about 10 minutes.

[127] In some embodiments, protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), derived from one or more microorganisms described herein, is processed to produce a food product or ingredient thereof, in a process that includes combining the protein product with one or more additional protein source (such as, but not limited to, pea, rice, glutinous rice, wheat, gluten, soy, hemp, canola, insects, algae, and/or buckwheat), heating the mixture (e.g., at about 150° F to about 400° F., and subjecting the mixture to a shearing force with an extruder to create a textured product with desired textural and/or functional characteristics (e.g., chewy, crunchy, crispy, resists dispersion in water, etc.).

[128] In some embodiments, free amino acids are included, either as part of the protein product or supplemental to the protein product, to impart a desired flavor. In one non-limiting embodiment, glutamic acid is included, thereby imparting a umami flavor to the food product.

[129] In some embodiments, for example, in a meat substitute or meat analogue product, a hydrogel, lipogel, and/or emulsion is included in the composition, for example, as an agent release system (e.g., for release of a coloring agent, a flavor agent, a fatty acid, a leavening agent, a gelling agent (e.g., bicarbonate (e.g., potassium bicarbonate), calcium hydroxide, and/or alginate (e.g., sodium or potassium alginate))), wherein the agent(s) may be released during cooking of the food product to simulate animal meat).

[130] In some embodiments, a food product includes one or more plant protein source such as, but not limited to, pea, rice, glutinous rice, wheat, gluten, soy, hemp, canola, insects, algae, and/or buckwheat, in combination with a protein product produced by microorganisms as described herein (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), wherein the protein product imparts a flavor to the food composition, such as, for example, a meat-like flavor (including a livestock, game, poultry, or seafood meat-like flavor).

[131] In some embodiments, a food product, for example, a meat substitute or meat analogue product, includes a heme compound, such as a heme-containing polypeptide. In one embodiment, the food product includes heme (e.g., heme-containing polypeptide) from the microorganism from which the protein product is derived. In certain such embodiments the said microorganism is a *Cupriavidus* microorganism, such as *Cupriavidus necator*.

[132] In some embodiments, a meat substitute or artificial or imitation meat product (e.g., a livestock (e.g., beef, pork), game, poultry, fish, or seafood analogue product) includes a protein product produced by microorganisms as described herein (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof). In some embodiments, the meat analogue product is a vegan product that does not contain any ingredients from animal sources. In some embodiments, an enhanced meat product which contains animal protein (e.g., a beef, poultry, pork, fish, seafood, or egg product, in which a portion of the product is a protein product ingredient produced by microorganisms as described herein (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof)), is provided. For example, the protein product may be included as an extender in an enhanced meat product or in a meat analogue product, e.g., the protein product replaces any of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, or at least about 70% of the meat ingredient or an artificial or imitation meat ingredient (for example, a plant-based artificial or imitation meat analogue ingredient) to produce the enhanced meat product or meat analogue/imitation meat product, respectively. In some embodiments, the microorganisms are CO₂-grown or air-grown microorganisms, e.g., oxyhydrogen microorganisms. Nonlimiting examples of meat substitute products are provided in U.S. Patent Nos. 10,327,464, 10,314,325, 10,287,568, 10,273,492, 10,172,380, 10,172,381, 10,093,913, 10,087,434, 10,039,306, 9,943,096, 9,938,327, 9,833,768,

9,826,772, 9,808,029, 9,737,875, 9,700,067, and 9,011,949, which are incorporated by reference herein in their entireties.

[133] In some embodiments, at least a portion, all, or substantially all of the protein product in a food product described herein, including but not limited to, a meat substitute or meat analogue product, includes protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof) derived from a *Cupriavidus* microorganism, such as, but not limited to, *Cupriavidus necator*, e.g., DSM 531 or DSM 541.

[134] In some embodiments, at least a portion, all, or substantially all of the protein product in a food product described herein, including but not limited to, a meat substitute or meat analogue product, includes protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof) derived from a lactic acid bacterium, such as, but not limited to a *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium. In some embodiments, the lactic acid bacterium is a Generally Recognized as Safe (GRAS) bacterium.

[135] In some embodiments, at least a portion or all of the protein product in a food product described herein, including but not limited to, a meat substitute or meat analogue product, includes protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof) derived from a *Fusarium*, *Rhizopus*, or *Aspergillus* fungal microorganism, such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus oryzae*, or *Apergillus sojae*. In some embodiments, the fungal microorganism is a GRAS microorganism.

[136] In some embodiments, the microbial protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof, in which concentration of endotoxins, and optionally nucleic acids, has been reduced) is used as or incorporated into an animal feed product. In certain embodiments, the microbial protein product can be used as a nutritional additive with a high added value as animal feed, more particularly, for livestock feed (e.g., cattle, sheep, pigs, goats, etc.), and/or for aquaculture for fish and/or seafood, and/or for insects (e.g., bees) or other invertebrates (e.g., red worms), and/or for heterotrophic fermentations, and/or for domestic animals or pets, and/or for direct human consumption.

[137] Optionally, a soluble hydrolysate fraction can undergo further concentration. In certain embodiments, following a liquid-solid separation step following protein hydrolysis, the soluble hydrolysate fraction is subject to a further concentration step in order to obtain a concentrated soluble hydrolysate fraction. The concentrated soluble hydrolysate fraction has at least about 40%, at least about 50%, at least about 55%, or about 50% to about 55% by weight of dry matter, or higher. Concentration may be performed by any conventional method known in the art, such as, for

example, by heating and vacuum using a rotary evaporator with a thermostatic bath or reverse osmosis, or any other suitable device. The concentrated soluble hydrolysate of certain embodiments has a composition making it suitable for agricultural and livestock purposes. In certain embodiments, it can be used as a nutritional additive for animal feed, more particularly, animal feed for livestock (e.g., cattle, sheep, pigs, goats, etc.), or aquaculture (e.g., finfish, shellfish), or insects (e.g., bees) or invertebrates (e.g., red worms), or for heterotrophic microorganisms (e.g., yeast; *E. coli*), or for domestic animals or pets. In some embodiments, it may be used as an additive or supplement for human nutrition.

[138] In certain embodiments, the insoluble fraction of the hydrolysate can undergo a final drying process to obtain a solid in the form of a paste or a powder, with a moisture content of about 10% to about 15% by weight, or lower. Such a drying process may be performed by any conventional method, such as by using hot air circulating ovens, or by freeze drying, for example. The dried or undried insoluble hydrolysate can be used as a nutritional additive with a high added value for animal feed, e.g., for livestock, aquaculture, insects, invertebrates, for heterotrophic microorganisms (e.g., yeast; *E. coli*), or for domestic animals or pets. In some embodiments, it may be used as an additive or supplement for human nutrition.

[139] Use of chemoautotrophic microorganisms for production of proteins and other components of food products is described in PCT Application Nos. PCT/US20/67555, filed December 30, 2020, PCT/US21/14795, filed January 22, 2021, PCT/US21/20147, filed February 28, 2021, and PCT/US21/23949, filed March 24, 2021, all of which are incorporated herein by reference in their entireties.

Meat analogue products

[140] Meat analogue products are provided that resemble and/or have the flavor of animal meat (e.g., livestock, game, poultry, fish, or seafood meat). The meat analogue product contains protein product (e.g., one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof, from which endotoxins, and optionally nucleic acids, have been removed (e.g., reduced or eliminated) as described herein), derived from one or more microorganism as described herein, and simulates texture and/or physical characteristics of animal meat, such as, for example, flavor, aroma, texture, appearance, etc. In some embodiments, the protein product that is incorporated into the meat analogue product is produced by a GRAS microorganism.

[141] In some embodiments, a meat analogue product includes at least about 10%, at least about 15%, at least about 20%, or at least about 25% by weight of microbial protein product as described herein, optionally bound together by one or more binding agents, to produce a food product that has one or more similar textural and/or functional characteristics in comparison to animal meat. In some embodiments, the meat analogue product resembles animal meat, for example, ground animal meat (e.g., ground beef, ground pork, ground turkey). In some embodiments, the meat analogue product

is principally or entirely composed of ingredients derived from non-animal sources. In alternative embodiments, the meat analogue product is composed of ingredients partially derived from animal sources but supplemented with ingredients derived from non-animal sources. In some embodiments, the meat analogue product further includes one or more agent release systems and/or other ingredients. In various embodiments, meat analogue products herein may be sliced, cut, ground, shredded, grated, or otherwise processed, or left unprocessed. Examples of sliced forms include but are not limited to dried meats, cured meats, and sliced lunch or deli meats. In some embodiments, the meat analogue food products provided herein are shredded and then bound together, chunked and formed, ground and formed, or chopped and formed, for example, to produce a product similar in appearance and/or texture to animal jerky.

[142] In some embodiments, the meat analogue products are vegan. In some embodiments, the meat analogue products comprise no GMO ingredients. In some embodiments, the meat analogue products comprise no ingredients derived from nuts. In some embodiments, the meat analogue products comprise less than about 0.6% or less than about 0.5% by weight of sodium. In some embodiments, the meat analogue products comprise less than about 500 mg, 400 mg, or 140 mg per 85 g serving, or comprise no or substantially no sodium. In some embodiments, the meat analogue products comprise less than about 5.0 g, less than about 3.5 g, or less than about 2.5 g fat per 85 g serving, e.g., less than about 6%, less than about 4% or less than about 3% by weight. In some embodiments, the meat-like food products comprise no gluten or substantially no gluten. In some embodiments, the meat-like food products comprise no soy or substantially no soy. In some embodiments, the meat-like food products comprise no artificial ingredients, such as, but not limited to, xanthan or methylcellulose.

[143] In some embodiments, the meat analogue food product may contain any of at least about 2%, at least about 5%, at least about 10%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, or at least about 90%, or any of about 2% to about 5%, about 2% to about 10%, about 5% to about 10%, about 5% to about 20%, about 10% to about 40%, about 20% to about 50%, about 40% to about 80%, or about 50% to about 90% microbial protein product, as described herein, by weight of the meat analogue product.

[144] In some embodiments, the meat analogue food products provided herein comprise about 3% to about 6%, about 3% to about 30%, or about 5% to about 30% by weight of lipid, e.g., about 3% to about 10%, about 5% to about 10%, about 10% to about 15%, about 15% to about 20%, about 20% to about 30%, about 5% to about 15%, about 10% to about 20%, about 20% to about 30%, about 3% to about 15%, about 5% to about 15%, about 15% to about 30%, about 3% to about 25%, about 5% to about 25%, or about 10% to about 30% by weight of lipid.

[145] In some embodiments, the lipid (including, but not limited to, fatty acids and/or oil) is produced by a microorganism, which may be the same as or different from the microorganism from

which the protein product is derived. The microorganism may be grown in autotrophic culture conditions, heterotrophic culture conditions, or a combination of autotrophic and heterotrophic culture conditions, as described *infra*. In some embodiments, the lipid is produced by the microorganism from which the protein product is derived and is incorporated into the meat analogue product as a component of the protein product. In other embodiments, the lipid is produced by the microorganism from which the protein product is derived, and is isolated or extracted from the microorganism or from the protein product produced by the microorganism, and is then incorporated into the meat analogue product. In further embodiments, the lipid is produced by a different microorganism than the microorganism from which the protein product is derived (for example, produced by any of the microorganisms disclosed herein), and is isolated or extracted from this microorganism or a protein product thereof as described herein, and is then incorporated into the meat analogue product. Nonlimiting examples of lipid-producing microorganisms, such as chemoautotrophic microorganisms that produce lipids, and growth, production, and extraction of lipids thereof, are described in U.S. Patent Nos. 9,085,785, 9,556,462, 9,879,290, and 9,957,534, and in U.S. Publication No. 2013/0078690, all of which are incorporated by reference herein in their entireties. In certain nonlimiting embodiments, the microorganism source of lipids is a *Rhodococcus* species (such as, but not limited to *Rhodococcus opacus*), *e.g.*, DSM 44193, DSM 43205, DSM 43206, DSM 3346, or a *Cupriavidus* species (such as, but not limited to, *Cupriavidus necator* or *Cupriavidus metallidurans*), *e.g.*, DSM 531, DSM 541, DSM 2839). Growth of chemoautotrophic microorganisms on gaseous carbon and/or energy sources is described in U.S. Patent Nos. 9,085,785, 9,157,058, 9,556,462, 9,879,290, and 9,957,534, and 10,696,941, all of which are incorporated by reference herein in their entireties, and in U.S. Publication No. 2013/0078690, and in the disclosure herein, *infra*.

[146] In some embodiments, the meat analogue products comprise about 0.5% to about 10% by weight of total carbohydrate, *e.g.*, about 0.5% to about 1%, about 1% to about 5%, about 5% to about 10%, about 2% to about 8%, or about 3% to about 6% by weight of total carbohydrate.

[147] In some embodiments, the meat analogue products comprise about 0.5% to about 5% by weight of edible fiber, *e.g.*, about 0.5% to about 1%, about 1% to about 5%, about 5% to about 10%, about 2% to about 8%, or about 3% to about 6% by weight of edible fiber.

[148] The meat analogue products provided herein comprise a moisture content (MC) of at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90% by weight. In some embodiments, the meat analogue products comprise a similar MC as animal meat (*e.g.*, livestock (*e.g.*, beef, pork), game, poultry (*e.g.*, chicken, turkey, duck), fish, or seafood (*e.g.*, shrimp, crab, lobster, scallop)).

[149] In some embodiments, the meat analogue products comprise one or more coloring agents. In some embodiments, the meat analogue products comprise one or more color enhancers. In some embodiments, the meat-like food products comprise mixtures of two or more coloring agents, color stabilizers, and/or color enhancers. Non-limiting examples of such mixtures include beet extract

and annatto, beet extract and turmeric, beet extract and saffron, beet extract and purple carrot, beet extract and grape seed extract, beet extract and tomato extract, beet extract and lycopene, beet extract and beta carotene, beet extract and anthocyanin, beet extract and anthocyanin and annatto, beet extract and annatto and lycopene, beet extract and ascorbic acid, anthocyanin and annatto, beet extract and annatto and ascorbic acid, beet extract and annatto and beta carotene, beet extract and turmeric and ascorbic acid, and anthocyanin and lycopene and annatto. In some such embodiments, the coloring agents, color stabilizers, and/or color enhancers are present at equal weight ratios. In other such embodiments, the coloring agents, color stabilizers, and/or color enhancers are present at unequal weight ratios (e.g., 55:45, 60:40, 65:35, 2:1, 70:30, 75:25, 80:20, 5:1, 85:15, 90:10, 20:1, 95:5, or 99:1). In some embodiments, the meat analogue products comprise browning agents, such as, but not limited to, pentose (e.g., ribose, arabinose, xylose), hexose (e.g., glucose, fructose, mannose, galactose), dextrins, and commercial browning agents (e.g., red arrow dextrose, wood-derived agents).

[150] In some embodiments, a meat analogue product herein includes one or more plant protein source such as, but not limited to, pea, rice, glutinous rice, wheat, gluten, soy, hemp, canola, insects, algae, and/or buckwheat, in combination with a protein product produced by microorganisms as described herein, wherein the protein product imparts a meat-like flavor to the composition.

[151] In some embodiments, a meat analogue product herein includes a heme compound, such as a heme-containing polypeptide. For example, the heme compound (e.g., heme-containing polypeptide) may be from the microorganism from which the protein product is derived. In certain such embodiments the heme compound is derived from a *Cupriavidus* microorganism, for example, *Cupriavidus necator*. In certain such embodiments the heme compound is a hemoglobin or flavohemoglobin.

[152] A meat analogue product as described herein may include: one or more protein source (e.g. microbial protein product as described herein, and optionally, plant-based protein; one or more fats (e.g. plant-based oil, such as, but not limited to, sunflower, canola, coconut, palm, and/or vegetable oil; and/or microbially produced fat, lipid, and/or oil; one or more binding agents (e.g., carrageenan, gum cellulose, egg or milk protein, and/or starch); and one or more other ingredients, such as, but not limited to, spices and/or flavors (e.g., salt, spice(s), and/or aroma-producing compound(s)), vitamins (e.g., vitamin B12), and coloring agents. In some embodiments, the meat analogue product includes microbial protein product as described herein and one or more plant-based protein source, such as, but not limited to, protein from peas (e.g., pea protein isolate), mung beans, and/or fava beans, and/or protein from one or more of whey, soy, adzuki, chickpea, lupin, lentil seed, cashew nut, almond seed, walnut, peanut, wheat gluten, rice bran, oat, and seaweed (e.g., brown seaweed). In some embodiments, the meat analogue product includes microbial protein product as described herein and one or more plant-based protein source that is similar to the microbial protein product in one or more functional properties, such as oil holding capacity (OHC) (g/g), water holding

capacity (WHC) (g/g), emulsion stability (min), foaming stability (%), foaming capacity (%), and gelation temperature (°C), such as, but not limited to, protein from peas (*e.g.*, pea protein isolate), mung beans, and/or fava beans.

[153] In one embodiment, a chicken analogue composition contains, *e.g.*, in decreasing order of weight percentage: water; wheat gluten; microbial protein product as described herein, and optionally, plant-based protein; oil; salt; flavoring agents, spices (*e.g.*, onion powder, garlic powder), seasonings (*e.g.*, nutritional yeast). In another embodiment, a chicken analogue composition contains, *e.g.*, in decreasing order of weight percentage: water; wheat gluten; microbial protein product as described herein, and optionally, plant-based protein; oil; seasonings (*e.g.*, nutritional yeast); salt; flavoring agents, spices (*e.g.*, onion powder, garlic powder); salt. In another embodiment, a seafood analogue composition contains, *e.g.*, in decreasing order of weight percentage: water; flour; binding agent; base; starch; microbial protein product as described herein, and optionally, plant-based protein. In another embodiment, a fish analogue composition contains, *e.g.*, in decreasing order of weight percentage: water; microbial protein product as described herein, and optionally, plant-based protein; first plant-derived oil, edible fiber, second plant-derived oil, filler/thickening agent, spices (*e.g.*, onion powder, garlic powder); salt. It will be apparent to one of skill in the art that different ingredients may be substituted for those listed, additional ingredients may be added, listed ingredients may be deleted, and/or proportions of ingredients may be modified to achieve a desired texture, nutritional profile, and/or flavor of the meat analogue product.

[154] In some embodiments, a meat analogue product includes microbial protein product as described herein in combination with cultured meat (meat produced by *in vitro* culturing of animal cells). For example, a cultured meat product may include any of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90% microbial protein product as described herein (*e.g.*, one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), with the remainder cultured meat cells and other ingredients suitable for production of a meat analogue product, as described herein. In some embodiments, the microbial protein product, such as, but not limited to, a microbial protein hydrolysate, or one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof, includes components that improve the flavor of the cultured meat product. In certain embodiments, the protein product, *e.g.*, microbial protein hydrolysate, or one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof, stimulates the development of flavor-enhancing elements in the cultured meat product. "Enhancing the flavor" of a food product, *e.g.*, a meat analogue product, such as a cultured meat product, includes rendering the product more palatable, or imparting one or more flavor components that are found in the naturally-produced counterpart of the cultured product (*i.e.*, in a meat product from the animal from which the cultured cells are derived). In some embodiments, the

cultured meat product includes lipid (including, but not limited to, fatty acids and/or oil) from the protein product as described herein, from the same or different microorganism as described *supra*, from animal cells, or from a plant-based source.

