

## **METHODS FOR CULTIVATING CELLS, PREPARATION OF HYDROLYSATE FROM CELLS AND APPLICATIONS THEREOF**

### **Technical Field**

**[0001]** Embodiments discussed herein generally relate to methods for cultivating cells and the preparation of hydrolysate from cells. Embodiments discussed herein also generally relate to the applications of the hydrolysate from cultivated cells.

### **Background**

**[0002]** Animal meat is high in protein, and supplies all the amino acids needed to build the protein used to support body functions. Meat for consumption is traditionally obtained from animals or fish that are reared on farms. However, agriculture and aquaculture for producing animal meat require a large amount of energy and resources, and have a high carbon footprint. Meat produced by agriculture or aquaculture may pose a public health risk as the production processes may expose the meat to diseases, pollutants, and toxins. A number of concerns such as a growing population, increasing demand for meat, environmental concerns, limited land and water resources, biodiversity loss, and the negative perception associated with animal slaughter have led scientists to develop techniques to produce meat by alternative processes.

**[0003]** In vitro meat production is the process by which muscle tissue or organ tissue from animals are grown in laboratories using cell culture techniques to manufacture meat and meat products. As used herein, in vitro meat and meat products includes animal protein products as well as non-meat products including soluble forms and solid forms. While still in an early stage of development, in vitro meat and meat products may offer a number of advantages over traditional meat products such as health and environmental advantages, and benefits to animal welfare. It is a next-generation and emerging technology that operates as part of a wider field of cellular agriculture, or the production of agricultural products from cell cultures.

**[0004]** Cells for the production of in vitro meat may be cells (e.g., muscle cells, somatic cells, stem cells, etc.) taken from animal biopsies, which may then be grown separately from the animal in culture media in a bioreactor or other type of sterile environment. The cells may grow into a semi-solid or solid form mimicking an animal organ by attaching to an edible three-dimensional scaffold that is placed in the bioreactor. The starter cells may be primary cells directly obtained from the

animal's tissues, or continuous cell lines. If grown under the right conditions in appropriate culture media, primary cells will grow and proliferate, but only a finite number of times that is related to the telomere length at the end of the cell's DNA. Continuous cell lines, on the other hand, can be cultured in vitro over an extended period. Cell biology research has established procedures on how to convert primary cells into immortal continuous cell lines. Primary cells may be transformed into continuous cell lines using viral oncogenes, chemical treatments, or overexpression of telomerase reverse transcriptase to prevent the telomeres from shortening.

**[0005]** The culture media may contain components necessary for cell proliferation such as amino acids, salts, vitamins, growth factors, and buffering systems to control pH. Current methods add fetal bovine serum (FBS) to the media prior to use as it provides vital macromolecules, growth factors, and immune molecules. However, FBS is derived from unborn calves and, therefore, is incompatible with the objective of being free from animal products. Growing the cells in an animal component-free medium is an important factor considered by scientists involved in in vitro meat production research. Some growth factors may be derived from human sources.

**[0006]** Current in vitro meat production covers most commodity meat types, such as, cell-based beef, pork and poultry meats. However, these types of meats have a complex tissue organization involving multiple cell types that are difficult and costly to produce using current biomedical technology techniques. There is also a lack of non-GM methods to increase the protein level and biomass yield in meat produced by cell culture techniques. Furthermore, as explained above, current cell culture technologies may rely on animal components (e.g., FBS) as a nutrient source, as well as expensive non-food grade growth factors.

**[0007]** In addition to human consumption, meat is often used to produce animal-derived raw materials for skincare, cosmetic and wound care products. For example, skincare, cosmetic, and wound care products often incorporate growth factors, cytokines, and extracellular matrix (ECM) proteins which stimulate skin tissue repair and regeneration. They can include hyaluronic acid derivatives to moisturize the skin and decrease wrinkles; antioxidants to protect against aging-associated oxidative stress. The active ingredients are mostly derived from wild animals and wild plants. However, creating derived raw materials from wild animals and wild plants has the following drawbacks.

- [0008]** First, it requires a constant supply of animal and plant sources. An increase in the demand for animal products causes more animals to suffer and be killed in farms and slaughterhouses. The growth in keeping livestock may also provide an additional burden to the environment or ecosystem. Further, it may also give rise to animal abuse and welfare issues.
- [0009]** Second, only targeted proteins are extracted and the rest of other functional proteins produced in the wild animals or wild plants are lost. Usually, the derived raw materials are created by extracting certain target/interested proteins from wild animals or wild plants. The rest of the other proteins or nutrients are not captured in the extraction process. However, it may be also desirable to also include those trace amounts of the functional proteins in the derived raw materials because such functional proteins could enhance the outcome for a certain purpose. For example, the total nutrition in oranges may be more beneficial to a human for a certain purpose compared to just vitamin C extracted from the orange. Moreover, raising animals or growing plants only to obtain some usable parts to generate derived raw materials is highly inefficient.
- [0010]** Third, it involves the use of harmful chemicals. For example, extracting collagen from fish skin involves a series of steps including the steps of mixing the fish skins with alkaline and acidic solutions. This provides an additional burden to the environment or ecosystem. Also, it may contribute to potential occupational hazards. The derived raw materials may also contain harmful chemicals.
- [0011]** Fourth, there are safety concerns due to the presence of environmental contaminants (heavy metal, antibiotics, microplastics, herbicides, fungicides, insecticides), adventitious agents (bacteria, viruses, fungi, transmissible spongiform encephalopathy agents), and allergens in the wild animals and wild plants.
- [0012]** Fifth, there are difficulties in controlling the molecular profile and determining the consistency of animal-derived (e.g. animal serum) and plant-derived (e.g. plant extract, plant hydrolysate) raw materials. Each batch of animal-derived or plant-derived raw materials is created from different batches of animal and plant, which can deviate significantly due to variations of temperature, time of harvest, types of animal feed and fertilizers, presence of pest and parasites, etc. Some molecules in a certain batch may trigger an allergic reaction in some individuals.

**[0013]** Sixth, further, if the animal-derived and plant-derived raw materials are obtained from wild animals and wild plants, it is very difficult to trace back to the origin.

**[0014]** Alternatively, derived raw materials may be created by using recombinant organisms. However, this method involves the use of genetically modified organisms, which could be harmful to the environment when such organisms are released accidentally. Furthermore, only one (1) protein may be produced per production line. This method is inefficient for multiple proteins derived from raw materials. In addition, the protein produced by the recombinant organisms has to be further isolated and purified. The isolation and purification typically involve multiple steps which lead to an increase in production cost. Moreover, there may be variations in the folding of the protein produced by this method. Some functions may be lost in some variations.

**[0015]** Alternatively, the spent medium may also be a source of functional protein(s). However, similar to the above methods, it has several drawbacks. Firstly, it requires a constant supply of animal and plant sources. The disadvantages thereof have been discussed in the foregoing and therefore not repeated here. Secondly, there are contamination concerns due to the presence of environmental contaminants (heavy metal, antibiotics, microplastics, herbicides, fungicides, insecticides), adventitious agents (bacteria, viruses, fungi, transmissible spongiform encephalopathy agents), and allergens in the wild animals and wild plants sources. Thirdly, unwanted metabolites and wastes in the spent medium may affect the purity of the final product. Additional purification steps would lead to an increase in production costs.

### **Summary**

**[0016]** The embodiments of the present disclosure apply methods for in vitro meat production for human consumption that provides a solution to the above challenges.

**[0017]** It is an objective of the present invention to provide an alternative method of obtaining animal-derived raw materials and/or plant-derived raw materials under a controlled process. The derived raw materials according to the present invention are free of environmental contaminants, adventitious agents and allergens. It also helps to limit animal suffering and sacrifice associated with the production of animal-derived materials.

**[0018]** Cell hydrolysate is often extracted from the derived raw materials and be applied as an active ingredient in skincare, wound care, cosmetic or food products. Cell hydrolysate includes but is not limited to hyaluronic acid. given the foregoing, it is further an objective of the present invention to provide a more efficient and environmentally friendly process in making various products including but not limited to skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications. It also provides improved batch-to-batch consistency and product traceability for such products.

**[0019]** It is also an objective of the present invention to create further values for consumers or manufacturers in various industries/products, including but not limited to the skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications, as it simultaneously solves all drawbacks encountered by the conventional derived raw material creation method (i.e. extracting from wild animals/plants, recombinant DNA organisms and spent medium). The present invention provides the following benefits:

- (1) no reliance on animal and/or plant sources;
- (2) no reliance on genetically modified organisms;
- (3) the capture of all functional proteins naturally produced by the subject cells;
- (4) Purity (no waste in the product);
- (5) No use of harmful chemicals;
- (6) simple downstream process, thereby lowering the cost; and
- (7) multi-functional instead of single-use/function/action.

