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METHOD FOR OPERATING A BIOREACTOR FOR CULTIVATED MEAT AND CORRESPONDING BIOREACTOR

Abstract

Method for the manufacturing of an elongated, e.g. microfibril structure for the fabrication of cultivated meat, wherein (a) a paste is extruded through an extrusion plate (**24**) and an adjacent attachment plate (**22**) with aligned nozzle openings (**42, 44**) into a space (**58**) downstream of the second nozzle plate (**22**), (b) paste located in said space (**58**) or in the attachment plate (**22**) is hardened, (c) said paste is continued to be extruded through said extrusion plate (**24**) while the distance between the attachment plate (**22**) and the extrusion plate (**22**) is increased under formation of said elongated, e.g. microfibril structure between said plates (**22,24**).

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Background/Summary

TECHNICAL FIELD

[0001] The present invention relates to a method for manufacturing elongated structures, typically fibrous, preferably microfibrillar structures for making cultivated meat, in particular for making efficiently large bundles of several tens or 100s or even many thousands of such elongated, typically fibrous structures in parallel, as well as to a reactor for implementing such a method most efficiently and reliably. It furthermore relates to corresponding cultured meat products obtained using the method and/or the respective reactor.

PRIOR ART

[0002] Meat is an important protein source of the human diet. Controversial animal welfare associated with the traditional meat industry along with the increasing global population and demand for meat products, sustainable production alternatives are indispensable. Cultivated meat technology provides an opportunity to produce edible sources of animal protein that are not associated with the environmental impact of animal farming. In meat products, aligned fibrous/fibrillar structures are present at the tissular, cellular, and molecular scale levels, and are responsible for texture, tenderness, organoleptic and nutritional characteristics of meat products. In its current status, cultivated meat production is challenged by the availability of scalable and cost-efficient processes to produce tissues with high anisotropic fibrous structuration characteristic of animal meat, and reactors to culture said tissues at very high density and capable of applying cyclic mechanical stimulations.

[0003] In order to replicate a multiscale anisotropic structuration in a cultured meat product, it is important to employ a scaffolding technique that produces fibrous/fibrillar structures that guide the muscle progenitor stem cells to maturely differentiate in aligned myofibers. Additionally, the application of cyclic mechanical stretching during culture can promote cell differentiation and anisotropic orientation. Like meat obtained from the flesh of slaughtered animals, cultivated meat products should be produced in very large quantities and to a reasonably low price. For these reasons, not only the scaffolding and cultivation techniques employed are expected to be safe for human and non-human consumption, but they should also be scalable and compatible with high production rates. Keeping these points in mind, prior art available in patents and scientific literature is discussed in the following paragraphs.

[0004] In WO-A-2020160533 fibrous scaffolds for cultivated meat production are disclosed. The scaffold consists of a porous fibrous mat. Cells are integrated in the product by seeding the scaffold with a cell suspension. The seeding step is the critical one, and it can last hours to days depending on the used cells. The seeding efficiency is controlled by the cell attachment kinetic and by the bioavailability of attachment sites. Low seeding efficiency can be experienced. The seeding is limited by the available surface area, and consequently, the cell density achievable in the final products is limited.

[0005] In MacQueen a al. (Muscle tissue engineering in fibrous gelatin: implications for meat analogs. npj Sci Food 3, 20 (2019)) the preparation of fibrous gelatin scaffold is reported by immersion rotary jet spinning at high rates (˜100 g/h, dry weight) and, depending on process conditions, the approach allows the fabrication of edible scaffolds for cultured meat production in a scalable manner. However, as the scaffold relies on post-production cell seeding, the achievable seeding density is limited by the surface available area as confirmed by the lack of a mature muscle architecture.

[0006] In U.S. Pat. No. 7,622,299, bioengineered tissue substitutes are reported. The invention reports the fabrication of muscle tissues with a high degree of anisotropy consisting of aligned microfibers array. In this invention, microfibers need to be prepared individually and then arranged

in arrays consisting of multiple fibers in parallel stacked in layers. Additionally, cells need to be seeded on the construct. Therefore, the same drawbacks mentioned for other porous scaffolds seeded post-fabrication will also apply here. Additionally, the disclosed invention requires an equipment with a large footprint compared to the amount of produced fibers and is thus not suitable for high scale production.