Flavoring Agents

[155] One or more flavoring agent may be included in a meat analogue product as described herein. For example, a flavoring agent or a combination of two or more flavoring agents may be included that are designed to mimic the natural flavor of a meat product, such as a beef, chicken, pork, fish, or seafood (*e.g.*, shrimp, crab, lobster) product.

[156] Precursors of meat flavor may include water soluble components (*e.g.*, amino acids, peptides, carbohydrates, nucleotides, thiamine) and/or lipid or water insoluble components. In some embodiments, meat flavor precursors include free sugars, sugar phosphates, nucleotides, free amino acids, peptides, and/or thiamine. Compounds such as 2-methyl-3-furanthiol, 2-furfurylthiol, methionol, 2,4,5-trimethyl-thiazole, nonanol, 2-trans-nonenal, may be incorporated into or produced upon cooking of a meat analogue product as described herein, thereby imparting a meat like flavor to the product. In some embodiments, reaction of cysteine and a sugar in a meat analogue product as described herein imparts a chicken or pork flavor. For example, reaction between a sulfur containing amino acid, such as cysteine or cystine, and ribose, results in production of 2-methyl-3-furanthiol, which imparts a meat like flavor, such as a chicken flavor.

[157] In some embodiments, a protein product as described herein, for example, a protein hydrolysate and/or free amino acids, derived from one or more microorganisms, may be reacted with thiamine and one or more monosaccharide (*e.g.*, ribose, xylose, arabinose, glucose) and/or polysaccharide to produce a flavoring agent that imparts a meat like flavor. In one embodiment, thiamine is reacted with xylose.

Protein products

[158] A protein product (*e.g.*, one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof), is derived from and/or includes biomass and/or protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, and/or oligopeptides derived from one or more microorganisms described herein.

[159] In accordance with an embodiment, the protein product comprises about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% amino acids, *e.g.*, free amino acids. In accordance with an embodiment, the protein product comprises peptides that comprise about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about

70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% oligopeptides. In accordance with an embodiment, the protein product comprises peptides that comprise about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising about 20 to about 50 amino acids (e.g., 21 to 50 amino acids). In accordance with an embodiment, the protein product comprises peptides that comprise about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising about 50 to about 200 amino acids (e.g., 51 to 200 amino acids). In accordance with an embodiment, the protein product comprises peptides that comprise about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising about 200 to about 500 amino acids (e.g., 201 to 500 amino acids). In accordance with an embodiment, the protein product comprises peptides that comprise about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% polypeptides comprising less than 1000, 900, 800, 700, 600, 500, 400, 300, 200, or 100 amino acids. In accordance with an embodiment, the protein product comprises about 50% to about 55%, about 55% to about 60%, about 60% to about 65%, about 65% to about 70%, about 70% to about 75%, about 75% to about 80%, about 80% to about 85%, about 85% to about 90%, about 90% to about 95%, about 95% to about 98%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 98% of a combination of free amino acids, oligopeptides, and polypeptides having 21 to 50 amino acids, wherein the ratio of free amino acids to oligopeptides to polypeptides having 21 to 50 amino acids is about 1:1:1, or about 0 to about

3:about 0 to about 3:about 0 to about 3, or about 3 to about 6:about 0 to about 3:about 0 to about 3, or about 0 to about 3:about 3 to about 6:about 0 to about 3, or about 0 to about 3:about 0 to about 3:about 3 to about 6, or about 3 to about 6:about 3 to about 6:about 0 to about 3, or about 0 to about 3:about 3 to about 6:about 3 to about 6, or about 3 to about 6:about 0 to about 3:about 3 to about 6, or about 6 to about 9:about 0 to about 3:about 0 to about 3, or about 0 to about 3:about 6 to about 9:about 0 to about 3, or about 0 to about 3:about 0 to about 3:about 6 to about 9, or about 6 to about 9:about 6 to about 9:about 0 to about 3, or about 0 to about 3:about 6 to about 9:about 6 to about 9, or about 6 to about 9:about 0 to about 3:about 6 to about 9.

[160] In some embodiments, the protein product includes free amino acids. In certain embodiments, amino acids are produced by, and in some embodiments may be secreted by a microorganism described herein. Nonlimiting examples of microbial amino acid production may be found in PCT Application No. WO2014/145194, which is incorporated herein by reference in its entirety.

[161] In some embodiments, the protein product exhibits water and/or oil absorption at a level that is suitable for incorporation into a food composition as described herein, such as, but not limited to a meat analogue or meat substitute composition. For example, the water holding capacity of the protein product may be about 1 to about 10, e.g., about 2 to about 4, times by weight (g/g). For example, the oil holding capacity of the protein product may be about 0.5 to about 10, or about 0.8 to about 5, times by weight (g/g).

[162] In one embodiment, the protein product includes heme (e.g., heme-containing polypeptide), which is produced by the microorganism from which the protein product is derived, such as a *Cupriavidus* microorganism, e.g., *Cupriavidus necator*.

[163] In some embodiments, at least a portion, all, or substantially all of the protein in a protein product described herein, including but not limited to, single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, and/or oligopeptides, is derived from a *Cupriavidus* microorganism, such as, but not limited to, *Cupriavidus necator*, e.g., DSM 531 or DSM 541.

[164] In some embodiments, at least a portion, all, or substantially all of the protein in a protein product described herein, including but not limited to, single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, and/or oligopeptides, is derived from a lactic acid bacterium, such as, but not limited to a *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium. In some embodiments, the lactic acid bacterium is a GRAS bacterium.

[165] In some embodiments, at least a portion, all, or substantially all of the protein product in a protein product described herein, including but not limited to, single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, and/or oligopeptides, is derived from a *Fusarium*, a *Rhizopus*, or an *Aspergillus* fungal microorganism, such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus*

oryzae, or *Aspergillus sojae*. In some embodiments, the fungal microorganism is a GRAS microorganism.

Protein concentrates

[166] In certain embodiments, methods are employed that extract non-protein fractions (e.g., lipids, nucleic acids, polysaccharides) without solubilizing the major protein fractions. The insoluble protein fractions that are recovered are protein concentrates. In certain embodiments, a protein concentrate is produced from protein-containing biomass produced by one or more microorganisms as described herein.

[167] In certain embodiments, a protein concentrate is produced via a solvent extraction process. In certain such embodiments, the solvent extraction process comprises an alcohol extraction or wash, such as, for example, an aqueous alcohol wash. In certain embodiments an acid treatment is utilized to produce a protein concentrate. In certain embodiments a protein concentrate is produced via a heat denaturation process.

[168] In certain embodiments, one or more of solvent extraction, acid treatment, and/or heat denaturation steps are deployed, and may be used sequentially or in parallel for production of a protein concentrate. In certain embodiments, a solvent extraction step is followed by a heat denaturation step in the production of a protein concentrate. In certain embodiments, a heat denaturation step is followed by a solvent extraction step in the production of a protein concentrate. In certain embodiment a heat denaturation step and an acid treatment are combined in the production of a protein concentrate. In certain such embodiments, an insoluble material resulting from heat + acid treatment is subjected to a solvent extraction step. In certain embodiments, a protein concentrate produced via one or more of solvent extraction, acid treatment, and/or heat denaturation step(s) is subjected to a water wash.

[169] In certain embodiments, a protein concentrate as described herein contains at least a portion, or most, of the oil and/or water soluble non-protein constituents that were present in the starting biomass removed by the protein concentrate process.

[170] In certain embodiments, a protein concentrate as described herein contains at least about 60%, at least about 70%, at least about 80%, or at least about 90% protein by weight, on a moisture free basis. The crude protein content may be defined as the total nitrogen weight percentage (%N) of a material multiplied by a Jones factor equal to 6.25, i.e., crude protein = 6.25*%N. In certain embodiments, a protein concentrate as described herein contains a crude protein content of at least about 60%, at least about 70%, at least about 80%, or at least about 90% protein by weight, on a moisture free basis. The determination of the total amino acid content of a proteinaceous material is well established in the science of biochemical analysis (e.g., using AOAC method 994.12). In certain embodiments, a protein concentrate as described herein contains a total amino acid content of at least about 60%, at least about 70%, at least about 80%, or at least about 90% by weight of the concentrate, on a moisture free basis. In certain embodiments, a protein concentrate produced

according to the present invention contains a higher protein content and/or higher crude protein content and/or higher total amino acid content, than a soy protein concentrate.

[171] In certain embodiments, a protein concentrate as described herein contains a carbohydrate content of less than about 20%, less than about 10%, less than about 5%, or less than about 1% by weight. In certain embodiments, a protein as described herein contains an ash content of less than about 10%, less than about 8%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% by weight. In certain embodiments, a protein concentrate as described herein contains a lipid content of less than about 10%, less than about 8%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, or less than about 1% by weight.

[172] In certain embodiments, a solvent extraction process is applied in the production of a protein concentrate. The solvent may include one or more alcohols or one or more alcohols in aqueous solution. The production of a protein concentrate using alcohol solvent is based on the ability of solutions comprising lower aliphatic alcohols (e.g., methanol, ethanol, isopropyl alcohol) to extract lipids and the soluble sugar fractions without solubilizing proteins and/or by rendering protein insoluble by denaturation. In certain embodiments, the concentration of alcohol used in the solvent in a solvent extraction process is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 95%, or at least about 99% by weight.

[173] Starting with wet or dry microbial biomass produced as described herein, in certain embodiments, the protein concentration process comprises one or more of the following steps: liquid-solid extraction, removal and recovery of a solvent from the liquid extract, removal and recovery of a solvent from the solid (e.g., the protein concentrate), and drying and grinding of the solid (e.g., the protein concentrate).

[174] In certain embodiments, solid-liquid extraction is performed batchwise or continuously. In certain embodiments, solid-liquid extraction is performing using one or more of: horizontal belt extractors; basket extractors; stationary extractors; and/or rotary cell extractors.

[175] Heat treatment may render sugars less soluble by binding them to proteins (e.g., Maillard reaction) or by caramelization. Such condensation reactions may make sugars less extractable by solvent. They may also result in darker colors for the concentrates that may be undesirable in certain applications. In certain embodiments, a heat treatment is not performed until after solvent extraction. In certain embodiments, the protein concentrate process utilized avoids the occurrence of the Maillard reaction.

[176] In certain embodiments, a non-polar solvent is utilized in a solvent extraction step. In certain embodiments, a non-polar solvent is utilized in combination with an alcohol solvent. In certain embodiments a non-polar solvent is utilized in combination with an aqueous alcohol solution. In certain embodiments, a non-polar solvent is utilized to extract neutral lipids from an extract produced using alcohol and/or an aqueous alcohol solution. In certain non-limiting embodiments, a non-polar

solvent is utilized that has a boiling point range (*i.e.*, distillation range) of 65 °C to 70 °C. In certain non-limiting embodiments, a non-polar solvent is utilized that consists primarily of six-carbon alkanes. In certain embodiments, hexane is utilized as a non-polar solvent. In certain such embodiments, the hexane utilized as a non-polar solvent complies with the strict quality specifications required for the extraction of edible oils from soybean and other plant-based sources, including but not limited to: boiling (distillation) range, maximum non-volatile residue, flash point, maximum sulfur, maximum cyclic hydrocarbons, color and specific gravity.

[177] In certain embodiments, "supercritical extraction" using liquid carbon dioxide under high pressure is utilized for solvent extraction.

[178] It is known that the rate-limiting process in solvent extraction of biological materials is often diffusion. For this reason, typically substantial effort and energy are directed toward size reduction of the biomass to be extracted. For example, soybeans and oilseeds are often rolled into thin flakes, thus reducing one dimension to facilitate diffusion. In certain embodiments, the microscopic dimensions of the single cell microorganisms as described herein obviate the need to perform any size reduction prior to solvent extraction. In certain embodiments, solvent extraction is more efficient, *i.e.*, requires less solvent and/or recovers a higher percentage of extractable solute, than comparable solvent extraction performed on higher plant or animal biomass.

[179] In certain embodiments, the cell mass, *i.e.*, microbial biomass produced as described herein is kept in liquid suspension when subjected to solvent extraction or if dried, is fed as a loose powder with open, porous structure into a solvent extraction process.

[180] In certain embodiments, the rate of extraction is increased by applying one or more of agitation and/or increasing the temperature. Higher temperature can result in higher solubility of the extractable material (*e.g.*, lipid), and/or higher diffusion coefficients.

[181] Water-free (absolute) low aliphatic alcohols, such as ethanol or isopropanol, are fairly good solvents for lipids at high temperature, but the solubility of oils in these solvents decreases drastically as the temperature is lowered. In certain embodiments, lipid extraction takes place at high temperature one or more alcohol, including but not limited to ethanol, isopropanol, and/or methanol. In certain such embodiments, the lipid extract is cooled, and lipid saturation occurs. In certain such embodiments, the excess lipid separates as a distinct phase, which can be recovered by a solid-liquid separation process, such as, but not limited to, centrifugation. In certain such embodiments, the solvent, *i.e.*, alcohol(s), is reheated and sent back for solvent extraction.

[182] When a concentration gradient is used to transfer the extractable substance out of a solid, keeping the gradient high can facilitate the extraction process. In certain embodiments, the principle of counter-current multistage extraction is utilized to exploit this effect. In certain embodiments, the solvent extraction process is divided into a number of contact stages. In certain embodiments, each stage comprises the mixing of solid, *e.g.*, microbial biomass and/or protein concentrate, and the solvent phases, and the separation of the two streams after extraction is achieved. In certain embodiments, in going from one stage to the next, the solids, *e.g.*, microbial biomass and/or protein

concentrate, and the solvent flow in opposite directions. Thus, microbial biomass and/or protein concentrate with the lowest extractable content (e.g., lipids) are contacted with the leanest solvent, resulting in higher extractable yield (e.g., lipid yield) and high driving force throughout the extractor.

[183] In certain embodiments, solvent extraction is performed using batch, semi-continuous and/or continuous solvent extractors.

[184] In batch processes, a certain quantity of microbial biomass and/or biological material is contacted with a certain volume of fresh solvent. In certain embodiments, the extract is drained off, distilled and the solvent is recirculated through the extractor until the residual extractable content (e.g., lipid content) in the batch of microbial biomass and/or biological material is reduced to a targeted level.

[185] In certain embodiments, a semi-continuous solvent extraction system is utilized that consists of several batch extractors connected in series. In certain such embodiments, the solvent and/or extract flows from one extractor to the next one in the series. In certain non-limiting embodiments, a French Stationary Basket Extractor is utilized.

[186] In certain embodiments, a continuous solvent extraction process is utilized in which microbial biomass and/or biological material and/or protein concentrate and solvent are fed continuously into an extractor. In certain embodiments, one or more of: belt extractors, such as but not limited to a De Smet extractor; moving basket extractors, such as but not limited to a Lurgi moving basket extractor or T.O.M. (Turning Over of Material) HLS extractor; and/or carousel extractors are utilized for solvent extraction.

[187] In certain embodiments, a protein concentrate produced as described herein invention has a residual lipid content of no more than about 0.6% by weight, about 0.25% to about 0.6% by weight, or no more than about 0.25% by weight. In certain embodiments of protein concentration production as described herein that utilize solvent extraction, the solvent losses per extraction are no more than about 0.3% per extraction, or about 0.07% to about 0.3% per extraction, or no more than about 0.07% per extraction.

[188] In certain embodiments, at least two streams leave the solvent extraction step, including: an extract (e.g., lipid extract) stream and a solid (e.g., protein concentrate) stream. In certain such embodiments, the solid stream contains solvent residues. In certain embodiments, one or more processes are utilized for removing and recovering the solvent from one or the other or both of the streams.

[189] In certain embodiments, alcohols are removed from the liquid extract by evaporation and rectified by distillation. In certain such embodiments, the alcohols are then brought to the proper concentration for further extraction. In certain such embodiments, the recovered solvent is recycled through an extractor. In certain embodiments the distillation residue includes lipids and/or an aqueous solution comprising nucleic acids, sugars, and/or other solubles. In certain embodiments the aqueous residue is concentrated to roughly 50% total soluble solids. In certain embodiments the lipids and/or aqueous residue are used as a caloric ingredient and/or as a binding agent in animal

feeds. In certain embodiments, the lipids and/or aqueous residues are fed back into a bioreactor. In certain embodiments the lipids and/or aqueous residues are fed back into a bioreactor where they may be used for mixotrophic growth. In certain such cases, the mixotrophic growth comprises the growth on H₂ and organic substrates, including but not limited to lipids and/or nucleic acids. In certain embodiments the extraction contains 30% or less lipids. In certain embodiments, for every ton of lipid recovered, roughly 2.5 tons of solvent is recovered by distillation. In certain embodiments, one or more methods of solvent removal are utilized such as, but not limited to: flash evaporation, vacuum distillation, and/or steam stripping.

[190] In certain embodiments, solvents are removed from the solid resulting from one or more solvent extraction steps. In certain embodiments, flash desolventizing is utilized to remove solvent residues. In certain embodiments, superheated vapors of an alcohol-water mixture are applied to protein concentrates produced as described herein. In certain embodiments, steam distillation is used to remove solvent residues or traces of solvent from solids resulting from solvent extraction. In certain such embodiments, the said solids recovered from solvent extraction are used to produce a protein concentrate. In certain embodiments, desolventizing of solids recovered from solvent extraction is performed via flash desolventizing (FD). In certain such embodiments, solids with solvent residues coming out of an extractor are fluidized in a stream of superheated solvent vapors where the superheat of the vapor provides the energy for the evaporation of solvent from the solids. In certain such embodiments, the turbulent nature of the solid-vapor flow facilitates rapid heat and mass transfer. In certain embodiments, a short stripping stage is utilized for complete solvent removal. In certain embodiments, rapid cooling follows the removal of residual solvents. In certain embodiments, any excess water left in protein concentrate after desolventizing is removed by methods such as but not limited to hot air drying, drying under hot inert gases, vacuum drying, or freeze drying.

[191] In certain embodiments, an acid treatment or acid-wash process is utilized to precipitate proteins from solution, and the precipitated protein is used to produce a protein concentrate. In certain embodiments, the pH of the broth or biomass or biomass lysate emerging from the bioreactor is roughly pH = 7, or roughly in the range of pH 6 to pH 8. Proteins generally exhibit minimum aqueous solubility at pH ranges corresponding to the isoelectric range of the protein. In certain embodiments, the isoelectric range of the proteins produced as described herein (*e.g.*, proteins produced utilizing a CO₂ carbon source) occurs at a pH less than pH = 6, or in a range of pH 3 to pH 6, pH 4 to pH 5, pH 4.2 to 4.5, or at pH of about 4, or around pH 4.2 or around pH 4.5. In certain embodiments, an acid, such as, but not limited to, one or more of: phosphoric acid, sulfuric acid, hydrochloric acid, acetic acid, and/or carbonic acid/CO₂ (aqueous), is utilized to lower the pH of the culture broth and/or biomass and/or biomass lysate to the isoelectric range. In certain embodiments, proteins are precipitated by lowering the pH to the isoelectric range, and nucleic acids, sugars, and/or other soluble non-proteins are extracted using as a solvent water to which an acid has been added, so as to keep the pH in the isoelectric region. In certain embodiments, rotary

vacuum filters or decanting centrifuges are used for solid-liquid separation with the solids comprising the precipitated protein.