**[0020]** The benefits of the present invention lead to the creation of new values that meet consumers' needs. It provides active ingredient of various products or the products itself (includes but not limited to skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications) the following characteristics:

- (1) clean label due to the high purity and non-existence of waste or harmful chemicals in the derived raw materials;
- (2) sustainability due to little or low reliance on animal and/or plant sources;
- (3) non-chemical synthesis due to the use of biomaterials;
- (4) multi-functional due to its complete molecular profile with all functional proteins naturally produced by the subject cells;
- (5) backed by scientific principles and test results.

**[0021]** According to some embodiments of the present disclosure, a method for meat production by in vitro cell culture includes isolating tissue from an animal or plant source and making a cell suspension of cells. The method further includes growing the cells on a food-grade scaffold in a culture medium such that the cells grow into a solid or semi-solid structure that mimics an animal organ. Additionally, the method further includes increasing expression of a protein in the growing cells by altering a level of one or more micro RNAs that regulate the expression of the protein.

**[0022]** According to another embodiment of the present disclosure, a method for meat production by in vitro cell culture includes isolating tissue from a plant or animal source and making a cell suspension of cells, and growing the cells on a food-grade scaffold in a culture medium such that the cells grow into a solid or semi-solid structure that mimics an animal organ. The method further includes co-culturing the cells with bioengineered cells that secrete nutrients, growth factors, and cytokines that support the growth of the cells.

**[0023]** According to another embodiment of the present disclosure, a method for generating cell hydrolysate by in vitro cell culture includes the steps of (i) lysing a cell by using sonication to release proteins in the cell; (ii) digesting the proteins by using protease to produce short peptides having a molecular size smaller than 500 daltons (Da); (iii) terminating the digestion step by either heating protease to 95 degree Celsius for 15 minutes or diluting the protease 100-fold using PBS; and (iv) sterile filtering the mix obtained from the digestion termination step through a membrane filter.

**[0024] Brief Description of the Drawings**

**[0025]** The disclosure may be better understood by reference to the detailed description when considered in connection with the accompanying drawings. The components in the figures are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the disclosure.

**[0026]** FIG. 1 is a flowchart of a method for meat production by in vitro cell culture, according to some embodiments of the present disclosure.

**[0027]** FIG. 2 is a schematic representation of a method for post-transcriptional enhancement of protein expression, according to some embodiments of the present disclosure.

- [0028] FIG. 3 is a schematic representation of a method for post-transcriptional enhancement of collagen, type 1, alpha 1 (COL1A1) expression, according to some embodiments of the present disclosure.
- [0029] FIG. 4 is a schematic representation of a method for post-transcriptional enhancement of collagen, type 1, alpha 2 (COL1A2) expression, according to some embodiments of the present disclosure.
- [0030] FIG. 5 is a schematic or conceptual cross-sectional view of a bioreactor used for in vitro meat production having a solid phase support, according to some embodiments of the present disclosure.
- [0031] FIG. 6 is a schematic or conceptual cross-sectional view of a bioreactor similar to FIG. 5 but having a second solid phase, according to some embodiments of the present disclosure.
- [0032] FIG. 7 is a flowchart of a method for generating cell hydrolysate from in vitro cell culture, according to some embodiments of the present disclosure.
- [0033] FIG. 8 is a flowchart of a method for evaluating the effect of cell hydrolysate on human skin cells, according to some embodiments of the present disclosure.
- [0034] FIG. 9 is a chart depicting skin cells laminin expression upon exposure to hydrolysate.

### **Detailed Description**

#### **[0035] In Vitro Meat Production**

- [0036] Referring now to the drawings, and with specific reference to FIG. 1, a method 10 for in vitro meat production is shown. As used herein, "in vitro meat production" refers to a cell-based meat production process or cell-based agriculture process in which tissues from animals and/or plants are grown in laboratories using cell culture techniques to manufacture meat and meat products. At a block 12, tissue from an animal or a plant is isolated. In some embodiments, the tissue is derived from bony fish of the class Osteichthyes including saltwater fish such as a grouper, sea bass, or a yellow cocker. In other embodiments, other types of animal tissue, such as cow tissue, may be isolated. In some embodiments, the block 12 may involve collecting organ tissue, such as a swim bladder, from a fish and making a cell suspension. Although the following description primarily describes tissues derived from fish sources, it will be understood that the concepts may be applied to tissues derived from other types

of animal sources and/or plant sources to provide other types of in vitro meat and/or animal protein products, and vegetarian meat and/or protein products.

**[0037]** Many of the isolated cells are adult cells, and can be made to proliferate continuously using various established methods in medical research (block 14). For example, specific genes, such as Yamanaka factors, may be used to reprogram the adult cells into stem cells, such as induced pluripotent stem cells (iPSCs). Alternatively, the isolated adult cells may be transformed into continuous cell lines by telomerase reverse transcriptase overexpression. In other embodiments, other types of cells may be isolated such as adult stem cells and embryonic stem cells. In this regard, it will be understood that the methods of the present disclosure include all sources of cell lines.

**[0038]** At a next block 16, the cells are grown into a solid or semi-solid structure mimicking an animal organ, such as a fish organ, by attaching/adhering to a food-grade biocompatible scaffold in a sterile chamber or container, such as a bioreactor. The sterile chamber or container may be temperature controlled, and may have inlets and outlets for introducing and removing substances such as chemicals, nutrients, and cells. The food-grade biocompatible scaffold becomes part of the final edible product, and is made of plant-based or fungi-based materials such as, but not limited to, agarose, alginate, chitosan, mycelium, and konjac glucomannan. Alginate is a biopolymer naturally derived from brown algae and is biocompatible. In addition, plant-based chitosan from fungi has antibacterial properties. In some embodiments, the block 16 is carried out in the absence of antibiotics or antimicrobial compounds in the sterile container. A block 18 involves supplying the culture medium to the bioreactor to support cell survival and growth. The culture medium may be a buffered solution containing components such as, but not limited to, inorganic salts (e.g., calcium chloride ( $\text{CaCl}_2$ ), potassium chloride (KCl), sodium chloride (NaCl), sodium bicarbonate ( $\text{NaHCO}_3$ ), sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ), magnesium sulfate ( $\text{MgSO}_4$ ), etc.), amino acids, vitamins (e.g., thiamine, riboflavin, folic acid, etc.), and other components such as glucose,  $\beta$ -mercaptoethanol, ethylenediaminetetraacetic acid (EDTA), and sodium pyruvate. Non-limiting examples of growth media include, but are not limited to, Leibovitz's L-15 medium, Eagle's Minimum Essential Media (MEM), Medium 199, Dulbecco's Modified Eagle Medium (DMEM), Ham's F12 Nutrient Mix, Ham's F10 Nutrient Mix,

MacCoy's 5A Medium, Glasgow Modified Eagle Medium (GMEM), Iscove's Modified Dulbecco's Medium, and RPMI 1640.

**[0039]** If plant tissues/cells are used, the culture medium may contain components necessary for plant cell proliferation such as amino acids, salts, vitamins, growth factors, carbohydrates, nitrate and buffering systems to control pH. The culture medium may be Murashige and Skoog (MS) medium, Linsmaier and Skoog (LS) medium, Gamborg (B5) medium, Nitsch and Nitsch (NN) medium and White's medium.

**[0040]** According to a block 20, food-grade growth factors and cytokines are introduced into the culture medium in the bioreactor to support cell growth and proliferation. The growth factors and cytokines may include, but are not limited to, insulin growth factor 1 (IGF-1), insulin, interleukin 6 (IL-6), interleukin 6 receptor (IL-6R), interleukin 11 (IL-11), fibroblast growth factor (FGF), epidermal growth factor (EGF), and transferrin. The block 20 may involve co-culturing bioengineered cells with the isolated cells in the absence of fetal bovine serum (FBS). The bioengineered cells are engineered to secrete the above growth factors and cytokines, and supply these biomolecules to the isolated cells as needed for growth and proliferation. As used herein, "bioengineered" cells are not equivalent to genetically-modified cells. The bioengineered cells have a specific gene that overexpresses one or more specific proteins. The bioengineered cells may be fish cells, or other types of animal cells, such as cow cells. The bioengineered cells are not present in the final meat product. As non-limiting examples, bioengineered fish cells may be co-cultured with isolated fish cells, or bioengineered cow cells may be co-cultured with isolated cow cells. The co-culturing method of the present disclosure eliminates the need for animal-derived fetal bovine serum (FBS) in the culture medium. Furthermore, the co-culturing method provides a continuous supply of food grade-specific growth factors and cytokines to the growing cells in situ, and simplifies and reduces the cost of the production process. However, in other embodiments, FBS or other serum may be used to supply growth factors, cytokines, and other nutrients to support cell growth during the block 16.