[0007] In Kang et al (Engineered whole cut meat-like tissue by the assembly of cell fibers using tendon-gel integrated bioprinting, NATURE COMMUNICATIONS|(2021) 12:5059), a scaffolding method based on cell-laden gels is reported. The reported method overcome some of the seeding issues mentioned for the above-mentioned prior art. The gel formulation is based on fibrinogen crosslinked by thrombin and embedding cell and collagen microfibers. Because of the composition of such formulation, the sol to gel transition has a slow kinetics and therefore to produce fibers by molding in a sacrificial gel. Because of these reasons, the disclosed method and compositions are hardly scalable to produce a large quantity of cultivated meat.

[0008] In Distler et al (3D printed oxidized alginate-gelatin bioink provides guidance for C2C12 muscle precursor cell orientation and differentiation via shear stress during bioprinting, Biofabrication 12 (2020) 045005) a gel paste formulation containing oxidized alginate and gelatin in combination to shear stresses are used to provide guidance to cell orientation and differentiation. The formulation of this prior art allows fast gelation which could be compatible high production capabilities. Nevertheless, bioprinting is slow process used for precisely depositing materials in space, most often from a single nozzle at a time, and is thus not a promising technology for mass production.

[0009] In Kessel et al (3D Bioprinting of Macroporous Materials Based on Entangled Hydrogel Microstrands, Adv. Sci. 2020, 7, 2001419) a method to prepare aligned hydrogel fiber bundles is reported. The method is used in a bioprinting setup by depositing the fibers on a substrate or in a molding process setup. Both processes are not scalable for the production and culture of high quantity of biological tissues in sterile conditions.

[0010] In Zhang et al (Creating polymer hydrogel microfibres with internal alignment via electrical and mechanical stretching, Biomaterials, Volume 35, Issue 10, March 2014, Pages 3243-3251), authors report a strategy for the generation of hydrogel microfibres with internal alignment induced by a combination of electrical and mechanical stretching. Cells can be loaded directly in the hydrogel formulation cells (i.e., a seeding step is not required). Nevertheless, the process requires the use of high voltage (3000-5000V). The use of high voltage in processing/manufacturing facilities would pose risk for electric arcs and poses specific requirements in terms of electric insulation of machines. In addition to that, the reported process is relatively slow.

[0011] WO-A-2019211189 discloses an apparatus for the production of tissue from cells. The apparatus comprises an elongate body having at least one circumferential groove and being operable to extend, by close-fitting relationship, centrally through at least one trough. The troughs are extending in a closed path, such that the at least one of the circumferential grooves open into an inner edge of a trough. Also disclosed is a process for production of tissue from cells, via a transitioning intermediate which transitions from the cells into the tissue.

[0012] US20210017485 discloses a cell expansion system for culturing and expanding cells in hydrogel tubes is disclosed herein. The cell expansion system allows for expanding cells that can significantly reduce the production time and cost, while increase the production capacity.

[0013] Hoesli et al (A novel alginate hollow fiber bioreactor process for cellular therapy applications in Cell Culture and Tissue Engineering, Vol. 25 (6), p. 1740-1751) report that gel-matrix culture environments provide tissue engineering scaffolds and cues that guide cell differentiation. For many cellular therapy applications such as for the production of islet-like clusters to treat Type 1 diabetes, the need for large-scale production is anticipated. The throughput of the commonly used nozzle-based devices for cell encapsulation is limited by the rate of droplet formation to ˜0.5 L/h. This work describes a novel process for larger-scale batch immobilization of