[192] In certain embodiments, lysed or defatted microbial biomass produced as described herein is mixed with acidified water in an agitation vessel. In certain such embodiments, the slurry is then fed to a decanter centrifuge, which separates the extracted solids from the extract. In certain such embodiments, the solids are continuously discharged from the decanter centrifuge. In certain such embodiments the discharged solids have about 10%, about 20%, about 30%, or about 10% to about 30% by weight dry matter content. In certain such embodiments, the solids recovered in this way are dried to yield an isoelectric protein concentrate. In certain embodiments, an isoelectric solid cake is resuspended in water and the acidity is neutralized, and a second step of centrifugal separation produces a cake of neutral protein concentrate. In certain such embodiments, the protein concentrate has a protein content of at least about 60%, at least about 70%, at least about 75%, or at least about 80% by weight on a dry matter basis.

[193] In certain embodiments, the protein solubility of the neutralized product as indicated by the nitrogen solubility index (NSI) is an NSI of at least about 40%, at least 50%, at least 60%, or an NSI value greater than about 60%.

[194] In certain embodiments, the liquid extract containing soluble components such as nucleic acids, sugars, minerals, and the protein fractions which are soluble at a pH of less than 6, pH 3 to pH 6, pH less than 5, pH about 4.5, or pH 4 to pH 5, are fed back to the original bioreactor and/or to another bioreactor for mixotrophic or heterotrophic microbial biomass production. In certain such embodiments, additional proteinaceous biomass is produced.

[195] In certain embodiments, a heat denaturation and/or water extraction process is used in the production of a protein concentrate. In certain embodiments, the proteins produced as described herein are rendered insoluble by thermal denaturation, using humid heat. In certain embodiments, microbial biomass produced as described herein is heated in boiling water or in a pressure cooker or in an autoclave. In certain embodiments, microbial biomass produced as described herein is submitted to a continuous high temperature-short time humid heat treatment, using for example, an extruder-cooker. In certain embodiments, the heat treatment of the microbial biomass involves subjecting the biomass to temperatures of at least about 90°C, at least about 100°C, at least about 110°C, at least about 120°C, at least about 130°C, or at least about 140°C, or temperatures of about 100°C to about 121°C, or temperatures of at most 150°C. In certain embodiments, the duration of heat treatment is at least about 5 minutes, at least about 10 minutes, at least about 15 minutes, at least about 30 minutes, at least about 45 minutes, or at least about one hour, at least about three hours, at least about five hours, or about 24 hours, or the duration is less than about 48 hours, or less than about 72 hours. In certain embodiments, heat-treated microbial biomass produced as described herein is extracted with hot water, which dissolves nucleic acids, sugars, and/or other non-protein solubles. In certain embodiments, following heat treatment and protein denaturation, solid-liquid separation steps well known in the science of producing protein concentrates from soy

and other plant-based sources of protein are utilized to separate the solid denatured proteins from the non-protein solubles. Examples of equipment and processes that may be used in the present invention to separate the protein-rich solids, from the protein-depleted liquid include but are not limited to one or more of: rotary vacuum filters, decanting centrifuges, continuous centrifuges, and belt presses.

[196] In certain embodiments, protein-rich solids (*i.e.*, cake) produced through one or more of the previously described processes, and specifically one or more of: solvent extraction, heat denaturation, and acid/isoelectric precipitation, are run through an extruder. In certain such embodiments, the extrudate is cooled and then ground.

[197] In certain embodiments, protein-rich solids (*i.e.*, cake) and/or extrudate, produced through one or more of the previously described processes, and specifically one or more of: solvent extraction, heat denaturation, and acid/isoelectric precipitation, optionally followed by extrusion, are dried using drying processes well established in the production of protein concentrates from soybeans and other plant-based sources. In certain embodiments, the protein-rich cake is wet-milled to a fine slurry. In certain such embodiments, the slurry is then spray dried. In certain embodiments, the protein-rich cake or extrudate is freeze dried. In certain embodiments, the protein-rich cake or extrudate is dried in forced circulation driers. In certain embodiments, the protein-rich cake or extrudate is dried to a moisture content of about 10% or less.

[198] In certain embodiments, a protein-rich cake or extrudate is ground into a fine powder. In certain such embodiments, at least about 97% of the ground product passes through a 100-mesh standard screen. In other embodiments of the present invention, the protein-rich cake or extrudate is converted to the form of grits, with a coarser granulation. In certain embodiments, a protein-rich cake or extrudate is converted to powder or grits using one or more of: hammer mills, pin mills, impact turbo mills and/or similar pulverizers. In certain said embodiments, not more than about 3% of the ground product is retained by a 100-mesh screen. In certain embodiments, an air classification system is used to separate fine product from coarse fractions. In certain embodiments, coarse fractions are recirculated back through the mill or pulverizer.

[199] In certain embodiments, the final form of the protein concentrate emerging from the process as described herein is granular, or flour-like, or spray dried, or texturized.

[200] In certain embodiments, a protein concentrate produced as described herein contains less than about 1% lipid by weight. In other embodiments, a protein concentrate produced as described herein contains less than about 20%, less than about 15%, less than about 10%, or less than about 5% lipid by weight. In certain embodiments, the lipid content of a protein concentrate produced as described herein varies from about 1% to about 10% by weight, or in certain embodiments about 4.5% to about 9% or about 5% to about 6%.

[201] In certain embodiments, a plant-based oil or fat is combined with a protein concentrate produced as described herein, where the combined lipid content of the protein concentrate and plant-oil or -fat formulation varies from about 4.5% to about 9%, about 5% to about 6%, about 9% to

about 15%, or about 15% and about 20%, by weight. In certain embodiments, the said formulation of protein concentrate and plant-oil or -fat has a total lipid content of about 15% by weight.

[202] In certain embodiments, a lecithin, such as, but not limited to, a soybean lecithin or an egg lecithin, is combined with a protein concentrate produced as described herein. In certain such embodiments, the addition of lecithin increases the dispersibility and emulsifying properties of the protein concentrate. In certain such embodiments, the lecithin content of the formulation comprising the protein concentrate and lecithin, varies up to about 15% by weight.

[203] In certain embodiments, the oil and/or phospholipid content of the microbial biomass produced as described herein possesses egg and/or shortening type effects and can act as an emulsifier.

[204] It is known that high lipid content in a protein concentrate can lead to poor storage stability. In certain embodiments, a low lipid protein concentrate is produced with increased storage stability.

[205] In certain embodiments, a protein concentrate produced as described herein can have an NSI of up to about 80%, about 70%, about 60%, about 50%, about 40%, about 30%, about 20%, or about 10%. In certain embodiments, a protein concentrate produced as described herein can have an NSI of about 10% to about 20%, about 10% to about 30%, about 20% to about 30%, or about 10% to about 15%.

[206] In certain embodiments, the dispersibility and functionality of the protein concentrate is increased by steam injection or jet-cooking, and/or by high-shear homogenization.

Protein hydrolysates

[207] In some embodiments, at least a portion, all, or substantially all of the protein product is produced by hydrolyzing protein (e.g., single cell protein, cell lysate, protein concentrate, protein isolate, and/or protein extract) from at least one microorganism described herein. For example, hydrolysis of cellular protein may produce peptides, oligopeptides, and/or free amino acids.

[208] Hydrolysis of microbial protein may be performed by acidic, basic, and/or enzymatic processes. Methods for hydrolyzing protein are well known in the art. Nonlimiting examples of microbial protein hydrolysis methods and hydrolysate compositions may be found in U.S. Provisional Application Nos. 62/901,169 and 62/943,754, and in PCT Application No. US20/50902, which are incorporated herein by reference in their entireties.

[209] In some embodiments, a hydrolysis method may include raising or lowering the pH of a proteinaceous suspension, e.g., a suspension of microbial biomass, thereby producing an alkaline or acidic suspension, respectively. The starting biomass suspension may include a suitable amount of the biomass in liquid, for example, microbial biomass in a growth medium. In some embodiments, the amount of the biomass, dried weight / reaction volume, is at least about .01%, at least about 0.2%, at least about 0.5%, at least about 1%, at least about 2%, or at least about 3%, or about 0.1% to about 8%, e.g., about 0.2% to about 8%, about 0.5% to about 6%, about 1% to about 6%, about

2% to about 6%, about 3% to about 5%, about 4% to about 8%, about 6% to about 8% about 5% to about 7%, or about 5% to about 8%.

[210] In some embodiments, microorganism cells within the biomass are subjected to lysis at the beginning of the process, *e.g.*, prior to raising or lowering the pH, to facilitate harvesting the protein from the biomass into a suspension composition.

[211] In certain embodiments, the alkaline or acidic suspension may be subjected to heat for a suitable amount of time, to generate a protein hydrolysate composition. The suspension may be concentrated, dried (*e.g.*, lyophilized), or utilized directly as a liquid suspension. In certain embodiments, the alkaline or acidic suspension is subjected to heat and elevated pressure, *e.g.*, by autoclaving the alkaline or acidic suspension, to generate a protein hydrolysate composition. In some embodiments, the suspension is neutralized with buffer to lower or raise the pH after the heat or heat/pressure treatment. In certain embodiments, the pH is lowered (for an alkaline suspension) or raised (for an acidic suspension) sufficiently to allow subsequent enzymatic treatment of the suspension with a hydrolytic enzyme, such as a protease (*e.g.*, alkaline protease, acid protease, or metalloprotease). After enzymatic hydrolysis, a protein hydrolysate composition is produced. In other embodiments, the biomass suspension is hydrolyzed with a proteolytic enzyme, such as a protease (*e.g.*, alkaline protease, acid protease, or metalloprotease), without prior alkaline or acid treatment.

[212] In certain embodiments, the hydrolyzed protein in the protein hydrolysate is predominantly in the soluble fraction of the suspension. The resulting suspension may be clarified, *e.g.*, by centrifuge, to obtain a supernatant fraction, which contains hydrolyzed protein. In some embodiments, the hydrolytic treatment (*e.g.*, alkaline or acid hydrolysis, optionally including enzymatic (*e.g.*, protease) treatment or enzymatic hydrolysis alone) is followed by clarification of the suspension (hydrolysate) to remove undissolved material in the suspension, *e.g.*, separation of soluble and insoluble fractions. The suspension may be clarified using any suitable method, such as centrifugation, filtration, etc. In some embodiments, after the suspension is clarified, *e.g.*, centrifuged, the supernatant may be separated from the pellet.

[213] In some embodiments, the clarified liquid composition (*e.g.*, soluble fraction, such as supernatant of separated suspension), which contains hydrolyzed protein, is dried, *e.g.*, lyophilized, to produce a dry or substantially dry composition. In some embodiments, the lyophilized composition has a water content of about 10% or less, *e.g.*, about 8% or less, about 6% or less, about 5% or less, or about 3% or less. In some embodiments, the lyophilized protein hydrolysate composition has a water content of about 1% to about 10%, *e.g.*, about 1% to about 8%, about 1% to about 6%, about 2% to about 5%, about 2% to about 6%, about 3% to about 5%, about 4% to about 8%, about 6% to about 8% about 5% to about 7%, or about 5% to about 8%.

[214] In some embodiments, the clarified liquid composition (*e.g.*, soluble fraction, such as supernatant of separated suspension) is dewatered or concentrated to lower the water content. In some embodiments, the concentrated composition has a water content of about 80% or less, *e.g.*,

about 75% or less, about 50% or less, about 40% or less, or about 30% or less; and in some embodiments, each of the foregoing water content ranges may be at least about 20%, at least about 25%, at least about 30%, at least about 40%, or at least about 50% (to the extent such foregoing ranges exceed such lower limits). In some embodiments, the dewatered product is dried, e.g., using heat and/or evaporation, employing a method such as, but not limited to, one or more of spray drying; drum drying; oven drying; vacuum drying; vacuum oven drying; drying under an inert gas such as N₂; and solar evaporation. In some embodiments, the clarified product is dewatered initially with a rotary evaporator, e.g., such that about 50% to about 65% or more of the moisture is removed. In some embodiments, further dewatering is achieved by lyophilization, e.g., such that the lyophilized protein hydrolysate composition has a water content from about 1% to about 10%, e.g., about 1% to about 8%, about 1% to about 6%, about 2% to about 5%, about 2% to about 6%, about 3% to about 5%, about 4% to about 8%, about 6% to about 8% about 5% to about 7%, or about 5% to about 8%.

[215] In some embodiments, at least a portion or all of the protein from which a protein hydrolysate is produced (e.g., single cell protein, cell lysate, protein concentrate, protein isolate, and/or protein extract) is from a *Cupriavidus* microorganism, such as, but not limited to, *Cupriavidus necator*, e.g., DSM 531 or DSM 541. In some embodiments, a protein hydrolysate composition (e.g., containing peptides, oligopeptides, and/or free amino acids) is derived from protein from a *Cupriavidus* microorganism, such as, but not limited to, *Cupriavidus necator*, e.g., DSM 531 or DSM 541.

[216] In some embodiments, at least a portion, all, or substantially all of the protein from which a protein hydrolysate is produced (e.g., single cell protein, cell lysate, protein concentrate, protein isolate, and/or protein extract) is from a lactic acid bacterium, such as, but not limited to a *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium. In some embodiments, a protein hydrolysate composition (e.g., containing peptides, oligopeptides, and/or free amino acids) is derived from protein from a lactic acid bacterium, such as, but not limited to, a *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium. In some embodiments, the lactic acid bacterium is a GRAS bacterium.

[217] In some embodiments, at least a portion or all of the protein from which a protein hydrolysate is produced (e.g., single cell protein, cell lysate, protein concentrate, protein isolate, and/or protein extract) is from a *Fusarium*, a *Rhizopus*, or an *Aspergillus* fungal microorganism, such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus oryzae*, or *Aspergillus sojae*. In some embodiments, a protein hydrolysate composition (e.g., containing peptides, oligopeptides, and/or free amino acids) is derived from protein from a *Fusarium*, a *Rhizopus*, or an *Aspergillus* fungal microorganism, such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus oryzae*, or *Aspergillus sojae*.

[218] In some embodiments, protein hydrolysates herein include peptides that comprise or consist of peptides that are of a size range that is typically non-allergenic, e.g., non-allergenic to humans. In

some embodiments, protein hydrolysates that are incorporated into food compositions as described herein include peptides and free amino acids, wherein the peptides are of a size range that is typically non-allergenic. In some embodiments, non-allergenic peptides are of a size range that is about 800 to about 1500 Da average molecular weight distribution. For example, peptides obtained by protein hydrolysis as described herein may be less than any of about 1500, 1400, 1300, 1200, 1100, 1000, 900, or 800 Da average molecular weight.

[219] In some embodiments, salts are removed from protein hydrolysates (for example, where acid or alkaline salts are used for hydrolysis), prior to incorporation of the hydrolysate into a food composition as described herein. For example, the protein hydrolysate may be purified by filtration (e.g., ultrafiltration) or dialysis to remove salts and/or other impurities.

Microorganisms

[220] Proteinaceous material (protein product as described herein) used in the methods and incorporated into the compositions described herein is derived from one or more microorganism. The microbial organisms from which single cell protein, cell lysate, protein concentrate, protein isolate, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof is derived may be photoautotrophic, heterotrophic, methanotrophic, methylotrophic, carboxydutrophic or chemoautotrophic organisms. In some embodiments, the microbial organisms include oxyhydrogen microorganism. The microbial organisms may be wild-type, or may be genetically modified (e.g., recombinant), or a combination thereof.

[221] Microbial biomass may be collected from a culture of one or more suitable microorganism, e.g., in a fermenter or bioreactor. Biomass may be collected using any suitable method, such as a centrifuge, to separate the cell mass from the culture medium. In some embodiments, the collected biomass may be used to produce a protein hydrolysate composition. In some embodiments, the collected biomass is spray dried or lyophilized to generate a dry biomass, which then may be used as an ingredient for production of a food composition as described herein or to produce a protein hydrolysate composition. In some embodiments, a protein product (e.g., single cell protein, cell lysate, protein extract, protein-containing extract, protein concentrate, protein isolate, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof) is produced from the collected biomass.

[222] In some embodiments, the microorganisms or protein product thereof includes a strain within the genus *Cupriavidus* or *Ralstonia* or *Hydrogenobacter*. In some embodiments, the microorganisms include the species *Cupriavidus necator* or *Cupriavidus metallidurans*. In some embodiments, the microorganisms include a strain of the species *Cupriavidus necator* DSM 531 or DSM 541. In some embodiments, the microorganisms includes the species *Cupriavidus metallidurans*. In some embodiments, the microorganisms include a strain of the species *Cupriavidus metallidurans* DSM 2839.

[223] In some embodiments, the microorganisms or protein product thereof includes a strain within the genus *Xanthobacter*. In some embodiments, the microorganisms includes the species *Xanthobacter autotrophicus*. In some embodiments, the microorganisms include a strain of the species *Xanthobacter autotrophicus* DSM 432.

[224] In some embodiments, the microorganisms or protein product thereof includes a *Rhodococcus* or *Gordonia* microorganism. In some embodiments, the microorganisms include *Rhodococcus opacus*. In some embodiments, the microorganisms include *Rhodococcus opacus* (DSM 43205) or *Rhodococcus sp.* (DSM 3346). In some embodiments, the microorganisms include *Rhodococcus opacus*; *Hydrogenovibrio marinus*; *Rhodopseudomonas capsulata*; *Hydrogenobacter thermophilus*; or *Rhodobacter sphaeroides*. In some embodiments, the microorganisms include a strain within the family burkholderiaceae.

[225] In some embodiments, the microorganisms or protein product thereof includes a lactic acid bacterium, such as, but not limited to a *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium. In some embodiments, the lactic acid bacterium is a GRAS bacterium.

[226] In some embodiments, the microorganisms or protein product thereof includes a *Fusarium*, a *Rhizopus*, or an *Aspergillus* fungal microorganism, such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus oryzae*, or *Aspergillus sojae*. In some embodiments, the fungal microorganism is a GRAS microorganism.

[227] In some embodiments, a consortium of microorganisms (*i.e.*, two or more microorganisms grown together) is used as a source of protein product in the methods and compositions described herein. The consortium may include one or more of any of the microorganism species or strains described herein or one or more microorganisms having one or more microorganism traits described herein. In some embodiments, the consortium includes two or more of any of the microorganism species or strains or microorganisms described herein or two or more microorganisms having one or more microorganism traits described herein.

[228] In some embodiments, a microorganism as described herein can accumulate protein to about 50% or more of the total cell mass by weight. In some embodiments, a microorganism as described herein can accumulate protein to about 60% or more of the total cell mass by weight. In some embodiments, the microorganism can accumulate protein to about 70% or more of the total cell mass by weight. In some embodiments, the microorganism can accumulate protein to about 80% or more of the total cell mass by weight. In some non-limiting embodiments, the microorganism exhibiting these traits is a *Cupriavidus* microorganism, for example, *Cupriavidus necator*, *e.g.*, *Cupriavidus necator* DSM 531 or DSM 541.