**[0041]** Additionally, according to a block 22, protein expression in the cells is increased to increase the biomass yield in the resulting meat product. As used herein, "biomass yield" refers to the amount of digestible material (e.g., proteins) in the resulting meat product that is available for energy production upon

consumption. More specifically, the block 22 involves increasing protein expression by altering micro RNA levels in the cells, with the manipulation of the cells being carried out prior to culturing. Micro RNAs are endogenous, short, non-encoding single-stranded RNA sequences involved in regulating post-transcriptional gene expression. The block 22 involves increasing the amount of up-regulating micro RNAs that increase protein expression by promoting messenger RNA (mRNA) translation, and/or decreasing the amount of down-regulating micro RNAs that decrease protein expression by suppressing mRNA translation. The micro RNA levels may be increased or decreased by introducing micro RNAs, micro RNA mimics, or micro RNA inhibitors into the cells. The micro RNA mimics have the same function as micro RNAs, but maybe more stable and efficient in modulating protein expression. In some embodiments, electroporation may be used to introduce episomal vectors into the cells that carry instructions to express specific micro RNAs. Alternatively or in combination with this, an adeno-associated virus may be used as a vehicle carrying episomal instructions to express specific micro RNAs. Decreasing the amount of targeted down-regulating micro RNAs may be achieved by introducing inhibitors for the targeted micro RNAs into the cells by transfection. It is noted here that the methods of increasing protein expression/biomass yield according to the present disclosure is carried out without modifying the genome of the cells.

**[0042]** Turning to FIG. 2, a method for post-transcriptional enhancement of protein expression in the cell lines is schematically depicted. One or more up-regulating micro RNAs (miRNAs) may be increased to increase mRNA translation and protein production of selected proteins. Alternatively or in combination with this, one or more down-regulating miRNAs may be blocked with inhibitors (anti-miRNAs) to increase mRNA translation and protein production of selected proteins.

**[0043]** Fish swim bladder primarily includes fibroblasts and collagen protein. Collagen type 1 (collagen I) is a dominant protein in the fish swim bladder, and increased expression of collagen I in cultured fish swim bladder cells may increase biomass yield. Collagen I in fish swim bladder cells includes collagen, type 1, alpha 1 (COL1A1) and collagen, type 1, alpha 2 (COL1A2). COL1A1 and COL1A2 expression is increased by up-regulating microRNA 21 (miR-21), such that increasing levels of miR-21 increase COL1A1 and COL1A2 production in fish swim bladder cells. Additionally, COL1A1 and COL1A2 expression being

decreased by down-regulating microRNA 29a (miR-29a), such that decreasing levels of miR-29a or blocking the action of miR-29a increases COL1A1 and COL1A2 production in fish swim bladder cells. FIGs. 3-4 show increasing COL1A1 (FIG. 3) and COL1A2 (FIG. 4) production by increasing miR-21 levels and by blocking the action of miR-29a with the use of inhibitors (anti-miR 29a). Increased COL1A1 and COL1A2 production results in increased biomass yield in the resulting meat product. Similar strategies may be applied to increase relevant protein levels in other types of animal cells.

**[0044]** Turning to FIG. 5, an exemplary bioreactor 30 used for culturing the isolated cells is shown. The cells attach to and grow on a solid phase support 32 provided by a food-grade scaffold 34 which is held in a sterile chamber 36 in the bioreactor 30. The scaffold 34 may dictate the shape of the meat product. The food-grade scaffold 34 is made of plant-based or fungi-based materials such as, but not limited to, agarose, alginate, chitosan, mycelium, and konjac glucomannan. The solid phase support 32 may be porous so that the cells may attach to and grow on inner surfaces of the support 32. The culture medium supplying nutrients to the cells is introduced into the bioreactor 30 through an inlet 38, and is emptied from the bioreactor 30 through an outlet 40.

**[0045]** FIG. 6 shows a bioreactor 50 similar to the bioreactor 30 of FIG. 5, but further includes a second solid phase 52 separated from the solid phase support 32 by a fine mesh 54. The second solid phase 52 may contain or support the bioengineered cells that secrete nutrients, growth factors, and cytokines for the cells growing on the solid phase support 32 in situ, and may physically separate the bioengineered cells from the cells on the solid phase support 32. The second solid phase 52 is made of plant-based materials, similar to the solid phase support 32. The mesh 54 is permeable to nutrients, growth factors, and cytokines, but is impermeable to cells. The bioreactor 50 of FIG. 6 allows the co-culturing of the bioengineered cells with the growing cells. In some embodiments, the bioreactors 30 and 50 of FIGs. 5 and 6 may be arranged in tandem. In other embodiments, several of the bioreactors 30, several of the bioreactors 50, or mixtures of the bioreactors 30 and 50 may be arranged in series for scaling up the process. The bioreactor 30 may be used mainly for biomass production, whereas the bioreactor 50 may be used for providing nutrients, growth factors, and cytokines to the growing cells.

**[0046]** The in vitro meat production method of the present disclosure provides meat products with a simple tissue organization of one cell type. The meat product with one cell type is easier to make, develop, and commercialize compared to other cultured meats having multiple cell types. Alternative embodiments of the present disclosure provide meat products with multiple cell types. Furthermore, Applicant has discovered a strategy to increase biomass/protein production by altering micro RNA levels or activity in the growing cells. In one example, two key micro RNAs (miR-21 and miR-29a) are targeted to increase the levels of the dominant protein (collagen I) found in fish swim bladder cells. As far as the Applicant is aware, alteration of micro RNA levels or activity to achieve an increased protein/biomass yield in cultured meat products has not been used by others in the field of cultured meat development. Targeting micro RNAs for increased protein production may cause less stress to the cells than known knock-in or knock-out methods. Bio-engineered cells are co-cultured with the growing animal cells to supply the growing fish cells with food-grade growth factors and cytokines for cell growth and proliferation in situ, reducing or eliminating the need for animal-derived FBS in the culture medium. The co-culturing technique simplifies the production process and reduces production costs.

**[0047]** Furthermore, the nutrients of the cultivated meat product may be customized to generate a healthier food product. For example, the cultured meat product may be customized according to diet recommendations from a dietician to from a personal genomic test. Healthy nutrients such as high-density cholesterol, polyunsaturated fatty acids, and monounsaturated fatty acids in the meat product may be enriched by culturing the cells in specific conditions. Alternatively, or in combination with this, nutrients known to be damaging to health such as low-density cholesterol and saturated fatty acids may be reduced by culturing the cells in specific conditions. Micronutrients, such as vitamins and minerals, may also be enhanced. Nutrient customization of the cultivated meat products may be achieved in various ways such as, but not limited to, 1) tailoring the nutrients fed to the growing cells during cell culture, and/or 2) controlling the proportions of layering scaffolds with different cells.

**[0048]** The production of the cultivated food product is under a clean, sterile and highly controlled process. Thus, undesirable degradation by microorganisms such as bacterial or fungi of the nutrients in the food product is minimized. Undesirable taste and smell from the breakdown of nutrients by microorganisms are also

minimized. This property of cultivated food enables new uses in cooking and helps creates novel recipes. One such application of cultivated food is cultivated fish maw derived from fish swim bladders. Traditional fish maw has an undesirable fishy taste and smell due to the degradation of amine by bacteria in the production process. This undesirable property limits the food ingredient to savory dishes served hot or warm. Cultivated fish maw produced from cell culture technology does not have an undesirable fishy taste and smell. In addition to hot and savory dishes, cultivated fish maw can be used in sweet dishes, as a dessert or in a ready-to-eat format served at chilled or at ambient temperature.

**[0049] Generating Cell Hydrolysate by In Vitro Cell Culture**

**[0050]** The products produced through in vitro meat production of the present invention may be used to produce animal-derived raw materials or plant-derived raw materials. Besides human consumption of such derived raw materials, those can be used in various products including but not limited to skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications. Animal-derived raw materials and/or plant-derived raw materials from the products generated by in vitro meat production of the present invention offer a lot of benefits compared to those created from wild animals or plants.