mammalian cells in alginate-filled hollow fiber bioreactors (AHFBRs). A methodology was developed whereby (1) alginate obstruction of the intra-capillary space medium flow was negligible, (2) extra-capillary alginate gelling was complete and (3) 83±4% of the cells seeded and immobilized were recovered from the bioreactor. Chinese hamster ovary (CHO) cells were used as a model aggregate-forming cell line that grew from mostly single cells to pancreatic islet-sized spheroids in 8 days of AHFBR culture. CHO cell growth and metabolic rates in the AHFBR were comparable to small-scale alginate slab controls. Then, the process was applied successfully to the culture of primary neonatal pancreatic porcine cells, without significant differences in cell viability compared with slab controls. As expected, alginate-immobilized culture in the AHFBR increased the insulin content of these cells compared with suspension culture. The AHFBR process could be refined by adding matrix components or adapted to other reversible gels and cell types, providing a practical means for gel-matrix assisted cultures for cellular therapy.

[0014] Levi et al. (Trends in Food Science & Technology, Volume 126, August 2022, Pages 13-25) report that cultured meat aims to solve the current sustainability and environmental issues of conventional livestock husbandry by converging tissue engineering practices with food innovation science to recapitulate the components and structure of animal-derived meat. To this end, various scaffolding technologies were adopted and developed to support the cultivation, expansion, and differentiation of cells using edible, low-cost, and sustainable materials and methods. The cell-scaffold constructs can benefit different processing strategies that include structuring approaches and additives to generate final cultured meat products. In the paper, they elaborate on the main considerations for the design of scaffolds for cultured meat applications, review the leading scaffolding technologies, and discuss their current or potential application for the engineering of cultured meat. The findings are, that extensive research efforts in recent years attest to the boundless potential of novel cultured meat scaffolds. Moreover, different scaffolding technologies originally developed for tissue engineering can be adapted to cultured meat by using edible materials and avoiding toxic crosslinkers and reagents throughout all of the development steps, thus ensuring compliance with food and safety regulations. Altogether, the combination of tissue engineering and food science technologies holds the promise of technologically and commercially viable scaffolds towards the realization of the cultured meat vision.

[0015] US-A-2021017485 relates to a cell expansion system for culturing and expanding cells in hydrogel tubes. The cell expansion system allows for expanding cells that can significantly reduce the production time and cost, while increase the production capacity.

[0016] CN-A-112374607 discloses an anaerobic membrane bioreactor for sewage treatment. The anaerobic membrane bioreactor comprises a shell and a filter material layer arranged in the shell, and further comprises a high-pressure gas backflushing structure arranged above the filter material layer and comprising a high-pressure gas distribution structure and a plurality of high-pressure gas nozzles arranged on the high-pressure gas distribution structure, wherein openings of the high-pressure gas nozzles face the filter material layer; a low-pressure gas disturbance structure arranged below the filter material layer and comprising a low-pressure gas distribution structure and a plurality of low-pressure gas nozzles arranged on the low-pressure gas distribution structure, wherein openings of the low-pressure gas nozzles face the filter material layer; when the filter material layer is blocked, high-pressure gas sprayed by the high-pressure gas nozzles is used for backwashing the filter material layer; and when the filter material layer is not blocked, the low-pressure gas nozzles are used for spraying low-pressure gas near the filter material layer to improve the flowability and disturbance of sewage near the filter material layer, so that the scouring effect of the sewage on the filter material layer is improved, the blocking period of the filter material layer is prolonged, the treatment effect of the reactor is improved, and the backwashing frequency is reduced.

[0017] Seah et al. (CRITICAL REVIEWS IN BIOTECHNOLOGY, vol. 42, no. 2, 2022, pages 311-323) report that the cultured meat market has been growing at an accelerated space since the

first creation of cultured meat burger. Substantial efforts have been made to reduce costs by eliminating serum in growth media and improving process efficiency by employing bioreactors. In parallel, efforts are also being made on scaffolding innovations to offer better cells proliferation, differentiation and tissue development. So far, scaffolds used in cultured meat research are predominantly collagen and gelatin, which are animal-derived. To align with cell-based meat vision i.e. environment conservation and animal welfare, plant-derived biomaterials for scaffolding are being intensively explored. This paper reviews and discusses the advantages and disadvantages of scaffold materials and potential scaffolding related to scale-up solution for the production of cultured meat.