[229] In certain embodiments, the biomass that is produced (*e.g.*, microorganism cells) has a higher protein content and/or a lower fat content than soybeans. In certain embodiments the biomass produced (*e.g.*, microorganism cells) has a protein content higher than or at least about any of about 40%, about 50%, about 60%, about 70%, or about 80% by weight, and a fat content of about or lower than any of about 20%, about 15%, about 10%, or about 5% by weight. For

example, the biomass may have a protein content higher than or at least about 40% by weight and a fat content of about or lower than about 20% by weight, or a protein content higher than about or at least about 40% by weight and a fat content of about or lower than about 15% by weight, or a protein content higher than about or at least about 40% by weight and a fat content of about or lower than about 10% by weight, or a protein content higher than about or at least about 40% by weight and a fat content of about or lower than about 5% by weight, or a protein content higher than about or at least about 50% by weight and a fat content of about or lower than about 20% by weight, or a protein content higher than about or at least about 50% by weight and a fat content of about or lower than about 15% by weight, or a protein content higher than about or at least about 50% by weight and a fat content of about or lower than about 10% by weight, or a protein content higher than about or at least about 50% by weight and a fat content of about or lower than about 5% by weight, or a protein content higher than about or at least about 60% by weight and a fat content of about or lower than about 20% by weight, or a protein content higher than about or at least about 60% by weight and a fat content of about or lower than about 15% by weight, or a protein content higher than about or at least about 60% by weight and a fat content of about or lower than about 10% by weight, or a protein content higher than about or at least about 60% by weight and a fat content of about or lower than about 5% by weight, or a protein content higher than about or at least about 70% by weight and a fat content of about or lower than about 20% by weight, or a protein content higher than about or at least about 70% by weight and a fat content of about or lower than about 15% by weight, or a protein content higher than about or at least about 70% by weight and a fat content of about or lower than about 10% by weight, or a protein content higher than about or at least about 70% by weight and a fat content of about or lower than about 5% by weight, or a protein content higher than about or at least about 80% by weight and a fat content of about or lower than about 15% by weight, or a protein content higher than about or at least about 80% by weight and a fat content of about or lower than about 10% by weight, or a protein content higher than about or at least about 80% by weight and a fat content of about or lower than about 5% by weight. In some non-limiting embodiments, the microorganism exhibiting these traits is a *Cupriavidus* microorganism, for example, *Cupriavidus necator*, e.g., *Cupriavidus necator* DSM 531 or DSM 541.

[230] In some embodiments, a microorganism as described herein can naturally grow on H₂/CO₂ and/or syngas and/or producer gas. In some embodiments, the microorganism can naturally accumulate polyhydroxyalkanoate (PHA) (e.g., polyhydroxybutyrate (PHB)) to about 50% or more of the cell biomass by weight. In some embodiments, the microorganism has a native ability to direct a high flux of carbon through the acetyl-CoA metabolic intermediate, which can lead into fatty acid biosynthesis, along with a number of other synthetic pathways, for example, PHA, e.g., PHB, synthesis, and/or amino acid biosynthesis. In some embodiments, the microorganism exhibiting these traits is a *Cupriavidus* microorganism, for example, *Cupriavidus necator*, e.g., *Cupriavidus necator* DSM 531 or DSM 541). In some embodiments, the microorganism does not produce and/or accumulate PHA (e.g., PHB).

[231] In some nonlimiting embodiments, the microorganisms or protein product thereof includes *Corynebacterium autotrophicum*. In some nonlimiting embodiments, the microorganisms include *Corynebacterium autotrophicum* and/or *Corynebacterium glutamicum*. In some embodiments, the microorganisms include *Hydrogenovibrio marinus*. In some embodiments, the microorganisms include *Rhodopseudomonas capsulata*, *Rhodopseudomonas palustris*, or *Rhodobacter sphaeroides*.

[232] In some embodiments, the microorganisms or protein product thereof includes one or more of the following genera: *Cupriavidus*, *Rhodococcus*, *Hydrogenovibrio*, *Rhodopseudomonas*, *Hydrogenobacter*, *Gordonia*, *Arthrobacter*, *Streptomyces*, *Rhodobacter*, and/or *Xanthobacter*.

[233] In some embodiments, the microorganisms or protein product thereof includes a microorganism of the class Actinobacteria. In some embodiments, the microorganisms include a microorganism of the suborder corynebacterineae (*corynebacterium*, *gordoniaceae*, *mycobacteriaceae* and *nocardiaceae*). In some embodiments, the microorganisms include a microorganism of the family of Nocardiaceae. In some embodiments, the microorganisms include a microorganism drawn from one or more of the following classifications: *Corynebacterium*, *Gordonia*, *Rhodococcus*, *Mycobacterium* and *Tsukamurella*. In some embodiments, the microorganisms include a microorganism of the genus *Rhodococcus*, such as *Rhodococcus opacus*, *Rhodococcus aurantiacus*; *Rhodococcus baikonurensis*; *Rhodococcus boritolerans*; *Rhodococcus equi*; *Rhodococcus coprophilus*; *Rhodococcus corynebacterioides*; *Nocardia corynebacterioides* (synonym: *Nocardia corynebacterioides*); *Rhodococcus erythropolis*; *Rhodococcus fascians*; *Rhodococcus globerulus*; *Rhodococcus gordoniae*; *Rhodococcus jostii*; *Rhodococcus koreensis*; *Rhodococcus kroppenstedtii*; *Rhodococcus maanshanensis*; *Rhodococcus marinonascens*; *Rhodococcus opacus*; *Rhodococcus percolatus*; *Rhodococcus phenolicus*; *Rhodococcus polyvorum*; *Rhodococcus pyridinivorans*; *Rhodococcus rhodochrous*; *Rhodococcus rhodnii*; (synonym: *Nocardia rhodnii*); *Rhodococcus ruber* (synonym: *Streptothrix rubra*); *Rhodococcus* sp. RHA1; *Rhodococcus triatmae*; *Rhodococcus tukisamuensis*; *Rhodococcus wratislaviensis* (synonym: *Tsukamurella wratislaviensis*); *Rhodococcus yunnanensis*; or *Rhodococcus zopfii*. In some embodiments, the microorganisms include *Rhodococcus opacus* strain DSM 43205 or DSM 43206. In some embodiments, the microorganisms include strain *Rhodococcus* sp. DSM 3346.

[234] In some embodiments, the microorganisms or protein product thereof includes a microorganism (e.g., a microorganism of any of the microorganism genera or species described herein) that can naturally grow on H₂/CO₂ and/or syngas and/or producer gas, and that can naturally accumulate lipid to any of at least about 10% about, 20% about, 30%, about 40%, about 50%, about 60%, about 70%, about 80%, or more of the cell biomass by weight. In some embodiments, the microorganisms include a microorganism (e.g., a microorganism of any of the microorganism genera or species described herein) that has a native ability to send a high flux of carbon down the fatty acid biosynthesis pathway. In some embodiments, the microorganism exhibiting these traits is a *Rhodococcus* microorganism, for example, *Rhodococcus opacus* (e.g., *Rhodococcus opacus* DSM

43205 or DSM 43206 or DSM 44193), or a *Cupriavidus* microorganism, for example, *Cupriavidus necator* (e.g., *Cupriavidus necator* DSM 531 or DSM 541).

[235] In some embodiments, the microorganisms or protein product thereof include an oxyhydrogen or knallgas strain. In some embodiments, the microorganisms include one or more of the following knallgas microorganisms: *Aquifex pyrophilus*, *Aquifex aeolicus*, or other *Aquifex* sp.; *Cupriavidus necator* or *Cupriavidus metallidurans* or other *Cupriavidus* sp.; *Corynebacterium autotrophicum* or other *Corynebacterium* sp.; *Gordonia desulfuricans*, *Gordonia polyisoprenivorans*, *Gordonia rubripertincta*, *Gordonia hydrophobica*, *Gordonia westfalica*, or other *Gordonia* sp.; *Nocardia autotrophica*, *Nocardia opaca*, or other *Nocardia* sp.; purple non-sulfur photosynthetic bacteria, including but not limited to, *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, *Rhodopseudomonas capsulata*, *Rhodopseudomonas viridis*, *Rhodopseudomonas sulfoviridis*, *Rhodopseudomonas blastica*, *Rhodopseudomonas spheroides*, *Rhodopseudomonas acidophila*, or other *Rhodopseudomonas* sp.; *Rhodobacter* sp., *Rhodospirillum rubrum*, or other *Rhodospirillum* sp.; *Rhodococcus opacus* or other *Rhodococcus* sp.; *Rhizobium japonicum* or other *Rhizobium* sp.; *Thiocapsa roseopersicina* or other *Thiocapsa* sp.; *Pseudomonas facilis*, *Pseudomonas flava*, *Pseudomonas putida*, *Pseudomonas hydrogenovora*, *Pseudomonas hydrognothermophila*, *Pseudomonas palleronii*, *Pseudomonas pseudoflava*, *Pseudomonas saccharophila*, *Pseudomonas thermophile*, or other *Pseudomonas* sp.; *Hydrogenomonas pantotropha*, *Hydrogenomonas eutropha*, *Hydrogenomonas facilis*, or other *Hydrogenomonas* sp.; *Hydrogenobacter thermophiles*, *Hydrogenobacter halophilus*, *Hydrogenobacter hydrogenophilus*, or other *Hydrogenobacter* sp.; *Hydrogenophilus islandicus* or other *Hydrogenophilus* sp.; *Hydrogenovibrio marinus* or other *Hydrogenovibrio* sp.; *Hydrogenothermus marinus* or other *Hydrogenothermus* sp.; *Helicobacter pylori* or other *Helicobacter* sp.; *Xanthobacter autotrophicus*, *Xanthobacter flavus*, or other *Xanthobacter* sp.; *Hydrogenophaga flava*, *Hydrogenophaga palleronii*, *Hydrogenophaga pseudoflava*, or other *Hydrogenophaga* sp.; *Bradyrhizobium japonicum* or other *Bradyrhizobium* sp.; *Ralstonia eutropha* or other *Ralstonia* sp.; *Alcaligenes eutrophus*, *Alcaligenes facilis*, *Alcaligenes hydrogenophilus*, *Alcaligenes latus*, *Alcaligenes paradoxus*, *Alcaligenes ruhlandii*, or other *Alcaligenes* sp.; *Amycolata* sp.; *Aquaspirillum autotrophicum* or other *Aquaspirillum* sp.; *Arthrobacter* strain 11/X, *Arthrobacter methylotrophus*, or other *Arthrobacter* sp.; *Azospirillum lipoferum* or other *Azospirillum* sp.; *Variovorax paradoxus* or other *Variovorax* sp.; *Acidovorax facilis*, or other *Acidovorax* sp.; *Bacillus schlegelii*, *Bacillus tusciae*, other *Bacillus* sp.; *Calderobacterium hydrogenophilum* or other *Calderobacterium* sp.; *Dexia gummosa* or other *Dexia* sp.; *Flavobacterium autothermophilum* or other *Flavobacterium* sp.; *Microcycus aquaticus* or other *Microcycus* sp.; *Mycobacterium gordoniae* or other *Mycobacterium* sp.; *Paracoccus denitrificans* or other *Paracoccus* sp.; *Persephonella marina*, *Persephonella guaymasensis*, or other *Persephonella* sp.; *Renobacter vacuolatum* or other *Renobacter* sp.; *Seliberia carboxydohydrogena* or other *Seliberia* sp.; *Streptomyces coelicoflavus*, *Streptomyces griseus*, *Streptomyces xanthochromogenes*, *Streptomyces thermocarboxydus*, and other *Streptomyces* sp.; *Thermocrinis ruber* or other *Thermocrinis* sp.; *Wautersia* sp.; cyanobacteria including but not limited

to *Anabaena oscillarioides*, *Anabaena spiroides*, *Anabaena cylindrica*, or other *Anabaena* sp., and *Arthrospira platensis*, *Arthrospira maxima*, or other *Arthrospira* sp.; green algae including but not limited to *Scenedesmus obliquus* or other *Scenedesmus* sp., *Chlamydomonas reinhardtii* or other *Chlamydomonas* sp., *Ankistrodesmus* sp., and *Rhaphidium polymorphium* or other *Rhaphidium* sp. In some embodiments, a consortium of microorganisms that includes an oxyhydrogen microorganism, such as any of the above oxyhydrogen microorganisms, is used for production of protein product as described herein.

[236] In some embodiments, the microorganisms or protein product thereof includes one or more of the following genera: *Cupriavidus*; *Xanthobacter*; *Dietzia*; *Gordonia*; *Mycobacterium*; *Nocardia*; *Pseudonocardia*; *Arthrobacter*; *Alcanivorax*; *Rhodococcus*; *Streptomyces*; *Rhodopseudomonas*; *Rhodobacter*; and *Acinetobacter*; or a consortium of microorganisms that includes one or more of these microorganism genera.

[237] In some embodiments, the microorganisms or protein product thereof includes one or more of the following: *Arthrobacter methylotrophus* DSM 14008; *Rhodococcus opacus* DSM 44304; *Rhodococcus opacus* DSM 44311; *Xanthobacter autotrophicus* DSM 431; *Rhodococcus opacus* DSM 44236; *Rhodococcus ruber* DSM 43338; *Rhodococcus opacus* DSM 44315; *Cupriavidus metallidurans* DSM 2839; *Cupriavidus necator* DSM 531; *Cupriavidus necator* DSM 541; *Rhodococcus aetherivorans* DSM 44752; *Gordonia desulfuricans* DSM 44462; *Gordonia polyisoprenivorans* DSM 44266; *Gordonia polyisoprenivorans* DSM 44439; *Gordonia rubripertincta* DSM 46039; *Rhodococcus percolatus* DSM 44240; *Rhodococcus opacus* DSM 43206; *Gordonia hydrophobica* DSM 44015; *Rhodococcus zopfii* DSM 44189; *Gordonia westfalica* DSM 44215, *Xanthobacter autotrophicus* DSM 1618; *Xanthobacter autotrophicus* DSM 2267; *Xanthobacter autotrophicus* DSM 3874; *Streptomyces coelicoflavus* DSM 41471; *Streptomyces griseus* DSM 40236; *Streptomyces* sp. DSM 40434; *Streptomyces xanthochromogenes* DSM 40111; *Streptomyces thermocarboxydus* DSM 44293; *Rhodobacter sphaeroides* DSM 158. In some embodiments, the microorganisms or protein product thereof includes a consortium of microorganisms that includes one or more of these microorganism strains, or one or more of any of the microorganism genera or species disclosed herein.

[238] A number of different microorganisms have been characterized that are capable of growing on carbon monoxide as an electron donor and/or carbon source (*i.e.*, carboxydotrophic microorganisms). In some cases, carboxydotrophic microorganisms can also use H₂ as an electron donor and/or grow mixotrophically. In some cases, the carboxydotrophic microorganisms are facultative chemolithoautotrophs [Biology of the Prokaryotes, edited by J Lengeler, G. Drews, H. Schlegel, John Wiley & Sons, Jul 10, 2009, which is incorporated herein by reference in its entirety]. In some embodiments, the microorganisms or protein product thereof includes one or more of the following carboxydotrophic microorganisms: *Acinetobacter* sp.; *Alcaligenes carboxydus* or other *Alcaligenes* sp.; *Arthrobacter* sp.; *Azomonas* sp.; *Azotobacter* sp.; *Bacillus schlegelii* or other *Bacillus* sp.; *Hydrogenophaga pseudoflava* or other *Hydrogenophaga* sp.; *Pseudomonas*

carboxydohydrogena, *Pseudomonas carboxydovorans*, *Pseudomonas compransoris*, *Pseudomonas gazotropha*, *Pseudomonas thermocarboxydovorans*, or other *Pseudomonas* sp.; *Rhizobium japonicum* or other *Rhizobium* sp.; and *Streptomyces* G26, *Streptomyces thermoautotrophicus*, or other *Streptomyces* sp. In some embodiments, the microorganisms or protein product thereof includes a consortium of microorganisms that includes carboxydophilic microorganisms, such as one or more of the above carboxydophilic microorganisms. In certain embodiments, a carboxydophilic microorganism that is capable of chemolithoautotrophy is used. In certain embodiments, a carboxydophilic microorganism that is able to utilize H₂ as an electron donor in respiration and/or biosynthesis is used.

[239] In some embodiments, the microorganisms or protein product thereof includes obligate and/or facultative chemoautotrophic microorganisms, such as one or more of the following: *Acetoanaerobium* sp.; *Acetobacterium* sp.; *Acetogenium* sp.; *Achromobacter* sp.; *Acidianus* sp.; *Acinetobacter* sp.; *Actinomadura* sp.; *Aeromonas* sp.; *Alcaligenes* sp.; *Alcaligenes* sp.; *Aquaspirillum* sp.; *Arcobacter* sp.; *Aureobacterium* sp.; *Bacillus* sp.; *Beggiatoa* sp.; *Butyrivibrio* sp.; *Carboxydotherrus* sp.; *Clostridium* sp.; *Comamonas* sp.; *Cupriavidus* sp.; *Dehalobacter* sp.; *Dehalococcoide* sp.; *Dehalospirillum* sp.; *Desulfobacterium* sp.; *Desulfomonile* sp.; *Desulfotomaculum* sp.; *Desulfovibrio* sp.; *Desulfurosarcina* sp.; *Ectothiorhodospira* sp.; *Enterobacter* sp.; *Eubacterium* sp.; *Ferroplasma* sp.; *Halothibacillus* sp.; *Hydrogenobacter* sp.; *Hydrogenomonas* sp.; *Leptospirillum* sp.; *Metallosphaera* sp.; *Methanobacterium* sp.; *Methanobrevibacter* sp.; *Methanococcus* sp.; *Methanococcoides* sp.; *Methanogenium* sp.; *Methanolobus* sp.; *Methanomicrobium* sp.; *Methanoplanus* sp.; *Methanosarcina* sp.; *Methanospirillum* sp.; *Methanothermus* sp.; *Methanotherx* sp.; *Micrococcus* sp.; *Nitrobacter* sp.; *Nitrobacteraceae* sp., *Nitrococcus* sp., *Nitrosococcus* sp.; *Nitrospina* sp., *Nitrospira* sp., *Nitrosolobus* sp.; *Nitrosomonas* sp.; *Nitrospira* sp.; *Nitrosovibrio* sp.; *Nitrospina* sp.; *Oleomonas* sp.; *Paracoccus* sp.; *Peptostreptococcus* sp.; *Planctomycetes* sp.; *Pseudomonas* sp.; *Ralstonia* sp.; *Rhodobacter* sp.; *Rhodococcus* sp.; *Rhodocyclus* sp.; *Rhodomicrobium* sp.; *Rhodopseudomonas* sp.; *Rhodospirillum* sp.; *Shewanella* sp.; *Siderococcus* sp.; *Streptomyces* sp.; *Sulfobacillus* sp.; *Sulfolobus* sp.; *Thermothrix* sp.; *Thiobacillus* sp.; *Thiomicrospira* sp.; *Thioploca* sp.; *Thiosphaera* sp.; *Thiothrix* sp.; *Thiovulum* sp.; sulfur-oxidizers; hydrogen-oxidizers; iron-oxidizers; acetogens; and methanogens; consortiums of microorganisms that include chemoautotrophs; chemoautotrophs native to at least one of hydrothermal vents, geothermal vents, hot springs, cold seeps, underground aquifers, salt lakes, saline formations, and soils; and extremophiles selected from one or more of thermophiles, hyperthermophiles, acidophiles, halophiles, and psychrophiles. In some embodiments, the microorganisms, or protein product thereof includes a consortium of microorganisms that includes chemoautotrophic microorganisms, such as one or more of the above chemoautotrophic microorganisms.