**[0051]** First, since the cells are grown under contaminant-free and disease-free conditions, the culture medium does not contain environmental contaminants (heavy metal, antibiotics, microplastics, herbicides, fungicides, insecticides) or adventitious agents (bacteria, viruses, fungi, transmissible spongiform encephalopathy agents). As a result, the animal-derived raw materials or plant-derived raw materials generated will be also contaminant-free and disease-free.

**[0052]** Second, an animal-component-free and chemically defined medium may be used to reduce the chance of triggering an allergic reaction of the user. This is achieved by substituting animal serum and animal-derived growth factors (e.g. bovine insulin) in the medium by recombinant growth factors. Plant extract/hydrolysate is not required in a chemically defined medium.

**[0053]** Third, the batch-to-batch consistency of the animal-derived raw materials or plant-derived raw materials may be significantly improved. This is because the nutritional profile (carbohydrates, amino acids, vitamins, minerals) of the basal medium is known and consistent and may be further refined using a chemically defined medium, i.e. a medium with known concentrations of all nutrients and growth factors.

- [0054]** Fourth, enhance traceability. Since the supply chain for every culture medium component is known, everything could be easily traced back to the origin.
- [0055]** Fifth, reduce animal suffering and sacrifice. Running the production process does not require a continuous supply of animal tissues from wild animals. Initially, the starter cells are purified from a small piece of animal tissue and developed into a cell line, which can be cryopreserved and propagated indefinitely in a culture medium. This limits animal suffering and sacrifice.
- [0056]** Sixth, reduce waste and enhance efficiency. Nutrients in the medium are directly supplied to cells for cell growth. Every cultivated cell is lysed to produce the cell hydrolysate. There is no waste of energy and nutrients for the growth of unused animal/plant parts, or life processes such as animal mating and locomotion.
- [0057]** Further, the present invention creates values for consumers or manufacturers of various industries/products, including but not limited to skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications, since it addresses all the drawbacks encountered in the conventional methods as mentioned in the background section. The present invention provides the following benefits:
- [0058]** First, it does not rely on a continuous feed of animal sources. For example, to produce more collagen-based cream via the conventional method, more animal body parts are needed which can result in more animal suffering or sacrifice. In the present invention, starter cells (not limited to stem cells, muscle cells, fibroblast cells, adipocytes) are purified from a small piece of animal tissue and established into a cell line. The cell line can be cryopreserved and stored in liquid nitrogen. When needed, the cell line can be thawed and propagated indefinitely under cell culture conditions to produce active ingredients (e.g. growth factors, ECM molecules). Therefore, the entire process is self-sustainable and causes little animal suffering/sacrifice.
- [0059]** Second, it does not rely on the animal source. In the present invention, starter cells (not limited to stem cells, muscle cells, fibroblast cells, adipocytes) are purified from a small piece of animal tissue and established into a cell line. The cell line is not genetically modified.
- [0060]** Third, the derived raw materials of the present invention are multi-functional because it includes a lot of trace amount of functional proteins in addition to the

key target protein. Such a trace amount of functional proteins may be essential for many cellular functions. A product created from derived raw materials with a functional protein profile closer to an extracted wild animal/plant part generally provides better performance than a product created from derived raw materials with a less complete functional protein profile. Even though functional proteins can be added into the derived raw materials through external replenishments, it is difficult to replenish a number of functional proteins. Also, the cost increases with the number of replenishments.

**[0061]** Fourth, the functional proteins created by the present invention do not contain waste since the ingredient in the growth medium is well controlled.

**[0062]** Fifth, the derived raw material created by the present invention does not contain harmful chemicals as the functional proteins are not extracted using harmful chemicals. The ingredient in the growth medium is well controlled.

**[0063]** Sixth, the functional proteins created by the present invention do not contain wastes and harmful chemicals, therefore, there is no need to further isolated and purified. Therefore, the production cost can be lower.

**[0064]** The benefits of the present invention allow the creation of new values that meet consumers' needs. It provides active ingredient of various product or the products itself (including but not limited to skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications) the following characteristics:

- (1) clean label due to the high purity and non-existence of waste or harmful chemicals in the derived raw materials;
- (2) sustainability due to little or low reliance on animal and/or plant sources;
- (3) non-chemical synthesis due to the use of biomaterials;
- (4) multi-functional due to its complete molecular profile with all functional proteins naturally produced by the subject cells;
- (5) backed by scientific principles and test results.

**[0065]** Referring now to the drawings, and with specific reference to FIG. 7, a method of producing cell hydrolysate from in vitro cell culture. In cell growing step 100, cells are first grown under controlled conditions using a culture medium which is either partially defined (i.e. defined basal medium supplemented with FBS/plant hydrolysates/human platelet lysate) or a chemically defined medium (i.e. medium having defined concentrations of all nutrients and growth factors) that is devoid of animal or plant components. In some embodiments, cells are seeded at  $4 \times 10^4$

cells/cm<sup>2</sup> in growth medium (DMEM/F12 + 10% FBS) and kept inside a humidified incubator (34°C; 5% CO<sub>2</sub>, 95% air). In some embodiments, the cells are grown using the in vitro meat production method 10 of the present invention. In yet some embodiments, at least one cell line may be used in the cell growing step 100. In some embodiments, the cell lines include stem cells, muscle cells, fibroblast cells, and adipocytes. In yet one specific embodiment, cultivated fish swim bladder cells are used in the cell growing step 100.

**[0066]** Once the cell confluence reaches at least 90%, detaching step 102 begins. In detaching step 102, the cells are detached from its adhered surface using trypsin/EDTA or any other recombinant protease (e.g. recombinant collagenase IV, TrypLe Express). In some embodiments, upon reaching 90% confluence, the growth medium is removed. Cells are washed once by PBS and detached by treatment with trypsin/EDTA inside the CO<sub>2</sub> incubator. When the cells have been dislodged, stop trypsin activity by adding a growth medium. Collect cell suspension into 50 ml tubes.

**[0067]** The mix obtained from the detaching step 102 is then centrifuged at 400 x g for 5 minutes in the centrifuging step 104. The supernatant is removed. The cell pellet is re-suspended in basal medium (e.g. DMEM/F12). The remaining cell suspension is transferred to a pre-weighed 50 ml tube. The cells are centrifuged at 400 x g for 5 minutes. The supernatant is then removed. The tube is weighted again. The number of cells and the mass of the cell pellet are recorded. In some further embodiments, the cell concentration is determined using a Countess II Automated Cell Counter and a portion of the cells are seeded for making the next batch of cell hydrolysates.

**[0068]** Upon the centrifugations, the cell pellet is obtained and may be stored at -80°C.

**[0069]** In the resuspension step 106, the cell pellet is suspended in PBS. In some embodiments, the cell pellet is suspended in 1ml PBS.

**[0070]** In lysis step 108, the cells from step 106 are lysed by sonication. In some embodiments, cells are lysed by sonicating the cell suspension using a Branson Digital Sonifier S450D (amplitude= 25%; 15s ON, 15s off, total= 10 cycles). Keep the tube on ice (i.e. sonication at about zero degree Celsius).

**[0071]** Following cell lysis by sonication, the soluble fraction is isolated from the cell debris. Proteins in the soluble fraction are enzymatically digested into short

functional peptides in digestion step 110. The desired protease of choice is added to the soluble fraction at 37 °C for 1-2 hours. In some embodiments, the protease is proteinase K. Short peptides are favored (i.e. having a molecular size smaller than 500 daltons (Da)) because molecules having a molecular size greater than 500 daltons (Da) do not effectively penetrate through the outermost epidermis and be absorbed by the underlying skin layers. In some embodiments, an appropriate amount of protease is added to the cell suspension to break down cellular proteins into smaller peptides. Perform the digestion for 1-2 hours and keep the tube inside a 37°C water bath. Swirl the tube every 10 minutes.

**[0072]** After the digestion step 110, the mix from digestion step 110 is then centrifuged at 20000 x g for 30 minutes in the second centrifuging step 112. In some embodiments, the contents are transferred to 1.5 ml microcentrifuge tubes. The supernatants from the microcentrifuge tubes are combined into a 50 ml tube. Avoid disturbing the pellets in the microcentrifuge tubes.

**[0073]** In the termination step 114, the supernatant is then heated to 95 °C for 15 minutes to stop the enzyme digestion activity. Alternatively, PBS is added to the supernatant to stop the enzyme digestion activity through dilution. In some embodiments, the dilution should be 100-fold.

**[0074]** In the filtration step 116, the hydrolysate in the mix from the termination step 114 is sterile filtered through a 0.2 µm PVDF membrane filter.