[0018] No part of this discussion of the prior art is to be construed as an admittance of prior art.

SUMMARY OF THE INVENTION

[0019] Indeed, such multiscale anisotropic elongated structures characteristic of animal meat made of muscle bundles (e.g., group of aligned muscle fascicles, myofibers and myofibrils) can only be achieved by producing scaffolds mimicking the aligned elongated, typically fibrous structure of meat and guiding the cells, normally stem cells, differentiation process with the help of a suitable microenvironment, as well as topological and mechanical cues. Particularly good scaffolds for the growth of muscle cells incorporate anisotropic microstructures, in the sense that they have an internal material structure with a preferred direction along the axis. The problem with the provision of such structures is that it requires demanding manufacturing processes under sterile conditions that restricts the process scalability or increases the equipment footprint.

[0020] In this invention, we disclose a method and compositions allowing the production of elongated structures, in particular of fibrous meat bundles with multiscale structural characteristics like those of animal meat, and reactors to cultivate said elongated structures, in particular fibre bundles, at high density and capable of applying mechanical stimulations. Such a reactor is designed for the cell seeding and to cultivate e.g. porous fibrous bundles or mats at high density, while optionally applying mechanical stretching.

[0021] The disclosed invention comprises a method and reactors for the in-vitro production of elongated structures, in particular of meat tissue or precursors thereof for cultured meat production allowing inter alia the scalable production of muscle bundles of high quality and in large quantities.

[0022] More specifically, the invention discloses (i) a method for the fabrication and optionally also the cultivation of fibrous cell-laden gel structures, (ii) a reactor for carrying out corresponding method and (iii) products obtained using the method/reactor. The elongated, in particular fibrous structures support cell differentiation e.g. into muscle bundles with fibrillar and highly anisotropic texture.

[0023] According to a first aspect of the present invention it therefore relates to a method for the manufacturing of an elongated structure, preferably fibrous structure (including microfibril structures) for the fabrication of cultivated meat, preferably for the parallel manufacturing of a multitude of such elongated, preferably fibrous structures or a bundle of such elongated, preferably fibrous structures.

[0024] The method is characterised by the steps as defined in claim **1**.

[0025] More specifically, according to the proposed method, [0026] (a) a paste is extruded through an extrusion plate with at least one nozzle opening (including round but also elongated openings or specifically shaped openings or a combination of differently shaped openings) and at least partly through and/or or into (into without fully through to extent at least sufficient for attachment after hardening for the drawing process) an essentially adjacent attachment plate (preferably with aligned nozzle openings), preferably into a space downstream of attachment plate, wherein preferably just enough paste is extruded through the two plates such that in or behind the attachment plate the front part is penetrating into the attachment plate, e.g. in that either a layer of the paste is formed or at least in or behind each nozzle opening or porosity of the attachment plate the paste is located and/or is flowing apart to a diameter larger than the nozzle opening, [0027] (b)