[240] In some embodiments, the microorganisms or protein product thereof include extremophiles that can withstand extremes in various environmental parameters, such as temperature, radiation,

pressure, gravity, vacuum, desiccation, salinity, pH, oxygen tension, and/or chemicals. Such microorganisms include hyperthermophiles, such as *Pyrolobus fumarii*; thermophiles, such as *Synechococcus lividis*; mesophiles and psychrophiles, such as *Psychrobacter*, and/or extremely thermophilic sulfur-metabolizers such as *Thermoproteus* sp., *Pyrodictium* sp., *Sulfolobus* sp., and *Acidianus* sp.; radiation tolerant organisms such as *Deinococcus radiodurans*; pressure tolerant microorganisms including piezophiles or barophiles; desiccant tolerant and anhydrobiotic microorganisms including xerophiles, such as *Artemia salina*; microbes and fungi; salt tolerant microorganisms including halophiles, such as *Halobacteriaceae* and *Dunaliella salina*; pH tolerant microorganisms including alkaliphiles, such as *Natronobacterium*, *Bacillus firmus* OF4, *Spirulina* spp., and acidophiles such as *Cyanidium caldarium* and *Ferroplasma* sp; gas tolerant microorganisms, e.g., tolerant to pure CO₂, including *Cyanidium caldarium*; and metal tolerant microorganisms (metalotolerants), such as *Ferroplasma acidarmanus* and *Ralstonia* sp.

[241] In certain embodiments, the microorganisms or protein product thereof, include a cell line selected from eukaryotic plants, algae, cyanobacteria, green-sulfur bacteria, green non-sulfur bacteria, purple sulfur bacteria, purple non-sulfur bacteria, extremophiles, yeast, fungi, proteobacteria, engineered organisms thereof, and synthetic organisms. In certain embodiments, *Spirulina* is utilized.

[242] In certain embodiments, the microorganisms or protein product thereof includes green non-sulfur bacteria, which include but are not limited to the following genera: *Chloroflexus*, *Chloronema*, *Oscillochloris*, *Heliothrix*, *Herpetosiphon*, *Roseiflexus*, and *Thermomicrobium*.

[243] In certain embodiments, the microorganisms or protein product thereof includes green sulfur bacteria, which include but are not limited to the following genera: *Chlorobium*, *Clathrochloris*, and *Prosthecochloris*.

[244] In certain embodiments, the microorganisms or protein product thereof includes purple sulfur bacteria, which include but are not limited to the following genera: *Allochromatium*, *Chromatium*, *Halochromatium*, *Isochromatium*, *Marichromatium*, *Rhodovulum*, *Thermochromatium*, *Thiocapsa*, *Thiorhodococcus*, and *Thiocystis*.

[245] In certain embodiments, the microorganisms or protein product thereof includes purple non-sulfur bacteria, which include but are not limited to the following genera: *Phaeospirillum*, *Rhodobaca*, *Rhodobacter*, *Rhodomicrobium*, *Rhodopila*, *Rhodopseudomonas*, *Rhodothalassium*, *Rhodospirillum*, *Rhodovibrio*, and *Roseospira*.

[246] In some embodiments, the microorganisms or protein product thereof include a methanotroph and/or a methylotroph. In some embodiments, the microorganism is in the genus *Methylococcus*. In some embodiments, the microorganism is *Methylococcus capsulatus*. In some embodiments, the microorganism is a methylotroph. In some embodiments, the microorganism is in the genus *Methylobacterium*. In some embodiments, the microorganisms include one or more of the following species: *Methylobacterium zatmanii*; *Methylobacterium extorquens*; *Methylobacterium chloromethanicum*.

[247] In some embodiments, the microorganisms or protein product thereof a hydrogen-oxidizing chemoautotroph and/or a carboxydutroph and/or a methylotroph and/or methanotroph.

[248] In certain embodiments, the microorganisms or protein product thereof includes microorganisms that can grow heterotrophically, utilizing multi-carbon organic molecules as carbon sources, such as, but not limited to sugars, for example, but not limited to, glucose and/or fructose and/or sucrose. In some embodiments, the microorganism is capable of growing on untreated crude glycerol and/or glucose and/or methanol and/or acetate as the sole electron donor(s) and carbon source(s). In some embodiments, the microorganism is able to grow mixotrophically, for example, mixotrophic growth on an organic carbon source and an inorganic energy source (e.g., inorganic electron donor).

[249] In certain embodiments, the microorganisms or protein product thereof includes one or more of eukaryotic plants, algae, cyanobacteria, green-sulfur bacteria, green non-sulfur bacteria, purple sulfur bacteria, purple non-sulfur bacteria, extremophiles, archaea, yeast, fungi, proteobacteria, engineered organisms thereof, and synthetic organisms.

[250] In some embodiments, the microorganisms comprise or consist of gram-positive bacteria. In other embodiments, the microorganisms comprise or consist of gram-negative bacteria.

[251] In certain embodiments, the microorganisms or protein product thereof includes naturally occurring and/or non-genetically modified (non-GMO) microorganisms and/or non-pathogenic and/or are grown in specific environmental conditions provided by the bioprocesses that are absent from the surrounding environment.

[252] In certain embodiments, the microorganisms or consortium of microorganisms are isolated from environmental samples and enriched with desirable microorganisms using methods known in the art of microbiology, for example, growth in the presence of targeted electron donors, including, but not limited to, one or more of: H₂, CO, syngas and/or methane, and/or electron acceptors including, but not limited to, one or more of O₂, nitrate, ferric iron, and/or CO₂, and/or environmental conditions (e.g., temperature, pH, pressure, dissolved oxygen (DO), salinity, the presence of various impurities and pollutants, etc.).

[253] In certain embodiments, the microorganisms or consortium of microorganisms include probiotic microorganisms. In certain embodiments, the microorganisms or consortium of microorganisms include "generally recognized as safe" (GRAS) microorganisms, e.g., bacterial and/or fungal GRAS microorganisms. In certain embodiments, the microorganisms or consortium of microorganisms include yeast, such as, but not limited to, one or more of the following: *Candida humilis*; *Candida milleri*; *Debaryomyces hansenii*; *Kazachstania exigua* (*Saccharomyces exiguous*); *Saccharomyces cerevisiae*; *Saccharomyces florentinus*; *Torulaspora delbrueckii*; *Trichosporon beigelli*; and/or include fungi, such as, but not limited to, one or more of the following: *Aspergillus oryzae*; *Aspergillus sojae*; *Fusarium venenatum* A3/5; *Neurospora intermedia* var. *oncomensis*; *Rhizopus oligosporus*; *Rhizopus oryzae*; *Aspergillus luchuensis*; and/or include bacteria, such as, but not limited to, one or more of the following: *Bacillus amyloliquefaciens*; *Bacillus subtilis*;

Bifidobacterium animalis (lactis); *Bifidobacterium bifidum*; *Bifidobacterium breve*; *Bifidobacterium longum*; *Lactobacillus acidophilus*; *Lactobacillus brevis*; *Lactobacillus casei*; *Lactobacillus delbrueckii subsp. Bulgaricus*; *Lactobacillus fermentum*; *Lactobacillus helveticus*; *Lactobacillus kefiranofaciens*; *Lactobacillus lactis*; *Lactobacillus plantarum*; *Lactobacillus rhamnosus*; *Lactobacillus reuteri*; *Lactobacillus sakei*; *Lactobacillus sanfranciscensis*; *Lactococcus lactis (Streptococcus lactis, Streptococcus lactis subsp. Diacetylactis)*; *Leuconostoc*; *Leuconostoc carnosum*; *Leuconostoc cremoris*; *Leuconostoc mesenteroides*; *Pediococcus*; *Propionibacterium freudenreichii*; *Arthrospira (Spirulina) platensis*; *Streptococcus faecalis*; *Streptococcus thermophilus*.

[254] The protein containing biomass from which the protein product is derived may be produced by a consortium of different species of microorganisms. The consortium may optionally include multi-cellular organisms. In some embodiments, the consortium includes one or more of: an oxyhydrogen microorganism; a carboxydrotroph; a methanotroph; a methylotroph; a chemoautotroph; a photoautotroph; and a heterotroph.

[255] In some embodiments, the protein product also includes one or more vitamin produced by the microorganisms from which the protein product was derived. In some non-limiting embodiments, the microorganisms include a *Cupriavidus* microorganism, for example, *Cupriavidus necator* (e.g., *Cupriavidus necator* DSM 531 or *Cupriavidus necator* DSM 541). In some non-limiting embodiments, the vitamin is a B vitamin, including but not limited to, vitamin B1, B2, and/or B12. In a non-limiting example, the B vitamin (e.g., B1, B2, and/or B12) may be produced by a *Cupriavidus* microorganism, for example, *Cupriavidus necator* (e.g., *Cupriavidus necator* DSM 531 or *Cupriavidus necator* DSM 541).

Microbial cultures

[256] Any suitable methods may be used to culture the microorganisms. The microorganism may be grown under any suitable conditions, in an environment that is suitable for growth and production of biomass. In some embodiments, the microorganism may be grown in autotrophic culture conditions, heterotrophic culture conditions, or a combination of autotrophic and heterotrophic culture conditions. A heterotrophic culture may include a suitable source of carbon and energy, such as one or more sugar (e.g., glucose, fructose, sucrose, etc.). An autotrophic culture may include C1 chemicals such as carbon monoxide, carbon dioxide, methane, methanol, formate, and/or formic acid, and/or mixtures containing C1 chemicals, including, but not limited to various syngas compositions or various producer gas compositions, e.g., generated from low value sources of carbon and energy, such as, but not limited to, lignocellulosic energy crops, crop residues, bagasse, saw dust, forestry residue, or food, through the gasification, partial oxidation, pyrolysis, or steam reforming of said low value carbon sources, that can be used by an oxyhydrogen microorganism or hydrogen-oxidizing microorganism or carbon monoxide oxidizing microorganism as a carbon source and an energy source. Suitable methods and apparatus for culturing the microorganisms and generating a biomass for use in the present methods are described, e.g., in PCT Application Nos.

US2010/001402, US2011/034218, US2013/032362, US2014/029916, US2017/023110, US2018/016779, and U.S. Patent No. 9,157,058, each of which is hereby incorporated by reference herein in its entirety. In some embodiments, the organism may be grown photosynthetically in a bioreactor, in a hydroponics system, in a greenhouse, or in a cultivated field, or may be collected from waste or natural sources.

[257] The liquid cultures used to grow microorganism cells described herein can be housed in culture vessels known and used in the art. In some embodiments, large scale production in a bioreactor vessel can be used to produce large quantities of a desired molecule and/or biomass.

[258] In certain embodiments, bioreactor vessels are used to contain, isolate, and/or protect the culture environment. The culture vessels include those that are known to those of ordinary skill in the art of large scale microbial culturing. Such culture vessels include but are not limited to one or more of the following: airlift reactors; biological scrubber columns; bubble columns; stirred tank reactors; continuous stirred tank reactors; counter-current, upflow, expanded-bed reactors; digesters and in particular digester systems, for example, such known in the art of bioremediation; filters including but not limited to trickling filters, rotating biological contactor filters, rotating discs, soil filters; fluidized bed reactors; gas lift fermenters; immobilized cell reactors; loop reactors; membrane biofilm reactors; pachuca tanks; packed-bed reactors; plug-flow reactors; static mixers; trickle bed reactors; and/or vertical shaft bioreactors.

[259] Microbial culturing aimed at the commercial production of biomass and/or organic compounds, *e.g.*, protein product as described herein, specifically single cell protein, cell lysate, protein extract, protein-containing extract, protein concentrate, protein isolate, protein hydrolysate, free amino acids, peptides, oligopeptides, or combinations thereof, and/or other nutrients, such as, but not limited to vitamins (*e.g.*, B vitamins, for example, B1, B2, and/or B12) may be performed in bioreactors at large scale (*e.g.*, 500 L, 1,000 L, 5,000 L, 10,000 L, 50,000 L, 100,000 L, 1,000,000 L bioreactor volumes and higher).

[260] In certain embodiments, chemoautotrophic and/or heterotrophic and/or carboxydophilic and/or methanotrophic and/or methylotrophic microorganisms are grown in a liquid media inside a bioreactor using methods described herein.

[261] In some embodiments, the bioreactor containing the microorganisms is constructed of opaque materials that keep the culture in near or total darkness. Bioreactors constructed out of opaque materials such as steel and/or other metallic alloys and/or reinforced concrete and/or fiberglass and/or various high strength plastic materials can be designed to have large working volumes. In some embodiments, fermenters constructed of steel or other metallic alloys that are 50,000 liters and greater in volume are utilized. In some embodiments, bioreactors capable of containing positive headspace pressures above ambient pressure are utilized. In some embodiments, egg-shape or cylindrical digesters or vertical shaft bioreactors 3,000,000 liters and greater in volume are utilized. In some embodiments, the bioreactor comprising the microorganism does not allow light to penetrate part or most or all of its contained liquid volume. In certain non-

limiting embodiments, the microorganism used in the CO₂-fixation step is not photosynthetic. In certain non-limiting embodiments, the bioreactor design does not confine the culture in thin layers or have transparent walls so as to have light available to all parts, as is generally necessary with photosynthesis. In some embodiments, the microorganism is cultured without significant or any exposure to light. In certain such embodiments, net CO₂ consumption still occurs in the absence of light due to chemoautotrophic metabolism and conditions. In certain embodiments, converting electricity to artificial light is not required in a biological system for CO₂ capture and conversion.

[262] In certain embodiments, the lack of light dependence facilitates continuous CO₂ capture operations, day and night, year-round, in all weather conditions, without the need for any artificial lighting.

[263] In some embodiments, the microorganisms are grown and maintained in a medium containing a gaseous carbon source, such as but not limited to syngas, producer gas, or gas mixtures containing H₂ and CO₂, in the absence of light; where such growth is known as chemoautotrophic growth.

[264] In some embodiments, syngas, for example, generated from gasification of organic matter is utilized by the microorganisms for chemoautotrophic growth. The organic matter may be, for example, from an agricultural source (*e.g.*, corn stover, bagasse).

[265] In some embodiments, food grade CO₂ and/or air that goes through a direct air capture unit is utilized by the microorganisms for chemoautotrophic growth. Non-limiting examples of direct air capture may be found in U.S. Publication No. 2017/0106330 and Keith, D., et al. (2018) *Joule* 2(8):1573-1594, which are incorporated by reference herein in their entireties. In some embodiments, CO₂ is provided from an industrial source, and optionally may be concentrated via a gas separation procedure, thereby resulting in high concentration food grade CO₂.

[266] In certain embodiments, an increase in system capacity is met by vertical scaling, rather than only scaling horizontally. This is in contrast to phototrophic approaches using algae, cyanobacteria, or higher-plants for CO₂ capture. Although various vertical farming schemes have been proposed for photosynthetic systems, practically and economically speaking, phototrophic systems must expand horizontally, for example in shallow ponds or photobioreactors in the case of algae. This results in large geographic footprints and many negative environmental impacts.

[267] An algal or higher plant system grown with artificial lighting is challenged by inefficient utilization of light energy, and by inefficient conversion of electrical energy to light energy. In certain embodiments, a comparable algal or high-plant culture grown under artificial lighting will require more electrical power than the CO₂ capture and/or biomass production system described herein, in terms of CO₂ capture and/or biomass production. In certain embodiments, a comparable algal or higher-plant culture grown under artificial lighting will require at least ten times more electrical power than the CO₂ capture and/or biomass production system described herein, in terms of power per unit CO₂ capture and/or biomass production. For algae or higher-plants grown on artificial lighting, the heat rejection requirement is almost in direct proportion to the electrical input. In certain

embodiments of the methods described herein, the heat rejection requirements are lower than for a comparable algal or higher plant system, in terms of CO₂ capture and/or biomass production when grown on artificial lighting. In certain embodiments, the heat rejection requirements are at least ten times lower than for a comparable algal or higher plant system, in terms of CO₂ capture and/or biomass production when grown on artificial lighting.

[268] In an exemplary but nonlimiting embodiment, a bioreactor containing nutrient medium is inoculated with production cells. Generally, there will follow a lag phase prior to the cells beginning to double. After the lag phase, the cell doubling time decreases and the culture goes into the logarithmic phase. The logarithmic phase is eventually followed by an increase of the doubling time that, while not intending to be limited by theory, is thought to result from either a mass transfer limitation, depletion of nutrients including nitrogen or mineral sources, or a rise in the concentration of inhibitory chemicals, or quorum sensing by the microbes. The growth slows down and then ceases when the culture enters the stationary phase. In certain embodiments, there is an arithmetic growth phase preceding the stationary phase. In order to harvest cell mass, the culture in certain embodiments is harvested in the logarithmic phase and/or in the arithmetic phase and/or in the stationary phase.

[269] The bioreactor or fermenter is used to culture cells through the various phases of their physiological cycle. A bioreactor is utilized for the cultivation of cells, which may be maintained at particular phases in their growth curve. The use of bioreactors is advantageous in many ways for cultivating chemoautotrophic growth. For certain embodiments, protein-rich cell mass, which is used to produce proteins or protein hydrolysates, is grown to high densities in liquid suspension. Generally, the control of growth conditions, including control of dissolved carbon dioxide, oxygen, and other gases such as hydrogen, as well as other dissolved nutrients, trace elements, temperature and pH, is facilitated in a bioreactor. For certain embodiments, protein-rich cell mass, which is used to produce amino acids, peptides, proteins, hydrolysates, extracts, or whole cell products, is grown to high densities and/or grown at high productivities, in liquid suspension within a bioreactor.

[270] Nutrient media, as well as gases, can be added to the bioreactor as either a batch addition, or periodically, or in response to a detected depletion or programmed set point, or continuously over the period the culture is grown and/or maintained. For certain embodiments, the bioreactor at inoculation is filled with a starting batch of nutrient media and/or one or more gases at the beginning of growth, and no additional nutrient media and/or one or more gases are added after inoculation. For certain embodiments, nutrient media and/or one or more gases are added periodically after inoculation. For certain embodiments, nutrient media and/or one or more gases are added after inoculation in response to a detected depletion of nutrient and/or gas. For certain embodiments, nutrient media and/or one or more gases are added continuously after inoculation.

[271] For certain embodiments, the added nutrient media does not contain any organic compounds.

[272] In certain embodiments, a small amount of microorganism cells (*i.e.*, an inoculum) is added to a set volume of culture medium; the culture is then incubated; and the cell mass passes through lag, exponential, deceleration, and stationary phases of growth.

[273] In batch culture systems, the conditions (*e.g.*, nutrient concentration, pH, etc.) under which the microorganism is cultivated generally change continuously throughout the period of growth. In certain non-limiting embodiments, to avoid the fluctuating conditions inherent in batch cultures, and to improve the overall productivity of the culture system, the microorganisms that are used for the production of protein and/or vitamins and/or other nutrients are grown in a continuous culture system called a chemostat. In such systems. The culture may be maintained in a perpetual exponential phase of growth by feeding it with fresh medium at a constant rate [F] while at the same time maintaining the volume [V] of the culture constant. In certain embodiments, a continuous culture system ensures that cells are cultivated under environmental conditions that remain roughly constant. In certain embodiments, the cells are maintained in a perpetual exponential phase through the use of a chemostat system. In certain cases, the culture is maintained in a steady state with a roughly fixed amount of standing biomass maintained in the bioreactor over time. In such a case the dilution rate (D) of the culture equals the growth rate of the microorganism, and is given by: $D = F/V$. The growth rate of a microorganism in continuous culture may be changed by altering the dilution rate. In certain embodiments, the growth rate of the microorganism is changed by altering the dilution rate. In certain non-limiting embodiments, cells are grown in a chemostat at a dilution rate of around 0.2

h^{-1} . In certain embodiments, the continuous bioreactor is maintained as a turbidostat, where a fixed amount of standing biomass is maintained in the bioreactor over time, and where all surplus biomass that is produced beyond that necessary to maintain the fixed amount of standing biomass within the bioreactor, is harvested continuously from the bioreactor.