**[0075]** In the final step 118, the hydrolysate obtained from the filtration step 116 may be applied as a topical agent to skincare, cosmetic, and wound care products. The hydrolysate may also be used as an active ingredient in various products, including but not limited to, skincare, wound care, cosmetic, food products, supplements, drugs and other medicinal applications. If the hydrolysate is not used immediately, in some embodiments, store it at -20 degrees Celsius.

**[0076]** The hydrolysate from cultivated cells is rich in nutrients, contains multiple protein factors that stimulate skin cell repair and regeneration, and have a molecular size smaller than 500 daltons (Da). The hydrolysate of the present invention can reach and take effect on the deep skin layers (dermis, hypodermis) as the hydrolysate of the present invention is small enough to pass through the stratum corneum and also maintains the key protein domains of growth factors and cytokines to elicit their functional activities.

**[0077]** The efficacy of the hydrolysate is further tested on human skin cells.

**[0078]** Referring now to the drawings, and with specific reference to FIG. 8, a method for evaluating the effect of cell hydrolysate on human skin cells.

**[0079]** In the seeding step 200, human cells are seeded in a complete growth medium. In some embodiments, the human keratinocyte cell line (HaCaT cells) or the human foreskin fibroblast cell line (BJ cells) can be used for this purpose. Cells are seeded onto 24 well/96 well plates in complete medium (e.g. DMEM/F12 + 10% FBS) such that they may reach 80 - 90% confluence the following day.

**[0080]** On at least one day after the seeding step 200, cell hydrolysate is added to the human cells in the hydrolysate addition step 202. This step 202 is performed when the human cells reach at least 80% confluence. Preferably, this step 202 is performed when the human cells reach at least 90% confluence. In some embodiments, cell hydrolysate is diluted to the desired concentration(s) range from 0.1% to 10%, preferably 1% using complete medium or serum-free medium (e.g. DMEM/F12). In yet some embodiments, the old medium in the wells is replaced with a control medium (i.e. vehicle control) or medium containing the diluted cell hydrolysate.

**[0081]** After step 202, the cells are returned to the humidified incubator for 6-24 hours in the incubation step. In some embodiments, the cells are returned to the humidified incubator (37°C; 5% CO<sub>2</sub>, 95% air) for 6 - 24 hours, preferably for 18 hours.

**[0082]** Then, total RNAs are harvested from cells to study target gene expression using RT-PCR. Alternatively, cell proliferation after treatment by hydrolysates can be studied by the CyQUANT Cell Proliferation Assay (ThermoFisher Scientific).

**[0083]** Assessing target gene expression

**[0084]** The following steps are performed to assess the target gene expression from the skin cells.

**[0085]** In the total RNA extraction step 206, the following sub-steps are performed:

1. At 6 - 24 hours post-transfection, extract all RNA from the cell monolayer using PureLink RNA Mini Kit (ThermoFisher Scientific) according to the manufacturer's instructions.
2. Medium from the well is removed. Wash cells once with PBS and remove the PBS.
3. Add 300 µl Lysis Buffer containing 1% 2-mercaptoethanol to the well. Lyse cells by pipetting up and down 10 times.

4. Transfer the lysate to a clean 1.5 ml microcentrifuge tube. Vortex the tube for 1 minute at maximum speed.
5. Centrifuge the tube at 2600 × g for 5 minutes. the supernatant is collected and transferred to a clean 1.5 ml microcentrifuge tube.
6. Add 1 volume of 70% ethanol to each volume of cell lysate. Vortex the mixture and transfer it to a Spin Cartridge (with a collection tube).
7. Centrifuge at 12,000 × g for 15 seconds at room temperature. Discard the flow-through. Reinsert the Spin Cartridge into the same Collection Tube.
8. Add 350 µl Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 × g for 15 seconds at room temperature. Discard the flow-through and the Collection Tube. Place the Spin Cartridge into a new Collection Tube.
9. Remove genomic DNA contamination by on-column DNase treatment using Purelink DNase. Prepare the DNase mixture as shown in Table 1 below. Add 80 µl of the DNase mixture to the column membrane and incubate at room temperature for 15 minutes.

**Table 1**

| <b>Components</b>                    | <b>Volume per sample</b> |
|--------------------------------------|--------------------------|
| DNase I reaction buffer              | 8 µl                     |
| Reconstituted Purelink DNase (3U/µl) | 10 µl                    |
| RNase-free water                     | 62 µl                    |
| <b>Total</b>                         | <b>80 µl</b>             |

10. Add 350 µl Wash Buffer I to the Spin Cartridge. Centrifuge at 12,000 × g for 15 seconds at room temperature. Discard the flow-through and the Collection Tube. Place the Spin Cartridge into a new Collection Tube.
11. Prepare Wash Buffer II in advance by adding 60 ml molecular biology grade 100% ethanol per 15 ml of Wash Buffer II. Add 500 µl Wash Buffer II containing ethanol to the Spin Cartridge.
12. Centrifuge at 12,000 × g for 15 seconds at room temperature. Discard the flow-through. Reinsert the Spin Cartridge into the same Collection Tube.
13. Repeat sub-steps 9-10 once.

14. Centrifuge at 12,000 × g for 2 minutes at room temperature to dry the membrane with the attached RNA. Discard the collection tube and place the Spin Cartridge into a recovery tube.

15. Add 30 µl RNase-Free water to the center of the Spin Cartridge membrane. Wait for 2 minutes. Centrifuge at 12,000 × g for 2 minutes at room temperature to collect the RNA eluate.

16. Quantify the concentration of RNA with Nanodrop 2000. If the purified RNA is of good quality, it should have A260/A280 and A260/A230 ratios of >1.8. Moreover, it should have an RNA integrity number (RIN) of ≥7 (RIN=10 for intact RNA) when analyzed by an Agilent 2100 bioanalyzer (Agilent).

17. Keep the RNA on ice until proceeding to first-strand cDNA synthesis.

**[0086]** Upon completion of step 206, first-strand cDNA synthesis step 208 is performed, which includes the following sub-steps:

1. Synthesize first-strand cDNA from total RNA using MMLVRT (RNase H-) enzyme (Excell Bio) similarly as described in the manufacturer's protocol.
2. For each reverse transcription reaction, mix total RNA, oligo (dT)<sub>20</sub> primer, and RNase-free water in a PCR tube on ice as shown in Table 2.

**Table 2**

| <b>Components</b>                     | <b>Volume per reaction (µl)</b> |
|---------------------------------------|---------------------------------|
| Total RNA                             | Up to 2 µg                      |
| 50 µM oligo (dT) <sub>20</sub> primer | 1 µl                            |
| RNase-free water                      | <b>Top up to 10 µl</b>          |

3. (Optional) Set up an extra reaction of one of the samples to serve as a no RT control. This can be used to test for genomic DNA contamination after quantitative real-time PCR.

4. Heat the reaction mixture at 70 °C for 10 minutes. Meanwhile, set up the RT master mix and no RT control mix as shown in Table 3. Remember to prepare at least 1 extra reaction of the RT master mix to make up for pipetting error.

**Table 3**

| Components                        | RT master mix (per reaction)        | No RT control mix                   |
|-----------------------------------|-------------------------------------|-------------------------------------|
| 5x M-MuLV reaction buffer         | 4 $\mu$ l                           | 4 $\mu$ l                           |
| 0.1M DTT (10x)                    | 2 $\mu$ l                           | 2 $\mu$ l                           |
| dNTP (10 mM)                      | 1 $\mu$ l                           | 1 $\mu$ l                           |
| RNaseOut (40U/ $\mu$ l)           | 0.5 $\mu$ l                         | 0.5 $\mu$ l                         |
| MMLVRT (RNase H-) (200U/ $\mu$ l) | 1 $\mu$ l                           | 0 $\mu$ l                           |
| RNase-free water                  | 1.5 $\mu$ l                         | 2.5 $\mu$ l                         |
|                                   | <b>Total = 10 <math>\mu</math>l</b> | <b>Total = 10 <math>\mu</math>l</b> |

5. After 10 minutes of heating, chill the sample reaction tube on ice for 2 minutes. Collect the tube contents to the bottom of the tube by performing a quick spin using a tabletop centrifuge.
6. Add 10  $\mu$ l RT master mix to each sample and add 10  $\mu$ l of no RT control mix to the no RT control.
7. Incubate the reaction tubes at 50 °C for 1 hour for reverse transcription. Inactivate the MMLVRT (RNase H-) enzyme by incubating the reaction tube at 70 °C for 15 minutes, followed by chilling the reaction tubes on ice.
8. Dilute cDNA samples by adding 180  $\mu$ l autoclaved MQ water (10x dilution). Store the diluted cDNA samples at -20 °C.