paste located in said attachment plate and/or in said space downstream of attachment plate is at least partly hardened, leading to a quasi-automatic fixing or attachment of the paste in and/or behind of the attachment plate, [0028] (c) said paste is continued to be extruded through said extrusion plate while the distance between said extrusion plate and said attachment plate is continuously increased under formation of said elongated structure between said plates.
[0029] In other words in the final step (c) extrusion runs in parallel with the distancing of the plate from each other such that in a controlled manner the elongated structure or the bundle of elongated structures in case of a plurality of nozzle openings is generated.
[0030] Preferably, the distancing speed of the attachment plate from the extrusion plate is larger than the extrusion speed, so there is a certain degree of drawing of the elongated structure during extrusion.
[0031] Correspondingly according to a first preferred embodiment, the extrusion takes place at an ejection speed under the generation of a speed gradient downstream of the respective nozzle opening in the extrusion plate by way of a distancing speed between said extrusion plate and said attachment plate larger than the ejection speed to form said elongated structure. The ejection speed is defined as the past (e.g. hydrogel) input flow rate divided by the total nozzle opening area.
[0032] In step (c) the pulling rate, measured as the speed of the attachment plate relative to the extrusion plate (the relative speed is relevant, the extrusion plate can be stationary or attachment plate can be stationary or both can be moving relative to each other), is preferably in the range of 0.01-100 m/min, more preferably between 0.1-10 m/min.
[0033] To make sure that the above-mentioned attachment of the paste downstream of attachment plate is established, according to a further preferred embodiment in at least one of: step (b), step (c) and after step (c), the drawn/extruded paste is immersed in a hardening bath, preferably a hardening bath comprising divalent or polyvalent cations or an acidic bath or a bath with a temperature different from the gel paste, more preferably a hardening bath comprising divalent cations.
[0034] Said extrusion plate and/or said attachment plate preferably each comprise a plurality of nozzle openings, preferably at least 20, more preferably at least 100, most preferably at least 200 or in the range of 250-1000 nozzle openings, [0035] and/or the nozzle openings cover 5-90% or between 5% and 95% of the plate cross-section area, more preferably between 20% and 90% or 20-80%, most preferably between 40% and 70%, [0036] and/or the nozzle openings are provided in said extrusion plate and/or said attachment plate with a nozzle density of 1-5000 nozzles/cm2, preferably in the range of 5-400 nozzles/cm2.
[0037] Preferably the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, more preferably 100-1500 μm, in particular the above nozzle specifications in terms of numbers, % and/or densities are given in combination with nozzle sizes with nozzle openings having a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, more preferably 100-1500 μm.
[0038] The nozzle openings can be of circular cross section, oval, rectangular, square-shaped opening, and most preferably all with the same circular diameter, which are preferably arranged in an overlapping manner in the two plates, if also the attachment plate is provided with nozzle openings.
[0039] The attachment plate can be a mesh, a porous plate, or a plate with nozzle openings, preferably a plate with nozzle openings aligned to nozzle openings of the extrusion plate.
[0040] If it is a mesh, a porous plate, the structure of the porosity has to be such that partial penetration of the paste which is extruded through the extrusion plate into the attachment plate is possible, and that hardening of that paste in porosity of the attachment plate is possible, for example by flooding the space downstream of the attachment plate with a hardening agent or by irradiation. Like that it is for example possible to work in situations where the paste only partly penetrates into the attachment plate into the porosity without fully penetrating the attachment plate,