[274] In certain embodiments, inoculation of the culture into the bioreactor is performed by methods including but not limited to transfer of culture from an existing culture inhabiting another bioreactor, or incubation from a seed stock raised in an incubator. In certain embodiments, the seed stock of the strain may be transported and stored in forms including but not limited to a powder, liquid, frozen, or freeze-dried form as well as any other suitable form, which may be readily recognized by one skilled in the art. In certain non-limiting embodiments, the reserve bacterial cultures are kept in a metabolically inactive, freeze-dried state until required for restart. In certain embodiments, when establishing a culture in a very large reactor, cultures are grown and established in progressively larger intermediate scale vessels prior to inoculation of the full-scale vessel.

[275] For certain embodiments, the bioreactors have mechanisms to enable mixing of the nutrient media that include, but are not limited to, one or more of the following: spinning stir bars, blades, impellers, or turbines; spinning, rocking, or turning vessels; gas lifts, sparging; recirculation of broth

from the bottom of the container to the top via a recirculation conduit, flowing the broth through a loop and/or static mixers. The culture media may be mixed continuously or intermittently.

[276] In certain embodiments the microorganism-containing nutrient medium may be removed from the bioreactor partially or completely, periodically or continuously, and in certain embodiments is replaced with fresh cell-free medium to maintain the cell culture in an exponential growth phase, and/or in another targeted growth phase (e.g., arithmetic growth), and/or to replenish the depleted nutrients in the growth medium, and/or remove inhibitory waste products.

[277] The ports that are standard in bioreactors may be utilized to deliver, or withdraw, gases, liquids, solids, and/or slurries, into and/or from the bioreactor vessel enclosing the microbes. Many bioreactors have multiple ports for different purposes (e.g., ports for media addition, gas addition, probes for pH and DO, and sampling), and a given port may be used for various purposes during the course of a fermentation run. As an example, a port might be used to add nutrient media to the bioreactor at one point in time, and at another time might be used for sampling. Preferably, the multiple uses of a sampling port can be performed without introducing contamination or invasive species into the growth environment. A valve or other actuator enabling control of the sample flow or continuous sampling can be provided to a sampling port. For certain embodiments, the bioreactors are equipped with at least one port suitable for culture inoculation that can additionally serve other uses including the addition of media or gas. Bioreactor ports enable control of the gas composition and flow rate into the culture environment. For example, the ports can be used as gas inlets into the bioreactor through which gases are pumped.

[278] For some embodiments, gases that may be pumped into a bioreactor include, but not are not limited to, one or more of the following: syngas, producer gas, hydrogen gas, CO, CO₂, O₂, air, air/CO₂ mixtures, natural gas, methane, ammonia, nitrogen, noble gases, such as argon, as well as other gases. In some embodiments the CO₂ pumped into the system may come from sources including, but not limited to: CO₂ from the gasification of organic matter; CO₂ from the calcination of limestone, CaCO₃, to produce quicklime, CaO; CO₂ from methane steam reforming, such as the CO₂ byproduct from ammonia, methanol, or hydrogen production; CO₂ from combustion, incineration, or flaring; CO₂ byproduct of anaerobic or aerobic fermentation of sugar; CO₂ byproduct of a methanotrophic bioprocess; geologically or geothermally produced or emitted CO₂; CO₂ removed from acid gas or natural gas. In certain non-limiting embodiments, the CO₂ has been removed from an industrial flue gas, or intercepted from a geological source that would otherwise naturally emit into the atmosphere. In certain embodiments, the carbon source is CO₂ and/or bicarbonate and/or carbonate dissolved in sea water or other bodies of surface or underground water. In certain such embodiments the inorganic carbon may be introduced to the bioreactor dissolved in liquid water and/or as a solid. In certain embodiments, the carbon source is CO₂ captured from the atmosphere. In certain non-limiting embodiments, the CO₂ has been captured from a closed cabin as part of a closed-loop life support system, using equipment such as but not

limited to a CO₂ removal assembly (CDRA), which is utilized, for example, on the International Space Station (ISS).

[279] In certain non-limiting embodiments, geological features such as, but not limited to, geothermal and/or hydrothermal vents that emit high concentrations of energy sources (e.g., H₂, H₂S, CO gases) and/or carbon sources (e.g., CO₂, HCO₃⁻, CO₃²⁻) and/or other dissolved minerals may be utilized as nutrient sources for the microorganisms herein.

[280] In certain embodiments, one or more gases in addition to carbon dioxide, or in place of carbon dioxide as an alternative carbon source, are either dissolved into solution and fed to the culture broth and/or dissolved directly into the culture broth, including but not limited to gaseous electron donors and/or carbon sources (e.g., hydrogen and/or CO and/or methane gas). In certain embodiments, input gases may include other electron donors and/or electron acceptors and/or carbon sources and/or mineral nutrients such as, but not limited to, other gas constituents and impurities of syngas (e.g., hydrocarbons); ammonia; hydrogen sulfide; and/or other sour gases; and/or O₂; and/or mineral containing particulates and ash.

[281] In certain embodiments, one or more gases are dissolved into the culture broth, including but not limited to gaseous electron donors such as, but not limited to, one or more of the following: hydrogen, carbon monoxide, methane, hydrogen sulfide or other sour gases; gaseous carbon sources such as, but not limited to one or more of the following: CO₂, CO, CH₄; and electron acceptors such as, but not limited to, oxygen, either within air (e.g., 20.9% oxygen) or as pure O₂ or as an O₂-enriched gas. In some embodiments, the dissolution of these and other gases into solution is achieved using a system of compressors, flowmeters, and flow valves known to one skilled in the art of fermentation engineering, that feed into one of more of the following widely used systems for dispersing gas into solution: sparging equipment; diffusers including but not limited to dome, tubular, disc, or doughnut geometries; coarse or fine bubble aerators; venturi equipment. In certain embodiments, surface aeration and/or gas mass transfer may also be performed using paddle aerators and the like. In certain embodiments, gas dissolution is enhanced by mechanical mixing with an impeller or turbine, as well as hydraulic shear devices to reduce bubble size. Following passage through the reactor system holding microorganisms which uptake the gases, in certain embodiments the residual gases may either be recirculated back to the bioreactor, or burned for process heat, or flared, or injected underground, or released into the atmosphere. In certain embodiments herein utilizing H₂ as electron donor, H₂ may be fed to the culture vessel either by bubbling it through the culture medium, or by diffusing it through a hydrogen permeable-water impermeable membrane known in the art that interfaces with the liquid culture medium.

[282] In certain embodiments, the microorganisms grow and multiply on H₂ and CO₂ and other dissolved nutrients under microaerobic conditions. In certain embodiments, a C1 chemical such as but not limited to carbon monoxide, methane, methanol, formate, or formic acid, and/or mixtures containing C1 chemicals including but not limited to various syngas compositions generated from various gasified, pyrolyzed, or steam-reformed fixed carbon feedstocks, are biochemically converted

into longer chain organic chemicals (*i.e.*, C2 or longer and, in some embodiments, C5 or longer carbon chain molecules) under one or more of the following conditions: aerobic, microaerobic, anoxic, anaerobic, and/or facultative conditions.

[283] A controlled amount of oxygen can also be maintained in the culture broth of some embodiments, and in certain embodiments, oxygen will be actively dissolved into solution fed to the culture broth and/or directly dissolved into the culture broth. In certain aerobic or microaerobic embodiments that require the pumping of air or oxygen into the culture broth in order to maintain targeted DO levels, oxygen bubbles may be injected into the broth at an optimal diameter for mixing and oxygen transfer. In some embodiments, conditions suitable for growth of an oxyhydrogen microorganism are deployed, such as use of H₂ and O₂ gas substrates (electron donors and acceptors), and optionally a C1 gaseous carbon source, such as CO₂ and/or CO.

[284] In some embodiments, the microorganisms convert a fuel gas, including but not limited to syngas, producer gas, CO, CO₂, H₂, natural gas, methane, and mixtures thereof. In some embodiments, the heat content of the fuel gas is at least 100 BTU per standard cubic foot (scf). In some embodiments, a bioreactor that is used to contain and grow the microorganisms is equipped with fine-bubble diffusers and/or high-shear impellers for gas delivery.

[285] Introducing and/or raising the gas flow rate into a bioreactor can enhance mixing of the culture and produce turbulence if the gas inlet is positioned beneath the surface of the liquid media such that gas bubbles or sparges up through the media. In certain embodiments, mixing is enhanced through turbulence provided by gas bubbles and/or sparging and/or gas plugging up through the liquid media. In some embodiments, a bioreactor comprises gas outlet ports for gas escape and pressure release. In some embodiments, gas inlets and outlets are preferably equipped with check valves to prevent gas backflow.

[286] In certain embodiments where chemosynthetic reactions occur within the bioreactor, one or more types of electron donor and one or more types of electron acceptor are pumped or otherwise added as either a bolus addition, or periodically, or continuously to the nutrient medium containing chemoautotrophic organisms in the reaction vessel. The chemosynthetic reaction, driven by the transfer of electrons from electron donor to electron acceptor in cellular respiration, fixes inorganic carbon dioxide and/or other dissolved carbonates and/or other carbon oxides into organic compounds and biomass.

[287] In certain embodiments a nutrient media for culture growth and production is used, comprising an aqueous solution containing suitable minerals, salts, vitamins, cofactors, buffers, and other components needed for microbial growth, known to those skilled in the art [Bailey and Ollis, *Biochemical Engineering Fundamentals*, 2nd ed; pp 383-384 and 620-622; McGraw-Hill: New York (1986)].

[288] In certain embodiments, the chemicals used for maintenance and growth of microbial cultures as known in the art are included in the nutrient media. In certain embodiments, these chemicals may include but are not limited to one or more of the following: nitrogen sources such as

ammonia, ammonium (e.g., ammonium chloride (NH_4Cl), ammonium sulfate ($(\text{NH}_4)_2\text{SO}_4$)), nitrate (e.g., potassium nitrate (KNO_3)), urea or an organic nitrogen source; phosphate (e.g., disodium phosphate (Na_2HPO_4), potassium phosphate (KH_2PO_4), phosphoric acid (H_3PO_4), potassium dithiophosphate ($\text{K}_3\text{PS}_2\text{O}_2$), potassium orthophosphate (K_3PO_4), dipotassium phosphate (K_2HPO_4)); sulfate; yeast extract; chelated iron; potassium (e.g., potassium phosphate (KH_2PO_4), potassium nitrate (KNO_3), potassium iodide (KI), potassium bromide (KBr)); and other inorganic salts, minerals, and trace nutrients (e.g., sodium chloride (NaCl), magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) or magnesium chloride (MgCl_2), calcium chloride (CaCl_2) or calcium carbonate (CaCO_3), manganese sulfate ($\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$) or manganese chloride (MnCl_2), ferric chloride (FeCl_3), ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) or ferrous chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$), sodium bicarbonate (NaHCO_3) or sodium carbonate (Na_2CO_3), zinc sulfate (ZnSO_4) or zinc chloride (ZnCl_2), ammonium molybdate (NH_4MoO_4) or sodium molybdate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$), cuprous sulfate (CuSO_4) or copper chloride ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), cobalt chloride ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), aluminum chloride ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$), lithium chloride (LiCl), boric acid (H_3BO_3), nickel chloride ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), tin chloride ($\text{SnCl}_2 \cdot \text{H}_2\text{O}$), barium chloride ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$), copper selenate ($\text{CuSeO}_4 \cdot 5\text{H}_2\text{O}$) or sodium selenite (Na_2SeO_3), sodium metavanadate (NaVO_3), chromium salts). In certain embodiments, the mineral salts medium (MSM) formulated by Schlegel et al may be used [“Thermophilic bacteria”, Jakob Kristjansson, Chapter 5, Section III, CRC Press, (1992)].

[289] Microorganisms described herein can be cultured in some embodiments in media of any type (rich or minimal), including fermentation medium, and any composition. As would be understood by one of ordinary skill in the art, routine optimization would allow for use of a variety of types of media. The selected medium can be supplemented with various additional components. Some non-limiting examples of supplemental components include glucose, fructose, sucrose, starches, polysaccharides, protein hydrolysates, antibiotics, IPTG for gene induction, and ATCC Trace Mineral Supplement. Similarly, other aspects of the medium and growth conditions of the microorganisms described herein may be optimized through routine experimentation. For example, pH and temperature are non-limiting examples of factors which can be optimized. In some embodiments, factors such as choice of media, media supplements, and temperature can influence production levels of a desired molecule. In some embodiments, the concentration and amount of a supplemental component may be optimized. In some embodiments, how often the media is supplemented with one or more supplemental components, and the amount of time that the media is cultured before harvesting the desired molecule is optimized.

[290] In certain embodiments, the concentrations of nutrient chemicals (e.g., electron donors, electron acceptors, carbon sources, and/or various mineral nutrients), are maintained within the bioreactor close to or at their respective optimal levels for optimal carbon uptake and/or fixation and/or conversion and/or production of biomass and/or organic compounds, and in particular protein, which varies depending upon the microorganism utilized but may be routinely determined and/or optimized by one of ordinary skill in the art of culturing microorganisms.

[291] In certain embodiments, one or more of the following parameters are monitored and/or controlled in the bioreactor: waste product levels; pH; temperature; salinity; dissolved oxygen; dissolved carbon dioxide gas; liquid flow rates; agitation rate; gas pressure. In certain embodiments, the operating parameters affecting chemoautotrophic growth, and/or other types of growth (e.g., heterotrophic growth) are monitored with sensors (e.g., dissolved oxygen probe or oxidation-reduction probe to gauge electron donor/acceptor concentrations), and/or are controlled either manually or automatically based upon feedback from sensors through the use of equipment including but not limited to actuating valves, pumps, and agitators. In certain embodiments, the temperature of the incoming broth as well as of incoming gases is regulated by systems such as, but not limited to, coolers, heaters, and/or heat exchangers.

[292] In certain embodiments, the microbial culture and bioreaction is maintained using continuous influx and removal of nutrient medium and/or biomass, in steady state where the cell population and environmental parameters (e.g., cell density, pH, DO, chemical concentrations) are targeted at a constant level over time. In certain embodiments, the constant level is an optimal level for feedstock conversion and/or production of targeted organic compounds. In certain embodiments the targeted organic compounds comprise proteins and/or amino acids. In certain embodiments, cell densities can be monitored by direct sampling, by a correlation of optical density to cell density, and/or with a particle size analyzer. In certain embodiments, the hydraulic and biomass retention times can be decoupled so as to allow independent control of both the broth chemistry and the cell density. In certain embodiments, dilution rates can be kept high enough so that the hydraulic retention time is relatively low compared to the biomass retention time, resulting in a highly replenished broth for cell growth and/or feedstock conversion and/or production of organic compounds. In certain embodiments, dilution rates are set at an optimal technoeconomic trade-off between culture broth and nutrient replenishment and/or waste product removal, and increased process costs from pumping, increased inputs, and other demands that rise with dilution rates.

[293] In certain embodiments, the pH of the microbial culture is controlled. In certain embodiments, pH is controlled within an optimal range for microbial maintenance and/or growth and/or conversion of feedstock and/or production of organic compounds and/or survival. To address a decrease in pH, in certain embodiments a neutralization step can be performed directly in the bioreactor environment or prior to recycling the media back into the culture vessel through a recirculation loop. Neutralization of acid in the broth of certain embodiments can be accomplished by the addition of bases, including but not limited to one or more of the following: limestone, lime, sodium hydroxide, ammonia, ammonium hydroxide, caustic potash, magnesium oxide, iron oxide, alkaline ash.

[294] In certain embodiments, an aqueous suspension of chemoautotrophic microorganisms converts one or more electron donors and CO₂ into protoplasm. In certain embodiments the said protoplasm comprises proteins, peptides, and/or amino acids. In certain embodiments, an aqueous suspension of hydrogen-oxidizing microorganisms can be used to convert hydrogen and carbon

dioxide into microbial protoplasm. In certain embodiments, an aqueous suspension of carbon monoxide-oxidizing microorganisms can be used to convert carbon monoxide and hydrogen and/or water into protoplasm. In certain embodiments, an aqueous suspension of methane-oxidizing microorganisms can be used to convert methane into protoplasm. In certain embodiments, the microorganism in suspension is a bacterium or an archaeon. In certain non-limiting embodiments, an aqueous suspension or biofilm of H₂-oxidizing chemoautotrophic microorganisms converts H₂ and CO₂, along with some other dissolved mineral nutrients, into biochemicals and protoplasm. In certain embodiments the said biochemicals and/or protoplasm comprises proteins, peptides, and/or amino acids. In certain embodiments, the other dissolved mineral nutrients include, but are not limited to, a nitrogen source, a phosphorous source, and a potassium source. In certain embodiments, the protoplasm produced is of food value to humans and/or other animals and/or other heterotrophs. In certain embodiments, certain biochemicals may be extracted from the protoplasm and/or extracellular broth, which have nutrient value, and/or value in a variety of organic chemistry or fuel applications. In certain embodiments, the intracellular energy to drive this production of protoplasm is derived from the oxidation of an electron donor by an electron acceptor. In certain non-limiting embodiments, the electron donor includes, but is not limited to, one or more of the following: H₂; CO; CH₄. In certain non-limiting embodiments, the electron acceptor includes but is not limited to O₂ and/or CO₂. In certain non-limiting embodiments, the product of the energy generating reaction, or respiration, includes but is not limited to water. In certain embodiments, the intracellular energy derived from respiration used to drive this synthesis of biochemicals and protoplasm from CO₂ is stored and carried in biochemical molecules including, but not limited to, ATP. For the knallgas microbes used in certain embodiments herein, the electron acceptor is O₂ and the product of respiration is water.

[295] In some embodiments the protein production and/or distribution of amino acid molecules produced is optimized through one or more of the following: control of bioreactor conditions, control of nutrient levels, and/or genetic modifications of the cells. In certain embodiments, pathways to amino acids, or proteins, or other nutrients, or whole cell products are controlled and optimized for the production of chemical products by maintaining specific growth conditions (e.g., levels of electron donors, nitrogen, oxygen, phosphorous, sulfur, trace micronutrients such as inorganic ions, and if present any regulatory molecules that might not generally be considered a nutrient or energy source). In certain embodiments, dissolved oxygen (DO) may be optimized by maintaining the broth in aerobic, microaerobic, anoxic, anaerobic, or facultative conditions, depending upon the requirements of the microorganisms. A facultative environment is considered to be one having aerobic upper layers and anaerobic lower layers caused by stratification of the water column. The biosynthesis of amino acids, or proteins, or other nutrients, or whole cell products by the microbes disclosed herein can happen during the logarithmic phase, the arithmetic phase, or afterwards during the stationary phase when cell doubling has stopped, provided there is sufficient supply of carbon and energy and other nutrient sources.