**[0087]** At last, the RT-PCR step 210 is performed. It includes the following sub-steps:

1. Quantify relative target gene expression between samples by quantitative real-time PCR (qPCR) using the Roche LightCycler 480 System (Roche Diagnostics). Set up the PCR reaction master mix with reagents from the PowerUp SYBr Green Mastermix (ThermoFisher Scientific) and primers specific to the gene of interest. The components of the mix are shown in Table 4.

**Table 4**

| Components                   | Volume per reaction (µl) |
|------------------------------|--------------------------|
| PowerUp SYBr Green Mastermix | 10 µl                    |
| Forward primer (8 µM)        | 1 µl                     |
| Reverse primer (8 µM)        | 1 µl                     |
|                              | Total= 12 µl             |

Table 5 shows examples of genes to be tested:

**Table 5**

| Gene of interest                                           | Primer sequence                                                    | Amplicon |
|------------------------------------------------------------|--------------------------------------------------------------------|----------|
| <i>Collagen Type I α1</i><br>(Col1a1)                      | F: 5'-ACTGGTGAGACCTGCGTGTA-3'<br>R: 5'-ATCCATCGGTCATGCTCTCG-3'     | 118 bp   |
| <i>Collagen Type I α2</i><br>(Col1a2)                      | F: 5'-CATGGATGAGGAGACTGGCA-3'<br>R: 5'-TGCCCTCAGCAACAAGTTCA-3'     | 80 bp    |
| <i>Tissue inhibitor of metalloproteinase 1</i> (Timp-1)    | F: 5'-ATCCATCCCCTGCAAAGTGC-3'<br>R: 5'-TGGAAGCCCTTTTCAGAGCC-3'     | 81 bp    |
| <i>Sirtuin 1</i> (Sirt1)                                   | F: 5'-CCAGCTAGGACCATTACTGCC-3'<br>R: 5'-ACTGCATTCTTTAGTGTAGCCAA-3' | 107 bp   |
| <i>Glyceraldehyde 3-phosphate dehydrogenase</i><br>(Gapdh) | F: 5'-TCAAGAAGGTGGTGAAGCAGG-3'<br>R: 5'-CGTCAAAGGTGGAGGAGTGG-3'    | 117 bp   |
| <i>Eukaryotic translation elongation factor 1 α1</i>       | F: 5'-CCACTGGAAGCAGGAATGAGT-3'<br>R: 5'-TGGTGCTCAAGCCACAGTTG-3'    | 100 bp   |

2. Perform 5-fold serial dilutions ( $5^1$  to  $5^5$  serial dilutions) of a reference cDNA sample by adding 5 µl cDNA to 20 µl autoclaved MQ water. There will be six standards ( $5^0$ ,  $5^1$ ,

5<sup>2</sup>, 5<sup>3</sup>, 5<sup>4</sup>, 5<sup>5</sup> -fold dilutions) for determining the standard curve in the qPCR run. The reference cDNA sample can be an undiluted 20 µl cDNA sample.

3. Write down what is added to each well of the qPCR plate on an experiment record sheet.

4. Add 12 µl of PCR reaction master mix per well of a LightCycler Multiwell plate 96 (Roche). Avoid introducing bubbles.

5. Add 8 µl of diluted cDNA sample, standard, or MQ water (PCR negative control) into each well according to the experiment record sheet. Avoid introducing bubbles.

6. Cover the qPCR plate with a sealing foil supplied in the LightCycler Multiwell plate 96 pack (Roche). Make sure all the wells are tightly sealed by the sealing foil to prevent evaporation during qPCR.

7. Collect the well contents to the bottom of the wells by centrifuging at 200 x g inside a Beckman Coulter Allegra 25R Centrifuge (Beckman Coulter Life Sciences).

8. Place the qPCR plate into the Roche LightCycler 480. Set the PCR conditions as follows:

(i) UDG activation

50°C 2 minutes (Ramp Rate 4.4°C/sec)

1 cycle

(ii) Initial Denaturation:

95°C 2 minutes (Ramp Rate 4.4°C/sec)

1 cycle

(iii) PCR

Analysis Mode: Quantification

95°C 15 seconds (Ramp Rate 4.4°C/sec)

55°C 15 seconds (Ramp Rate 2.2°C/sec)

72°C 30 seconds (Ramp Rate 4.4°C/sec, Acquisition Mode: Single)

40 cycles

(iv) Melting curve analysis

Analysis Mode: Melting Curves

95°C 20 seconds (Ramp Rate 4.4°C/sec)

55°C 1 minutes (Ramp Rate 2.2°C/sec)

95°C (Ramp Rate 0.11°C/sec, Acquisition Mode: Continuous, Acquisitions: 5 per °C)

1 cycle

(v) Cooling

4°C ∞ (Ramp Rate 2.2°C/sec)

1 cycle

9. Begin the qPCR run.
10. After the qPCR run is complete, take the qPCR plate out. Verify the validity of the qPCR run by the following criteria:
  - (i) The amplification efficiency is 90 - 105%,
  - (ii) The coefficient of determination ( $R^2$ ) of the standard curve is  $> 0.99$ ,
  - (iii) There is a single peak in the melting curve analysis,
  - (iv) The PCR product should yield a single band of the right amplicon size after gel electrophoresis.
11. Record the amplification efficiency. The relative gene target expression is normalized to the relative housekeeping gene expression (i.e. Gapdh), and can be calculated via the Pfaffl method, as illustrated below:

Relative target gene expression of B compared to A

$$= \frac{(1 + \text{amplification efficiency of target gene})^{C_t(A) - C_t(B)}}{(1 + \text{amplification efficiency of Gapdh})^{C_t(a) - C_t(b)}}$$

Where

$C_t(A)$  =  $C_t$  value of the target gene in sample A;

$C_t(B)$  =  $C_t$  value of the target gene in sample B;

$C_t(a)$  =  $C_t$  value of the Gapdh in sample A;

$C_t(b)$  =  $C_t$  value of the Gapdh in sample B.

**[0088]** Cell Proliferation assessment by the CyQUANT Cell Proliferation Assay

**[0089]** The following steps are performed to assess cell proliferation after treatment by hydrolysates by the CyQUANT Cell Proliferation Assay.

**[0090]** In the removing step 212, the following sub-steps are performed:

1. The CyQUANT Cell Proliferation Assay (Thermofisher Scientific) can be used to determine cell growth by indirectly measuring nucleic acid content. Cells should be seeded onto a 96-well plate. After cell treatment for 16- 24 hours, remove the culture medium as much as possible.
2. Freeze the plate at -70°C. The plate can be stored for up to 4 weeks at -70°C.

**[0091]** Upon completion of step 212, CyQUANT step 214 is performed, which includes the following sub-steps:

1. Thaw the assay kit components at room temperature. Prepare the CyQUANT GR working solution as shown in Table 6. Mix it by pipetting.

**Table 6**

| <b>Components</b>    | <b>Volume per well (μl)</b> |
|----------------------|-----------------------------|
| Component A          | 0.5 μl                      |
| Component B          | 10 μl                       |
| MilliQ water         | 189.5 μl                    |
| <b>Total= 200 μl</b> |                             |

2. Thaw the plate at room temperature.
3. Add 200 μl CyQUANT GR working solution to each sample well, and 200 μl to a blank well (no cell control). Incubate for 5 minutes.
4. Read the plate in a fluorescence plate reader (e.g. Molecular Devices SpectraMax iD5). Set the excitation filter at 480 nm, and the emission filter at 520 nm.
5. Subtract all sample fluorescence readings by the no cell control reading. Tabulate the results.

**[0092]** Referring to FIG. 9, the chart illustrates the difference in skin cells laminin expression between skin cells being treated with cell hydrolysate and skin cells

not being treated with cell hydrolysate. The laminin expression of skin cells being treated with cell hydrolysate is about 140%, where the laminin expression of skin cells not being treated with cell hydrolysate is about 100%.

**[0093]** The above description is illustrative and is not restrictive. Many variations of embodiments may become apparent to those skilled in the art upon review of the disclosure. The scope embodiments should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the pending claims along with their full scope or equivalents.

**[0094]** One or more features from any embodiment may be combined with one or more features of any other embodiment without departing from the scope embodiments. A recitation of "a", "an" or "the" is intended to mean "one or more" unless specifically indicated to the contrary. Recitation of "and/or" is intended to represent the most inclusive sense of the term unless specifically indicated to the contrary.