and then the paste is hardened in that porosity in the attachment plate leading to an attachment of the strings of paste for the subsequent extrusion process.
[0041] As concerns the nozzle openings in the extrusion plate, and if the attachment plate comprises corresponding nozzle openings also and in the same way as these nozzles, these nozzle openings can be arranged in a density and pattern adapted to the desired bundle structure. It is for example possible in particular in case of larger extrusion/attachment plates, to have separate areas with nozzle openings suitable and adapted to form fibre bundles which correspond to a desired shape of the meat product, for example a string of artificial muscle, while having areas between these nozzle opening areas without nozzle openings, so that in one process several such fibre bundles similar to natural muscle strings are generated and, if needed and desired, directly subsequently cultivated. The above-mentioned densities and percentages preferably apply specifically only to such areas of nozzle openings.
[0042] Preferably, the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, more preferably 50-1000 μm.
[0043] Preferably the nozzle openings are of circular form and most preferably all with the same circular diameter, which are arranged in an overlapping manner in the two nozzle plates.
[0044] According to yet another preferred embodiment, extrusion in steps (b) and (c) takes place into a preferably closed sterile reaction container (bioreactor, cultivation vessel), and subsequent to step (c), if needed followed by a step of further hardening of the extruded elongated structure, the reaction container is filled with culturing growth media and the elongated structures are used for growing meat cells seeded onto said structures and/or already contained in said paste.
[0045] According to a particularly preferred embodiment, the proposed method is characterised in that [0046] (a) said paste is extruded through said extrusion plate and at least partly through an adjacent attachment plate with aligned nozzle openings, preferably into a space downstream of the attachment plate, [0047] (b) slowing down or stopping said extrusion and hardening paste located in said attachment plate and/or in said space by flooding said space downstream of the attachment plate with a hardening bath, preferably a hardening bath comprising divalent or polyvalent cations or an acidic bath, or a bath with a temperature different from the gel paste, preferably a bath of calcium chloride, [0048] (c) followed, if need be after removal of the hardening bath, or in the presence of the hardening bath, by continued extrusion of said paste through said extrusion plate while distancing said attachment plate with a relative distancing speed larger than the extrusion speed from said extrusion plate under formation of said elongated structure, preferably of a multiscale anisotropic structure between said nozzle openings, [0049] (d) slowing down or stopping said distancing and extrusion, and hardening the paste in the form of microfibrils in the space between the two plates, by flooding said space downstream of the attachment nozzle plate with a hardening bath if not done so already during step (c), preferably a hardening bath comprising divalent or polyvalent cations or an acidic bath or a protein cross-linking bath, preferably a bath of calcium chloride, and, optionally, hardening the paste in a space upstream of the extrusion plate, [0050] (e) replacing what is in the space between the plates (typically the hardening bath or the cross-linking bath) with culturing media and using the elongated structures, preferably fibre bundles (e.g. microfibrils) for growing meat cells seeded onto said elongated structures and/or already contained in said paste.
[0051] The corresponding process can be controlled by controlling the composition of the media, the temperature, pH, the supernatant gas atmosphere, et cetera.
[0052] According to a further preferred embodiment, during culturing the two plates can be oscillated relative to each other stimulating and influencing the growth process in the anisotropic microfibrillar structures.
[0053] Preferably, said paste is a paste for the fabrication of cultivated meat, comprising of consisting of the following components: [0054] (A) at least one polysaccharide that can form a

solidified gel by the action of divalent or polyvalent cations, thermal gelling, light-induced addition reaction or light induced condensation reaction, or a combination thereof, preferably in a concentration in the paste in the range of 0.01-200 g per L of component (D); [0055] (B) preferably at least one protein, preferably assembling with the polysaccharide of component (A) via supramolecular or covalent interaction or a combination thereof, preferably in a concentration in the paste in the range of 0.001-500 g per L of component (D) [0056] (C) cells selected from mammalian cells, fish cells, crustaceous cells or a combination thereof, in a concentration in the paste in the range of 0-300 billion cells per L of component (D) [0057] (D) water or a water-based culturing medium; [0058] (E) additives different from (A)-(D), preferably selected from the group consisting of crosslinking kinetic modifier in a concentration in the paste in the range of 0-500 mM, flow modifier in a concentration in the paste in the range of 0-200 g per L of component (D), or a combination thereof.

[0059] In fact, preferably a paste as described in European application EP 22 164 793.6 is preferably used in this method. The disclosure of European application EP 22 164 793.6 is included into the specification as concerns the paste and methods of treating the paste as well as corresponding products as defined in that European application.

[0060] The major features of this paste and its use in the method are described below: [0061] (i) The gel paste composition in combination with the method of production allows the fabrication of cell-laden composite gels characterized by unique anisotropic elongated, preferably fibrillar structure at the microscale. [0062] (ii) The production process to develop such elongated, preferably fibrillar structure is fast and scalable and therefore compatible with the high production rate required for cultivated meat production. [0063] (iii) The elongated, preferably fibrillar structure of the composite gel promotes a superior differentiation of muscle progenitor stem cells into muscle tissue with the features typical of meat.