[296] In some embodiments, the growth medium for a microorganism described herein includes a protein and/or nutrient source from another microorganism (e.g., cell lysate, protein hydrolysate, peptides, oligopeptides, and/or amino acids, and/or organic molecules and/or other nutrients from a different microorganism). In some embodiments, the microorganism in the growth medium is a GRAS microorganism. In one embodiment, the growth medium for a lactic acid bacterium, such as, but not limited to, a *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium (for example, a GRAS lactic acid bacterium, such as a GRAS *Lactococcus*, *Lactobacillus*, *Enterococcus*, *Streptococcus*, or *Pediococcus* bacterium), includes cell lysate, protein hydrolysate, peptides, oligopeptides, and/or amino acids, and/or organic molecules and/or other nutrients from a different microorganism, such as, but not limited to, a *Cupriavidus* microorganism, such as, but not limited to *Cupriavidus necator*, for example, *Cupriavidus necator* DSM 531 or DSM 541. In another embodiment, growth medium for a fungal microorganism, such as a *Fusarium* or *Rhizopus* or *Aspergillus* fungal microorganism (for example, a GRAS fungal microorganism, such as a GRAS *Fusarium* or *Rhizopus* or *Aspergillus* fungal microorganism), such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus oryzae*, or *Aspergillus sojae* includes whole cell biomass, cell lysate, protein hydrolysate, peptides, oligopeptides, and/or amino acids, and/or organic molecules and/or other nutrients from a different microorganism, such as, but not limited to, a *Cupriavidus* microorganism, such as, but not limited to *Cupriavidus necator*, for example, *Cupriavidus necator* DSM 531 or DSM 541.

[297] In some embodiments, a fungal microorganism that is capable of lysing bacterial cells and/or hydrolyzing bacterial protein is cultured in the presence of such bacterial cells or nutrients derived from such bacterial cells. For example, bacterial biomass may be isolated and optionally dewatered or optionally deactivated, and then fungal microorganisms inoculated onto the bacterial biomass, or fungal microorganisms may be cultured in a growth medium as described herein, in the presence of bacterial biomass and/or bacterially derived nutrients. In certain nonlimiting embodiments, the fungal microorganisms include *Fusarium* or *Rhizopus* or *Aspergillus* microorganisms, such as but not limited to, *Fusarium venenatum*, *Rhizopus oligosporus*, *Rhizopus oryzae*, *Aspergillus oryzae*, or *Aspergillus sojae*. In certain non-limiting embodiments, the edible fungal species *Agaricus bisporus* is cultivated on media comprising protein-rich cells and/or nutrients produced according to the present invention. In certain embodiments, *Agaricus bisporus* lyses cells produced according to the present invention. In certain such embodiments the *Agaricus bisporus* utilizes proteins, amino acids, and/or other nutrients released by the said lysis of cells for nutrition and growth.

[298] The specific examples of bioreactors, culture conditions, heterotrophic and chemotrophic growth, maintenance, and amino acids, or proteins, or other nutrients, or whole cell product production methods described herein can be combined in any suitable manner to improve efficiencies of microbial growth and amino acid, or protein, or other nutrient, or whole cell production.

Electron donors and acceptors

[299] In certain non-limiting embodiments, microorganisms described herein are grown chemoautotrophically. For example, the microorganism growth may utilize biosynthetic reduction of CO₂, utilizing O₂ electron acceptor and/or H₂ electron donor. In certain embodiments, O₂ and H₂ are generated by the electrolysis of water. In certain non-limiting embodiments, part of the O₂ generated by electrolysis of water, and all of the H₂, is fed to an aqueous suspension of microorganisms as described herein. In certain non-limiting embodiments, the molar ratio of H₂ fed to an aqueous suspension of microorganisms to the moles of O₂ is greater than 2:1. In certain non-limiting embodiments where O₂ electron acceptor and H₂ electron donor are generated by the electrolysis of water, there is a surplus of O₂ remaining after all of the metabolic requirements for H₂ and O₂ of the microorganisms described herein have been met. In certain such embodiments the surplus O₂ may be supplied to humans and/or other aerobic lifeforms and/or to hydroponic systems for root aeration and/or is used in a gasification or partial oxidation or combustion process and/or is stored and sold as a chemical co-product.

[300] In certain embodiments that utilize molecular hydrogen as an electron donor, there can be a chemical co-product formed in the generation of molecular hydrogen using a renewable and/or CO₂ emission-free energy input. In certain embodiments, the oxyhydrogen reaction used in respiration is enzymatically linked to oxidative phosphorylation. In certain embodiments, the ATP and/or other intracellular energy carriers thus formed are utilized in the anabolic synthesis of amino acids and/or proteins. In certain embodiments, the oxygen produced by water-splitting in excess of what is required for respiration in order to maintain optimal conditions for carbon fixation and organic compound production by the knallgas microorganisms, may be processed into a form suitable for sale through process steps known in the art and science of commercial oxygen gas production.

[301] Certain embodiments apply hydrogen-oxidizing and/or CO-oxidizing and/or CH₄ oxidizing microorganisms that use more electronegative electron acceptors than CO₂ in energy conserving reactions for ATP production (e.g., respiration), such as but not limited to O₂. For example, hydrogenotrophic oxyhydrogen or knallgas microbes that couple the oxyhydrogen reaction, $2\text{H}_2 + \text{O}_2 \rightarrow 2\text{H}_2\text{O}$, to ATP production, can produce more ATP per H₂ and/or other electron donor consumed for respiration, than acetogens or methanogens that use CO₂ as an electron acceptor in respiration. For example, knallgas microorganisms can produce at least two ATP per H₂ consumed in respiration [L. Bongers (1970) "Energy generation and utilization in hydrogen bacteria" *Journal of bacteriology* 104(1):145-151 (<http://b.asm.org/content/104/1/145.abstract>), which is incorporated herein by reference in its entirety], which is eight times more ATP produced per H₂ consumed in respiration than what can be produced in microorganisms undergoing methanogenesis or acetogenesis, using H₂ as electron donor and CO₂ as electron acceptor in respiration. For this reason, using microorganisms that can utilize more electronegative electron acceptors in respiration and in the production of ATP, such as but not limited to knallgas microbes, for anabolic biosynthesis such as but not limited to amino acid or protein or fatty acid biosynthesis from syngas or H₂, can be

more efficient than using acetogens or methanogens, such as those which are currently used in biological gas-to-chemical (GTC) technologies for the production of short chain acids or alcohols (e.g., acetic acid or ethanol). In certain embodiments, the oxyhydrogen reaction used in respiration is enzymatically linked to oxidative phosphorylation. In certain embodiments, aerobic respiration is utilized by the microorganism cells described herein for the production of ATP. In certain embodiments, the ATP and/or other intracellular energy carriers thus formed are utilized in the anabolic biosynthesis of amino acids and/or proteins. In some embodiments, a knallgas and/or carboxydophilic and/or methanophilic and/or heterophilic microorganism or a composition or consortium comprising these microorganisms is utilized, wherein the microorganism expresses one or more enzymes that enables biosynthesis of useful carbon-based products of interest including but not limited to chemicals, monomers, polymers, proteins, polysaccharides, vitamins, nutraceuticals, antibiotics, or pharmaceutical products or intermediates thereof from a carbon-containing gas feedstock, including but not limited to syngas or producer gas or natural gas or biogas or CO₂ combined with renewable H₂ or CO or methane containing gases. In some embodiments, these said carbon-based products of interest can be biosynthesized heterotrophically from an organic multi-carbon feedstock, such as, but not limited to glucose, fructose, sucrose, and other sugars. In some non-limiting embodiments, a microorganism, or a composition comprising a microorganism is utilized, wherein the microorganism requires less than 4H₂ or NADH to produce one ATP through respiration. In other non-limiting embodiments, a microorganism is utilized that produces more than one ATP per H₂ or NADH consumed through respiration. In other non-limiting embodiments, a microorganism is utilized that produces at least two ATP per H₂ or NADH consumed through respiration, or at least 2.5 ATP per H₂ or NADH consumed through respiration.

[302] An additional feature of certain non-limiting embodiments regards the source, production, or recycling of the electron donors used by chemoautotrophic microorganisms to fix carbon dioxide and/or other C1 feedstocks into organic compounds. The electron donors used for carbon dioxide capture and carbon fixation can be produced or recycled in certain embodiments electrochemically or thermochemically using power from a number of different renewable and/or low carbon emission energy technologies including but not limited to: photovoltaics, solar thermal, wind power, hydroelectric, nuclear, geothermal, enhanced geothermal, ocean thermal, ocean wave power, tidal power. Many of the reduced inorganic chemicals upon which chemoautotrophs can grow (e.g. H₂, CO, H₂S, ferrous iron, ammonium, Mn²⁺) can be readily produced using electrochemical and/or thermochemical processes well known in the art and science of chemical engineering that can be powered by a variety carbon dioxide emission-free or low-carbon emission and/or renewable sources of power including but not limited to photovoltaics, solar thermal, wind power, hydroelectric, nuclear, geothermal, enhanced geothermal, ocean thermal, ocean wave power, or tidal power.

[303] The production of hydrogen from renewable energy sources is gradually replacing the generation from fossil feedstock systems, and the technical advances in the energy sector are expected to lower the prices of green hydrogen production in the near future. For instance, electrical

energy efficiencies up to 73% are already achieved by commercial and industrial grade electrolyzers, and research on new materials and electrolyzer configurations have shown possible efficiencies as high as 96%. Certain embodiments utilize a commercially available electrolysis technology with electrical energy efficiency of over 70% for the generation of H₂ electron donor and/or O₂ electron acceptor. Certain embodiments use electrolysis technologies with 73% or higher energy efficiency, and/or up to 96% energy efficiency, or higher.

[304] In certain embodiments that use molecular hydrogen as electron donor, the H₂ is generated by methods well known to art and science of chemical and process engineering, including but not limited to one or more of the following: through electrolysis of water including but not limited to approaches using Proton Exchange Membranes (PEM), liquid electrolytes such as KOH, alkaline electrolysis, Solid Polymer Electrolyte electrolysis, high-pressure electrolysis, high temperature electrolysis of steam (HTES), two-step electrochemical–chemical cycles such as those utilizing nickel oxide and nickel hydroxide electrodes, and/or through the thermochemical splitting of water through methods including but not limited to the iron oxide cycle, cerium(IV) oxide-cerium(III) oxide cycle, zinc zinc-oxide cycle, sulfur-iodine cycle, copper-chlorine cycle, calcium-bromine-iron cycle, hybrid sulfur cycle; and/or electrolysis of hydrogen sulfide; and/or thermochemical splitting of hydrogen sulfide; and/or other electrochemical or thermochemical processes known to produce hydrogen with low- or no- carbon dioxide emissions including but not limited to: carbon capture and sequestration (CCS) enabled methane reforming or biomass gasification. In certain embodiments, the approach to generating H₂ includes but is not limited to electrolysis powered by renewable electrical energy and/or electricity from a low-GHG source. In certain embodiments, electrolysis is powered by one or more of the following: solar, including but not limited to, photovoltaics and/or solar thermal; wind power, hydroelectric; nuclear; geothermal; enhanced geothermal; ocean thermal; ocean wave power; tidal power.

[305] Worldwide there are enormous wind energy resources, of which only a tiny percentage is utilized. The low current utilization is mainly attributed to the intermittent nature of wind resources, resulting in varying electricity generation over time, and underutilization of capacity to meet energy demand at most hours. The common mismatch between wind power supply and grid demand is manifested in examples from around the world, such as in Scotland where wind farms have been paid to shut down turbines due to oversupply [<http://www.mnn.com/earth-matters/energy/blogs/blown-away-wind-turbines-generate-enough-energy-to-power-every-home-in>], and in parts of Texas where electricity has been provided for free at night when wind power is high and grid demand is low [http://www.nytimes.com/2015/11/09/business/energy-environment/a-texas-utility-offers-a-nighttime-special-free-electricity.html?_r=2]. This challenge may be resolvable by utilizing wind power produced during off-peak demand hours to produce H₂ feedstock for the process in certain embodiments herein.

[306] Currently, hydrogen is increasingly regarded as a possible energy storage system in the so-called “power-to-gas” approach. The inherent instability of renewable energy production (particularly

solar and wind energy), and excess grid electricity (off-peak energy) may be mitigated by the production of hydrogen through water electrolysis. According to most current schemes, the produced hydrogen gas may then be converted back to electricity, by fuel cells and/or gas turbines, during periods of peak demand. Or alternatively the H₂ may be fed into the gas grid, or converted to methane via methanation. Furthermore, the hydrogen may be used as a raw material in the chemical, petrochemical, metallurgy and food industries. Certain embodiments provide new options within the power-to-gas framework, by enabling the H₂ to be used in a wider range of products, including biochemicals and in particular proteins, amino acids, fertilizers, and biostimulants. In certain embodiments, hydrogen produced using excess grid electricity and/or off-peak energy is used as an electron donor for one or more metabolic pathways occurring in hydrogen-utilizing microorganisms. In certain embodiments, the hydrogen and/or the oxygen needed for the microbial biosynthesis by hydrogen-oxidizing bacteria and/or aerobic bacteria is generated by water electrolysis using renewable energy, and in particular off-peak electricity, *i.e.*, electrical power available when the energy supply exceeds demand, and which, in the current situation, is often wasted.

[307] In certain embodiments, onsite storage of H₂ and CO₂ gases enables diversion of power from the grid only during periods when renewable generation exceeds electrical demand. In certain embodiments, power is allowed to flow as usual into the grid during periods of higher demand. In certain embodiments, the process does not disrupt renewable power supply, but rather facilitates more complete utilization of renewable generation capacity such as, but not limited to, wind and solar. Certain embodiments allow continued renewable operation and generation even during periods when electrical generation exceeds grid demand (*e.g.*, off-peak wind or solar generation).

[308] In certain embodiments, hydrogen electron donors are not necessarily generated with low- or no- carbon dioxide emissions. However, in certain such embodiments the hydrogen is generated from sustainable or low value sources of energy and/or carbon using methods known in the art of chemical and process engineering. Such methods include but are not limited to gasification, pyrolysis, steam-reforming, or autothermal reforming of feedstock such as but not limited to one or more of the following: agricultural materials, wood, methane hydrates, straw, sea weed and kelp, and low value, highly lignocellulosic biomass in general. In certain embodiments, a synthesis gas or producer gas containing H₂ and/or CO and/or CO₂ is utilized as an electron donor and/or as a carbon source. In certain embodiments, the H₂ and/or CO and/or CO₂ contained in a syngas or producer gas is supplemented by H₂ generated using a renewable and/or low-GHG energy source and conversion process such as one or more of those described herein.

[309] In certain non-limiting embodiments, reduction of CO₂ occurs and/or synthesis of cellular material that can be utilized as a food or nutrition source.

In certain embodiments, the ratio of hydrogen to carbon monoxide in syngas or producer gas may be adjusted through the water gas shift reaction and/or carbon capture, prior to the gas being delivered to the microbial culture. In certain embodiments, C₁ compounds are generated through

methane steam reforming of methane or natural gas, and particularly stranded natural gas, or natural gas that would be otherwise flared or released to the atmosphere, or biogas, or landfill gas, and provided as a syngas and/or producer gas or liquid stream of C1 compounds to the culture of microorganisms, where in certain embodiments the ratio of hydrogen to carbon monoxide in the syngas or producer gas may be adjusted through the water gas shift reaction and/or carbon capture, prior to the gas being delivered to the microbial culture.

[310] The following examples are intended to illustrate, but not limit, the invention.

EXAMPLES

Example 1 - Protein Concentrates, Isolates, or Hydrolysates from *Cupriavidus necator* grown on H₂ and CO₂ with reduced lipopolysaccharides

[311] *Cupriavidus necator* strain DSM 541 was grown on H₂ and CO₂ gas substrates and aqueous minimum salts medium in a 2-liter continuous stirred tank reactor (CSTR). Culture broth was harvested continuously from the CSTR and temporarily stored at 4°C. The biomass was then separated from the liquid broth by centrifugation, the supernatant was poured off, and the dewatered. Wet biomass (approximately 20% solids content / 80% moisture content) was collected and stored at -80°C.

[312] The frozen wet biomass was later thawed and treated in several different manners to remove or reduce the lipopolysaccharide (LPS) content. In some cases, the frozen biomass was freeze dried and then treated to remove LPS. The treatments applied were as described below and compared against the starting biomass (Case 0).

[313] Case 1: The wet biomass was resuspended in water to an 8% solids content using a Turrax stick and hand blender to make a smooth homogeneous slurry. This slurry was then placed in an autoclave. The autoclave was heated to 110°C and held at that temperature for 30 minutes. Following heat treatment, the autoclaved slurry was cooled down in a water bath and then centrifuged at 13,000 g for 40 minutes. The supernatant was poured off, separated for analysis, and the wet solid resulting from centrifugation was collected. The wet solids produced by centrifugation were then freeze dried and ground, resulting in a fine beige powder protein concentrate. The heat treated protein concentrate (HTPC) was evaluated for LPS content.

[314] Case 2: 12 vials of 0.52 g dry biomass were resuspended in 40 mL 0.25 M acetic acid and subjected to ultrasonication for 40 minutes. It was then heated for 7 hours at 105°C, washed with 40 mL distilled water, freeze dried. The freeze dried material was then homogenized with a turrex stick and 35 mL 28-30% NH₄OH plus ethanol added to it. The whole mass was stirred at room temperature for 1 hr, centrifuged to remove the solvent layer and subsequently dried under N₂ and finally at 50°C. The freeze dried protein concentrate was evaluated for LPS content

[315] Case 3: Frozen biomass was diluted to 2% solids load (dry equivalent) with DI water, and cooled on ice before lysing with French press. French press cell was pre-cooled on ice. Cells were

lysed at high ratio at 1280 pressure gauge setting. The cell lysate was collected in collector tube kept on ice at 15-20 drops/min. The cell lysate pH was adjusted from pH 7 to a pH of 11 with 0.21M NaOH to solubilize proteins and then centrifuged to separate supernatant (solubilized proteins) from pellet fraction. The supernatant was freeze dried. This protein isolate was evaluated for LPS content.

[316] Case 4: A 2% solution of biomass was mixed with Turrex Stick at 15000 rpm for 1 min. pH was increased to 10.85 with the help of NH₄OH 28%-30% (pre-made) solution in a fume hood. This mixture was transferred to and autoclaved at 110°C, 10 min after which the pH of the solution was decreased to pH 9 with CO₂ bubbling by inserting a cannula/18G needle for 10-20 min. Enzyme digestion was done with bacterial alkaline protease (Sigma P8038) at 55°C, 110 rpm overnight. Supernatant was separated from the pellet and contained soluble hydrolyzed proteins. This protein hydrolysate was freeze dried and evaluated for LPS content.

[317] Case 5: A 8% solution of biomass was mixed with Turrex Stick at 15000 rpm for 1 min. Strong phosphoric acid (14.8M) was added to make final reaction concentration at 0.5M and the mixture was autoclaved at 110°C, 10 min in pressure tube after which the pH of the solution was increased to pH 9 using Ca(OH)₂. The precipitate was removed and the supernatant was hydrolyzed further using bacterial alkaline protease (Sigma P8038) enzyme for 12-14 hrs. Supernatant was separated from the pellet and contained soluble hydrolyzed proteins. This protein hydrolysate was freeze dried and evaluated for LPS content.

[318] LPS content was evaluated using the gel clot Limulus Amebocyte Lysate (LAL) assay. The results were quantified on the basis of EU/mL. The fold reduction in endotoxin activity was quantified by comparing the EU/ml endotoxin quantification of the derivatives to the original whole cell biomass.

[319] Results are shown in Table 1.