**[0095]** While the present disclosure may be embodied in many different forms, the drawings and discussion are presented with the understanding that the present disclosure is an exemplification of the principles of one or more inventions and is not intended to limit anyone embodiment to the embodiments illustrated.

**[0096]** The disclosure, in its broader aspects, is therefore not limited to the specific details, representative system and methods, and illustrative examples shown and described above. Various modifications and variations may be made to the above specification without departing from the scope or spirit of the present disclosure, and it is intended that the present disclosure covers all such modifications and variations provided they come within the scope of the following claims and their equivalents.

**Exemplary Protocols****A. Development of a fish bladder cell line**

1. Obtain a healthy yellow crocker, sea bass, or fish of a similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove swim bladder from the fish under aseptic condition.
5. Wash the organ one or more times in hypochlorous acid.
6. Wash the organ one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the organ into small pieces (2-3 mm<sup>3</sup>).
8. Transfer the cut organ to a centrifuge tube containing 0.25% trypsin-EDTA in PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 pm mesh to remove undigested tissue.
11. Centrifuge the filtrate at 200g for 5 minutes.
12. Resuspend the cell pellet with complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
13. Seed the cell into a T25 flask.
14. Incubate at 24-28°C.
15. Remove cells that are not attached to the tissue culture flask the next day.
16. Replace half of the medium with fresh medium every 2-3 days.
17. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

**B. Development of a fish bladder cell line by tissue explant**

1. Obtain a healthy yellow crocker, sea bass, or fish of a similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove swim bladder from the fish under aseptic condition.
5. Wash the organ one or more times in hypochlorous acid.
6. Wash the organ one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the organ into small pieces (1-2 mm<sup>3</sup>).
8. Place organ pieces into a 24 well plate individually containing complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
9. Incubate at 24-28°C.
10. Replace half of the medium with fresh medium every 2-3 days without disturbing the tissue explant.
11. Incubate the tissue explant until adherent cells are observed.
12. Remove tissue explant.
13. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

**C. Development of a fish muscle cell line**

1. Obtain a healthy grouper, cod, sole, halibut, flounder, or fish of a similar category from a local fish market.

2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove muscle from the fish under aseptic condition.
5. Wash the tissue one or more times in hypochlorous acid.
6. Wash the tissue one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the tissue into small pieces (2-3 mm<sup>3</sup>).
8. Transfer the cut tissue to a centrifuge tube containing collagenase and dispase in PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 µm mesh to remove undigested tissue.
11. Centrifuge the filtrate at 200g for 5 minutes.
12. Resuspend the cell pellet with complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
13. Seed the cell into a T25 flask.
14. Incubate at 24-28°C.
15. Remove cells that are not attached to the tissue culture flask the next day.
16. Replace half of the medium with fresh medium every 2-3 days.
17. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

#### **D. Development of a fish muscle cell line from tissue explant**

1. Obtain a healthy grouper, cod, sole, halibut, flounder, or fish of a similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.
4. Remove muscle from the fish under aseptic condition.
5. Wash the tissue one or more times in hypochlorous acid.
6. Wash the tissue one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the muscle into small pieces (1-2 mm<sup>3</sup>).
8. Place muscle pieces into a 24 well plate individually containing complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum).
9. Incubate at 24-28°C.
10. Replace half of the medium with fresh medium every 2-3 days without disturbing the tissue explant.
11. Incubate the tissue explant until adherent cells are observed.
12. Remove tissue explant.
13. The cells are considered established when a complete monolayer is formed and the established cells are ready for subculture.

#### **E. Adult Stem cell isolation and culture**

1. Obtain a healthy grouper, cod, sole, halibut, flounder, or fish 6 months or younger of similar category from a local fish market.
2. Keep the fish on ice until cell isolation.
3. Immerse the fish in 10% bleach.

4. Remove muscle from the fish under aseptic conditions.
5. Wash the tissue one or more times in hypochlorous acid.
6. Wash the tissue one or more times in antibiotic medium (Leibovitz's L-15 or DMEM or EMEM with 400 IU/ml, penicillin, 400 pg/ml streptomycin).
7. After washing, cut the tissue into small pieces (2-3 mm<sup>3</sup>).
8. Transfer the cut tissue to a centrifuge tube containing collagenase and dispase in PBS.
9. Incubate at room temperature with continuous shaking for 1 hour.
10. Filter the supernatant with a 100 pm mesh to remove undigested tissue.
11. Centrifuge the filtrate at 200g for 5 minutes.
12. Resuspend the cell pellet with complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum, 100ng/ml basic fibroblast growth factor).
13. Plate the cells on an uncoated plate for 1 hour at 24-28°C.
14. Harvest the supernatant and place it on a plate coated with laminin, gelatin, Matrigel, or similar matrix.
15. Incubate at 24-28°C.
16. After 24 hours, wash away any loosely attached and non-adherent cells.
17. Replace medium every day with complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum, 100ng/ml basic fibroblast growth factor).

#### **F. Generating and culturing iPSC**

1. 2-4 days before transfection, plate cells in complete medium (L15 with 10% FBS) in a tissue culture flask. Cells should be approximately 75-90% confluent on the day of transfection (Day 0).
2. Aspirate the medium from gelatin-coated 6-well plates and replace them with 2 mL of fresh complete medium per well. Place the coated plates at 37°C until ready for use.
3. Thaw the Epi5™ vectors at 37°C and place them on wet ice until ready for use. Before use, briefly centrifuge the thawed vectors to collect them at the bottom of the tube.
4. Wash the cells in PBS.
5. Add 3 mL of 0.05% Trypsin/EDTA to the culture flask containing the cells.
6. Incubate the flask at room temperature for 3 minutes.
7. Add 5-8 mL of complete medium to each flask. Carefully transfer cells into an empty, sterile 15mL conical tube.
8. Check the viability by trypan blue dye exclusion cell viability assay
9. Centrifuge the cells at 200g for 2 min.
10. Carefully aspirate most of the supernatant and resuspend with complete medium.
11. Seed cells on gelatin-coated dishes plate 50,000 to 100,000 cells per well into a 6-well plate at 30-60% confluence in 2 mL complete medium and Incubate overnight at 24-28°C.
12. Prewarm Opti-MEM/Reduced-Serum Medium to room temperature and prepare Tube A and Tube B as described below.
13. Add 1.2 µL each of the two Epi5™ Reprogramming Vector mixes (2.4 µL total) to 118 µL Opti- MEM medium in a 1.5 mL microcentrifuge tube labeled Tube A. Add 4.8 µL of P3000™ Reagent and mix well.
14. Dilute 3.6 µL Lipofectamine 3000 reagent in 121 µL prewarmed Opti-MEM medium in a 1.5 mL microcentrifuge tube labeled Tube B.

15. To prepare a transfection master mix, add the contents of Tube A to Tube B and mix well.
16. Incubate the transfection master mix for 5 minutes at room temperature.
17. Mix one more time and add the entire 250  $\mu$ L of transfection master mix to each well.
18. Incubate overnight at 24-28°C.
19. 24 hours post-transfection, aspirate the medium from the plates. Add 2 mL N2B27 Medium (L15 with IX N-2 supplement, IX B27 supplement, 100 ng/mL bFGF to each well.
20. Change the N2B27 Medium every day for a total of 14 days by replacing the spent medium with 2 mL N2B27 Medium.
21. Aspirate the spent N2B27 Medium on Day 14 and replace it with a complete medium. Resume medium changes every day at 2 mL per well.
22. Observe the plates every other day under a microscope for the emergence of cell clumps, indicative of transformed cells. Within 15 to 21 days post-transfection, the iPSC colonies will grow to an appropriate size for transfer.
23. Colonies are distinct by Day 21 and can be picked for further culture and expansion.

#### **G. Method for subculturing cells**

1. Remove and discard the culture medium.
2. Briefly rinse the cell PBS to remove all traces of serum which contains trypsin inhibitor.
3. Add 2-3 mL of 0.25% Trypsin-EDTA solution to the flask.
4. Incubate at room temperature for 1 min.
5. Add 5-8 mL of complete growth medium.
6. Aspirate cells by gently pipetting.
7. Add appropriate aliquots of the cell suspension to new culture flasks at a subcultivation ratio of 1:2 to 1:3.
8. Incubate at 24-28°C.