Table 1

SAMPLE TYPE	CASE	ENDOTOXIN CONCENTRATION	ENDOTOXIN FOLD REDUCTION COMPARED TO WCB
Whole cell biomass (WCB)	0	1-2 x 10 ⁷ EU/ml (2 mg/ml solution)	Not applicable
HTPC using 110°C, 30 mins	1	6 x 10 ⁶ EU/ml	Little reduction (2-3 fold)
Protein concentrate using Acetic acid + heat treatment + Defatting	2	1.5 x 10 ⁴ EU/ml	666-1330X reduction
Protein isolate using cell lysis and protein solubilization at pH 11	3	6 x 10 ⁴ EU/ml	250-350 reduction

Protein Hydrolysate produced using a base	4	1×10^6 EU/ml	10-20X reduction.
Protein Hydrolysate produced using an acid	5	2.4×10^3 EU/ml	4000-6000X reduction

Example 2 – Continuous Axenic Culture of *Cupriavidus necator*

Bioreactor Apparatus and Setup

- [320] (1) pH measurement and control (Hamilton EasyFerm Plus K8 200 pH probe)
- [321] (2) Temperature control (capable of heating and cooling to maintain 30°C)
- [322] (3) Overhead stirring (or an alternative method of mixing) capable of generating high oxygen transfer rates (k_{La}) nominally between 1500 and 3000 hr⁻¹
- [323] (4) Dissolved oxygen (DO) measurement and control (Hamilton VisiFerm DO 225 optical DO probe)
- [324] (5) Multitude of pumps to deliver base, nutrient amendments, and antifoam (as needed)
- [325] (6) Automatic feed of base (NH₄OH)
- [326] (7) Liquid sampling apparatus for sterile sampling
- [327] (8) Liquid withdraw for sterile withdrawal of reactor contents
- [328] (9) Sparger (or similar gas diffuser)
- [329] (10) Gas inlet with sterile filter (0.2 µm) connected to the sparger or gas diffuser
- [330] (11) Septa for sterile injection/transfer of inoculum or media using either a syringe or peristaltic pump (alternatively: configured with pumps to perform sterile transfers of inoculum and media into the reactor)
- [331] (12) Autoclavable or capable of in-place cleaning and sterilization
- [332] (13) Mass flow controllers to accurately control gas flow rate and composition, with software to adjust flowrate and composition
- [333] (14) Optical density (OD) measurement (Eppendorf BioSpectrometer for offline measurement, or BugLab BE2100 or BE3000 for continuous measurement)
- [334] (15) Condenser prior to exhaust
- [335] (16) Overflow reservoir to capture and separate foam from the exhaust line
- [336] (17) Liquid level sensor and control (conductance-based sensor, optional at smaller scales)
- [337] (18) Foam detection and control (conductance-based, optional at smaller scales)
- [338] (19) Additional pumps for continuous media feed and broth withdrawal, to be used during continuous operation.
- [339] Suitable feedback and control loops used to monitor and control the bioreactor include:

[340] (1) Temperature control – One or more thermocouples are used to monitor the temperature of the reactor. A combination of a process heater and process cooling water are used to maintain a temperature of 30°C. A proportional integral (PI) or proportional integral derivative (PID) control system is used to control the temperature, while the PI or PID settings will be system dependent. During typical operation, primarily cooling is required due to the exothermic nature of the organism growth. Depending on scale and equipment used, process heating may be provided by an external electric heater or through a temperature-controlled jacket while process cooling is provided by cooling water run through a jacket or internal cooling loop.

[341] (2) pH control – pH probe is used to monitor the pH. A proportional integral (PI) loop is used to control a pump to add base as needed to maintain a pH of 7.0. During typical operation, only base addition is needed.

[342] (3) Dissolved oxygen (DO) – A dissolved oxygen probe is used to monitor the dissolved oxygen content relative to 100% saturation for either oxygen or air as calibration. A cascade feedback loop is used to maintain a DO setpoint by adjusting the following variables: (1) stirring or mixing rate, (2) total gas flow rate, and (3) oxygen concentration in the gas mixture. Note that a 100% DO reading using air as the calibration gas will be equivalent to a 21% DO reading if oxygen is used as the calibration gas instead.

[343] During the high growth phase, the actual reading of the dissolved oxygen probe is found to be around 0, which indicates that the oxygen is consumed at the maximum transfer rate. This results in DO readings that do not reflect the actual concentration of oxygen in the headspace. Depending on the DO control settings for allowable oxygen concentration of the inlet gas can result in undesirable potentially explosive mixtures of gases to arise in the headspace (i.e., >5% oxygen, with the remainder hydrogen and carbon dioxide.) In order to prevent this, control of the inlet gases to achieve a maximum safe concentration of oxygen in the headspace may be used (e.g., ≤ 5% oxygen and gas mixture on the H₂-rich side of the UEL).

[344] (4) Headspace oxygen control feedback – To prevent the system from operating in a potentially explosive regime (i.e., >5% oxygen), measurement of oxygen in the headspace can be used to control the composition of oxygen in the feed gas and/or to shutoff the oxygen feed.

[345] (5) Liquid level control – Due to the addition of base and media as well as the fact that the organism growth produces water, the liquid volume increases in the bioreactor over time and water must either be continuously or occasionally removed to maintain the working volume. A liquid level sensor provides input for a control loop (PI, PID, or on/off) to control a pump, which removes the excess liquid. A conductance-based sensor (or equivalent depending on process scale) is used to establish the liquid level and can be used to control the continuous or staged removal of liquid with a variable speed pump or a single speed pump, respectively.

[346] Manual liquid withdrawal is used for batch and fed-batch operation while the liquid withdrawal tube set at the desired liquid level combined with a pump is used for continuous operation.

[347] (6) Foam control – The growth of *C. necator* at high oxygen transfer conditions and high gas flow rates often results in foaming. A conductance-based sensor is used to measure the foam level either as a continuous measurement or point detection set at the maximum foam level. A basic feedback loop is used to deliver a small aliquot of antifoam as needed. Less preferred is regular manual addition of antifoam. The manual method can be used for lower-foaming system configurations, but still runs the risk of foaming over the bioreactor in between manual additions.

[348] (7) Continuous operation – During continuous operation, the flow rate of fresh media (minimal salt media (MSM)) into the reactor is determined by optical density (OD) trends in the broth. If the OD is being measured manually, a calculation is carried out to determine the proper flow rate setting to achieve or maintain the targeted OD. Inputs to this calculation include the current OD, the trend in OD since the last measurement, the liquid volume in the reactor, and the current estimated productivity. With the integration of a continuous OD sensor such as the BugLab BE2100 or 3000, the OD control can be automatic. The BugLab system can be set up to send an analog or digital output signal which varies based on the slope of recent optical density measurements. A peristaltic pump can be controlled via the analog output on the BugLab base unit. The peristaltic pump will then use the incoming signal to set the flow rate of media into the reactor, between user-defined upper and lower flow rate limits, to maintain a constant OD.

Media Preparation

Minimal Salt Media (MSM)

[349] The media used has the composition as shown in Table 2.

Table 2: Composition of MSM

Sodium phosphate dibasic dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$)	4.5 g/L
Potassium phosphate (KH_2PO_4)	1.5 g/L
Ammonium chloride (NH_4Cl)	1 g/L
Magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.2 g/L
Sodium bicarbonate (NaHCO_3)	0.5g/L
Ferric ammonium citrate ($\text{C}_6\text{H}_8\text{O}_7 \cdot x\text{Fe}^{3+} \cdot y\text{NH}_3$)	5 mg/L
Calcium chloride ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$)	10 mg/L
Zinc sulfate ($\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$)	100 µg/L
Manganese chloride tetrahydrate ($\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$)	30 µg/L
Boric acid (H_3BO_3)	300 µg/L
Cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$)	200 µg/L
Copper chloride dihydrate ($\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$)	10 µg/L
Nickel chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$)	20 µg/L
Sodium molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$)	30 µg/L

Base Preparation (2 N Ammonium Hydroxide)

[350] pH control is performed using 2 N NH_4OH .

[351] 2N Ammonium hydroxide is prepped from more concentrated solutions.

[352] *From 5 N NH₄OH*: For every 1 L of 2N NH₄OH required, combine 400 mL of 5 M NH₄OH with 600 mL of deionized water.

[353] *From concentrated (28%) NH₄OH*: For every 1 L of 2NH₄OH required, combine 137.6 mL of 28% NH₄OH with 862.4 mL deionized water.

Antifoam

[354] Antifoam is added to control the foaming that results from high oxygen transfer conditions. The primary antifoam used is polypropylene glycol.

Inoculum Preparation

[355] Fructose Media (MSM-F) Preparation

[356] The inoculum media is the same MSM as defined above, but with the addition of 40 g/L of D-fructose. The MSM is prepared per the protocol described above, D-fructose is added and dissolved, and the combined solution is filter sterilized. This media is referred to as MSM-F.

[357] For a 1-L preparation: to a 1-L volumetric flask add 40 g of D-fructose, then add MSM to the 1-L mark, stir the solution until the D-fructose is dissolved, and then filter sterilize the combined solution.

Inoculum Preparation from Glycerol Stock

[358] Inocula for the reactors are expanded in autoclaved Erlenmeyer flasks (250 or 500 mL).

[359] The glycerol stock can be: expanded frozen stock culture of *C. necator* grown at 30°C with shaking in tryptic soy broth; or gas-grown stock that had been prepared from pure material aseptically removed from a bioreactor during typical operation.

Preparation for Inoculating Small Bioreactors or Inoculum Train Bioreactor

[360] Small bioreactors are inoculated using ~50 mL of fructose grown inoculum (OD $\geq$ 20) per 1 L of MSM in the bioreactor to give a starting OD of >1.

Preparation for Inoculating Larger Bioreactors

[361] For larger bioreactors or several bioreactors in parallel, an inoculum train bioreactor is used to grow sufficient inoculum to enable starting at higher ODs. The inoculum train bioreactor is inoculated via the method described above and operated following the bioreactor operation conditions described below. The bioreactor is operated for 1-3 days with a target OD of >30. After this target is met, if the reactor is observed to be in the exponential growth phase, the inoculum train bioreactor is deemed ready to inoculate either a single larger bioreactor or multiple parallel bioreactors.

Bioreactor Operation

Preparation

[362] *Prior to autoclaving or sterilizing-in-place:* Calibrate pH probes with pH 4 and 7 standards. Assemble all reactor components as needed to ensure sterility. Prepare and sterilize all media. Calibrate mass flow controllers and pumps per their regular scheduled calibration procedure.

[363] *For the Eppendorf DASGIP reactors:* Install the DO probe, septum, liquid addition ports/lines, liquid withdrawal port (including sample vial), and condenser and check that fittings are hand-tight and O-rings are in good condition. Cover the pH probe port with a spare septum and tape over to close the port during autoclaving. Attach gas inlet tubing (with an inline sterile filter) to the sparger. Attach a vent/overflow bottle with flexible tubing to the condenser. Use cable ties to secure all connections to flexible tubing. Cover all exposed tubing ends with aluminum foil and secure with autoclave tape. Autoclave the reactors (dry) for one hour at 122°C. Separately autoclave the pH probes in buffer solution.

[364] *After autoclaving or sterilizing-in-place:* Follow appropriate procedures at each step to ensure sterility. As needed, connect all process lines (gas feed, gas exhaust, liquid feeds, liquid outlet, overflow reservoir, and temperature control) to the bioreactor.

[365] *For the Eppendorf DASGIP reactors:* Install the pH probes into the reactors in the biosafety cabinet. Install the reactors into the BioBlock and connect all process lines, turn on stirring, and start temperature control as described above. Use the DASGIP software to calibrate the DO probes. After calibration, start a new run in the DASGIP control software. Resume temperature control at 30°C. Start pH control at pH 7 and turn on liquid feed pumps. Set the DO controller to vary stirring rate (800-1200 rpm), gas inlet flow (0.5-2 VVM), and percentage of oxygen in the inlet gas.

Inoculation

[366] Following sterile transfer procedures appropriate for the equipment used, transfer sufficient inoculum from either a fructose grown inoculum or the inoculum train bioreactor into the bioreactor such that a starting OD of 1-5 is achieved. For smaller bioreactors, syringes and the liquid withdrawal line can be used to transfer the inoculum. For larger reactors, a sterile pump should be used to perform a sterile transfer.

Base Addition for pH Control

[367] Base in the form of 2N NH₄OH is added to the reactor to maintain a pH of 7.0.

Nutrient Amendments

[368] For a given amount of 2 N NH₄OH consumed for pH adjustment additional mineral nutrients from the MSM medium are proportioned in such that no mineral nutrient limitations arise.

[369] For the fed-batch protocol, the nutrient amendments are added continuously with the base addition based on ratios that prevent mineral nutrient limitations. In certain embodiments, individual pumps are used for each of the nutrient amendment solutions.

Media Feed for Continuously Stirred Tank Reactor (CSTR) Operation

[370] For continuous operation where the media is fed continuously to the reactor while broth is removed to maintain a set liquid volume, the media is the basal MSM described above.

Foam Control

[371] For small bioreactors, the antifoam is added in a sterile fashion via syringe and from an autoclaved vial of antifoam. For larger bioreactors, the antifoam should be pumped in as needed based on a conductivity-based foam sensor.

Fed-Batch Operation

[372] For the batch growth process, several of the manual interventions can be automated, such as base addition, nutrient amendments, DO control, foam control and OD measurement. As an independent check of the continuous OD measurement, samples can be withdrawn from the reactor at several time points over the course of a run. When taking samples, flush the line with sterile air and remove a purge sample of ~1 mL to clear the sample tube of old un-mixed culture prior to removing a sample for OD and any other tests (DNA etc.).

Continuously stirred tank reactor (CSTR) operation

[373] Continuous operation is similar to fed batch operation within the reactors, with automated base addition, nutrient amendments, and DO control, and manual or automated antifoam addition and OD measurement. However, there is additional handling required for the fresh media feed and the broth withdrawal. The fresh media (MSM described above) is prepared ahead of time in a sterile container with sufficient media for several days of operation and is aseptically attached to the reactor liquid inlet line. The fresh media feed rate should be controlled either manually or automatically as described above. A pump and liquid level control are used to maintain the set liquid level. The material withdrawn from the reactor is plumbed to a pre-sterilized, refrigerated (4°C) collection reservoir. Material is harvested by pumping out of this reservoir on a regular basis, typically daily. The volume and OD of each harvest is measured, and 2 replicate 10 mL aliquots of broth may be taken to measure the cell dry weight density so that the productivity for the period encompassed by the harvested material can be calculated. The remainder of the harvest is centrifuged.

[374] *For the Eppendorf DASGIP bioreactor system:* The fresh media is prepared and sterilized 6 liters at a time in 10-L carboys with attached tubing which is connected, as sterile as possible, to the reactor liquid inlet line. A peristaltic pump with dual pump heads is used for both fresh media feed and broth withdrawal.

Harvest

[375] Material removed from the reactor is centrifuged to remove the biomass from the supernatant. Depending on scale and operational method, the material can be centrifuged in either a batch or continuous method at the equivalent of 12,000 G for 15-45 minutes at 4°C. Larger sample volumes tend to take longer to be centrifuged. Ideally, the broth is centrifuged once it is removed from the bioreactor, however if centrifuging does not occur immediately, then the broth should be refrigerated at 4°C until centrifuging can occur. At this point additional downstream processing steps may be performed on the wet centrifuged biomass including LPS removal and/or endotoxin reduction as described above.

Continuous axenic growth of *C. necator* with CSTR operation

[376] An Eppendorf DASGIP bioreactor system was adapted for growing *C. necator* (DSM 531 or 541) on H₂ and CO₂, as described above. Nutrient amendments were provided continuously. After an initial growth in fed-batch operation, the bioreactor was operated continuously, with continuous addition of fresh media at flow rates adjusted as needed to maintain the culture within a target OD range, as shown in Fig. 5. Fig. 5 shows base consumption, OD₆₀₀, and fresh media flow rate for the continuous run started from a single inoculum of *C. necator* that lasted for over 1,000 hours with no contamination of the *C. necator* culture. Streak plates taken from the bioreactor broth after 1,077 hours of continuous operation confirmed there was no contamination, *i.e.*, the colony morphology supported pure culture *C. necator*.

[377] Although the foregoing invention has been described in some detail by way of illustration and examples for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced without departing from the spirit and scope of the invention. Therefore, the description should not be construed as limiting the scope of the invention.

[378] All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entireties for all purposes and to the same extent as if each individual publication, patent, or patent application were specifically and individually indicated to be so incorporated by reference.

CLAIMS

We claim:

1. A method for producing a food product, said method comprising:
 - (a) processing microorganism cells that are harvested from a culture medium to produce a protein product, wherein said protein product comprises one or more of single cell protein, cell lysate, protein concentrate, protein isolate, protein extract, protein hydrolysate, free amino acids, peptides, and oligopeptides, and wherein said protein product comprises endotoxins from the microorganism cells;
 - (b) processing the protein product to reduce the concentration of said endotoxins; and
 - (c) processing the protein product to produce a food product.
2. The method of claim 1, wherein the concentration of endotoxin in the protein product is reduced by at least about 2-fold.
3. The method of claim 2, wherein the concentration of endotoxin in the protein product is reduced by about 2-fold to about 6000-fold.
4. The method of claim 1, wherein said protein product comprises nucleic acids, and wherein the protein product is processed to reduce the concentration of said nucleic acids prior to step (c).
5. The method of claim 4, wherein the nucleic acid content of the protein product is less than about 9%.
6. The method of claim 1, wherein the food product is a food item, a food ingredient, a nutritional product, an animal feed product, or a pet food product.
7. The method of claim 1, wherein the food product is a meat analogue product.
8. The method of claim 6 or 7, wherein the food product comprises at least about 10% of the protein product by weight.
9. The method of claim 1, wherein the food product is a vegetarian or vegan food product.
10. The method of claim 1, wherein the food product is an organic food product, a pesticide-free food product, a herbicide-free food product, a fungicide-free food product, an antibiotic-free food product, or a non-genetically-modified (non-GMO) food product.
11. The method of claim 1, wherein the food product is a probiotic food product or a prebiotic food product.

12. The method of claim 1, wherein the food product comprises no animal protein or fat.
13. The method of claim 1, wherein the food product comprises a dairy product, a dairy replacement product, a bakery product, a confection, a health or protein bar, a protein powder, a sports and/or energy drink, a protein shake, or a smoothie.
14. The method of claim 1, wherein the food product comprises one or more plant protein source.
15. The method of claim 1, wherein the food product comprises an insect or algae protein source.
16. The method of claim 1, wherein the microorganism is a chemoautotrophic microorganism.
17. The method of claim 16, wherein the chemoautotrophic microorganism is an oxyhydrogen microorganism.
18. The method of claim 17, wherein the oxyhydrogen microorganism is a *Cupriavidus* microorganism.
19. The method of claim 18, wherein the oxyhydrogen microorganism comprises *Cupriavidus necator* DSM 531 or DSM 541.
20. The method of claim 1, wherein step (a) comprises: (i) freeing organic molecules from the microorganism cells, via excretion, secretion, or cell lysis, or a combination thereof, wherein the organic molecules comprise proteins; and (ii) treating the freed organic molecules to hydrolyze peptide bonds between at least a portion of amino acids in at least a portion of the proteins, thereby producing a hydrolysis protein product that comprises polypeptides comprising 20 to 50 amino acids, oligopeptides comprising 2 to 20 amino acids, and/or free amino acids.
21. The method of claim 1, wherein step (c) comprises combining the protein product with other edible ingredients to form the food product.
22. A food product prepared by the method of any of claims 1 to 21.