#### **H. Adaption to suspension culture**

1. Passage monolayer culture at a frequency appropriate for the cell in question by trypsinization.
2. At each passage, wash cell monolayer with PBS and overlay with 0.25% trypsin.
3. Incubate at room temperature for 5 min.
4. Inactivate the enzyme with a complete medium.
5. Harvest the cell suspension and check the viability by trypan blue dye exclusion cell viability assay.
6. Seed the cell suspension into another culture flask.
7. Repeat passaging until the viability of the suspended cells is equal to or more than 90%.
8. Establish a suspension culture with 50 ml complete medium in a spinner or shaker flask at a cell density of 0.1-0.5 million/ml.
9. Incubate the spinner or shaker flask suspension cultures in a CO<sub>2</sub> incubator under the same conditions of temperature, humidity, and atmosphere optimal for monolayer cultures.
10. Adjust the cell density to 0.1-0.5 million/ml with fresh medium every 2-3 days.
11. Check the viability by trypan blue dye exclusion cell viability assay.
12. Establish multiple parallel cultures at cell density that promote healthy cell growth.

13. Increase cell density gradually to 1 million/ml using part of the culture.
14. If increasing cell density leads to cell death, discard the high-density culture.
15. Restart high-density adaption using cell from step 12.
16. Scale up to a 3L bioreactor when cells are adapted to grow in suspension.

#### **I. Adaption to serum-free medium (plant hydrolysate)**

1. Culture cells in DMEM/F12 complete medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4mM glutamine, 10% FBS).
2. Prepare serum-free medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4mM glutamine, 20% plant hydrolysate e.g. soy, cottonseed, rapeseed, wheat, yeast, or equivalent).
3. When cells reach confluence, replace medium with adaption medium I (40% fresh complete medium, 40% conditioned media from the passage before, 20% serum-free medium).
4. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
5. If adaption leads to cell death, discard the culture, and repeat step 3.
6. When cells reach confluence, replace medium with adaption medium II (30% fresh complete medium, 30% conditioned media from the cells in step 1, 40% serum-free medium).
7. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
8. If adaption leads to cell death, discard the culture, and repeat step 6.
9. When cells reach confluence, replace medium with adaption medium III (20% fresh complete medium, 20% conditioned media from the cells in step 1, 60% serum-free medium).
10. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
11. If adaption leads to cell death, discard the culture, and repeat step 9.
12. When cells reach confluence, replace medium with adaption medium IV (10% fresh complete medium, 10% conditioned media from the cells in step 1, 80% serum-free medium).
13. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
14. If adaption leads to cell death, discard the culture, and repeat step 12.
15. When cells reach confluence, replace medium with serum-free medium.
16. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
17. If adaption leads to cell death, discard the culture, and repeat step 15.
18. The serum-free medium usage can be increased more gradually in each step, i.e. an increase of 20% or less in each step.

#### **J. Adaption to serum-free medium (chemically defined)**

1. Culture cells in DMEM/F12 complete medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4mM glutamine, 10% FBS).
2. Prepare serum free medium (1:1 mixture of DMEM medium and Ham's F12 medium, 2-4 mM glutamine, ascorbic acid 2-phosphate 65-130 ug/ml, NaHCO<sub>3</sub> 550-1100 ug/ml, sodium selenite 14-28 ng/ml, insulin 19-38 ug/ml, transferrin 11-22 ug/ml, FGF-2 100-200 ng/ml, TGF-beta 2-4 ng/ml).
3. When cells reach confluence, replace medium with adaption medium I (40% fresh complete medium, 40% conditioned media from the passage before, 20% serum-free medium).
4. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days

5. If adaption leads to cell death, discard the culture and repeat step 3
6. When cells reach confluence, replace medium with adaption medium II (30% fresh complete medium, 30% conditioned media from the cells in step 1, 40% serum-free medium).
7. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
8. If adaption leads to cell death, discard the culture and repeat step 6
9. When cells reach confluence, replace medium with adaption medium III (20% fresh complete medium, 20% conditioned media from the cells in step 1, 60% serum-free medium).
10. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
11. If adaption leads to cell death, discard the culture, and repeat step 9.
12. When cells reach confluence, replace medium with adaption medium IV (10% fresh complete medium, 10% conditioned media from the cells in step 1, 80% serum-free medium).
13. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
14. If adaption leads to cell death, discard the culture, and repeat step 12.
15. When cells reach confluence, replace medium with serum-free medium.
16. Check the viability by trypan blue dye exclusion cell viability assay every 2-3 days.
17. If adaption leads to cell death, discard the culture, and repeat step 15.
18. The serum-free medium usage can be increased more gradually in each step. For example, an increase of 20% or less in each step.

#### **K. Post-transcriptional enhancement of protein expression**

1. Culture cells in complete medium (Leibovitz's L-15 or DMEM or EMEM with 200 IU/ml, penicillin, 200 pg/ml streptomycin, 10% fetal bovine serum), or serum-free medium (DMEM/F12 with plant hydrolysate or chemically defined compounds).
2. Remove and discard the culture medium.
3. Briefly rinse the cell PBS to remove all traces of serum which contains trypsin inhibitor.
4. Add 2-3 mL of 0.25% Trypsin-EDTA solution to the flask.
5. Incubate at room temperature for 1 min.
6. Aspirate cells by gently pipetting.
7. Centrifuge cell at 200g for 2 min.
8. Resuspend cells in complete medium or serum-free medium.
9. Add 0.5 million cells to each well of a 6-well plate.
10. Incubate at 24-28°C overnight.
11. Transfect micro RNA oligonucleotides (miR-21, miR-29a, miR-21 mimic, miR-29a mimic, anti-miR-21, anti-miR-29a, or equivalent) into the cell using polyethylenimine, liposome, electroporation, or other methods.
12. Incubate at 24-28°C overnight.
13. Transfer the cells to a multi-layer flask, spinner flask, or shaker flask in a CO<sub>2</sub> incubator under the same conditions of temperature, humidity, and atmosphere optimal culture

#### **L. Scaffolding for cell culture (Konjac + gum)**

1. Boil water with a few pieces of saffron until the color becomes pale yellow.
2. Remove the saffron and rest the solution until warm.
3. Prepare all dry ingredient

- a. Konjac-1-3%
  - b. Baking soda - 0.7-1.4%
  - c. Locust Bean Gum - 0.5-1%
  - d. Perfected Xanthan Gum - 0.5-1%
4. Measure 100ml of saffron solution.
  5. Add Baking soda, Locust Bean Gum, Xanthan Gum sequentially. Stir the mixture well after adding each ingredient.
  6. Add Konjac by sprinkling little by little on top of the solution. Keep stirring. The solution should become mushy.
  7. Spread the konjac mixture into mold with approximately 1-15mm thickness.
  8. Cover the mold with the lid and rest under room temperature for more than 30 min.
  9. Put the mold in 4°C fridge for 4 hours.
  10. Steam the mold under low heat for 40 minutes.
  11. Rest the mold under room temperature for 2 hours.
  12. Dehydrate the scaffold at 45-55 °C for 15 minutes.

**M. Scaffolding for cell culture (Alginate + Glutinous Rice Flour)**

1. Weigh 1 g (1%) Sodium Alginate.
2. Add 100ml water into the blender.
3. Add the Alginate powder into the blender and blend the mixture until dissolved.
4. Cover the container with plastic film and put the Alginate solution into the refrigerator overnight to eliminate the gas bubbles.
5. Weigh 10% Glutinous Rice Flour and put in a mold.
6. Add Alginate solution into the mold with approximately 1-15mm thickness.
7. Stir the mixture until all flour dissolves.
8. Steam the mixture under low heat for 30 min until the shape is set.
9. Cover the mold with the lid and rest under room temperature for 30 min.
10. Weigh 1% Calcium Lactate and stir to dissolve in water.
11. Immerse the scaffold with 1% Calcium Lactate solution for at least 2.5 hours to allow the formation of the membrane around the scaffold.

**CLAIMS**

What is claimed is:

1. A method for generating cell hydrolysate by in vitro cell culture includes the steps of:
  - (i) lysing a cell by using sonication to release proteins in the cell;
  - (ii) digesting the proteins by using protease to produce short peptides having a molecular size smaller than 500 daltons (Da);
  - (iii) terminating the digestion step by either heating protease to a predetermined temperature for a predetermined period of time or diluting the protease to a predetermined concentration; and
  - (iv) sterile filtering the mix obtained from the digestion termination step through a membrane filter.
2. The method of claim 1, wherein the membrane filter is a 0.2  $\mu\text{m}$  PVDF membrane filter.
3. The method of claim 1, wherein the predetermined temperature and predetermined period of time in the digestion termination step are 95 degree Celsius and 15 minutes respectively.
4. The method of claim 1, wherein the predetermined concentration is 100-fold and the protease is diluted by PBS.
5. A topical formulation comprising cell hydrolysate generated by the method of claim 1.