[0064] Advantages resulting from the major features of the invention comprise the following aspects, alone or in combination: [0065] (1) Gel paste is composed of edible components for the formation of the microfibrillar [0066] (2) Differentiated cells assemble in muscle bundles with realistic size. Muscle bundles have a tunable diameter within 0.01 mm and 2 mm and a length tunable in size above 1 mm. [0067] (3) Size of the muscle bundle can be tuned by playing on different process parameters. This allows the production of muscle fibers with a texture that mimics that of meat of different species. [0068] (4) High cell density can be achieved, comparable to animal muscle tissue. Cell density up to hundreds of million cells per cm3 can be achieved [0069] (5) Cell seeding is efficient (with virtually 100% seeding efficiency) and homogeneous throughout the whole scaffold volume [0070] (6) Gel paste composition and process parameters can be tuned to promote cell spreading and differentiation by fulfilling biomechanical requirements of any specific cell type. [0071] (7) Fibrous gels embedding cells withstand culturing conditions for several weeks. [0072] (8) Gel paste is converted into a mechanically stable gel within hundreds of seconds with cells being trapped and protected inside, making gel fibers easy to handle and compatible with a high production rate. Muscle bundles production can be completely automated, and production can happen directly inside the differentiation bioreactor. [0073] (9) As a consequence of the fibrous texture of the gel entrapping cells, the resulting muscle bundles develop superior aligned fibrillar structure even without active tension during culture. [0074] (10) High increase of protein content is observed upon differentiation as a consequence of the highly anisotropic gel microstructure and cell differentiation [0075] (11) Mild processing conditions [0076] (12) Optionally, muscle bundles are physically clamped during production in order to straightforwardly apply external stimuli (e.g. mechanical stretching and/or electrical field) to further push myofibrillar/myoglobin protein production. [0077] (13) Optionally, non-proteinaceous components used as processing aids in the preparation of the gel paste (e.g., polysaccharide components) can be degraded/dissolved at the end of the differentiation process to maximize of the protein content and minimize the ingredient list. [0078] (14) Optionally, bioactive molecule can be

immobilized in the gel to support differentiation and spreading. [0079] (15) Optionally, chromophores, flavour and aroma molecules can be added to the gel paste to improve overall sensory attributed of the produced meat. [0080] (16) Optionally, fibroblasts can be seed on and/or in the muscle bundle to deposit connective tissue and further tune the texture of the final meat product [0081] (17) Optionally, the crosslinking bath used for the gelation of the gel paste can contain iron ions to increase the iron content of the final meat product. [0082] (18) Optionally, post-cultivation, muscle bundles can be glued together by using microbial transglutaminase. Furthermore, plant proteins and animal proteins can be added to tailor texture and protein content/source. [0083] (19) Optionally, the produced muscle bundle, can be combined with plant, animal and cultivated fat to produce meat-based foodstuffs. [0084] (20) Optionally, micro/nano fibres can be added in the gel paste formulation to increase the toughen the hydrogel and modulate texture.

[0085] So the present invention discloses methods and compositions for the fabrication of a cell-laden composite gel which can be formed on-demand with fibrillar structure. The invention is composed of different elements: a composition for a gel paste, and a method to produce gel fibers from the gel paste and the use of it to produce cultivated meat products.

[0086] According to a preferred embodiment of such a paste, the at least one polysaccharide of component (A) is naturally crosslinkable and/or chemically modified to promote crosslinking, wherein preferably the chemical modifications include acrylation, methacrylation, epoxidation, allylation, or a combination thereof.

[0087] According to a further preferred embodiment of such a paste, the at least one polysaccharide of component (A) is selected from the group consisting of alginates, pectins, carrageenans, chondroitin sulfate, dermatan sulfate, heparin, heparin sulfate, as well as derivatives thereof and combinations thereof, preferably the polysaccharide component of component (A) is at least one alginate or alginate derivative.

[0088] Preferably, the polysaccharide(s) of component (A) is present in a concentration in the paste in the range of 0.1-100 g per L of component (D).

[0089] According to yet another preferred embodiment the at least one protein of component (B) takes the form of a native protein, denatured protein, protein hydrolysate, or a combination thereof.

[0090] Preferably the at least one protein of component (B) is bioactive with the ability to modulate a function and/or characteristic of the cells of component (C), in particular to promote cell attachment by providing integrin binding motifs.

[0091] The at least one protein of component (B) can be preferably selected from the group consisting of gelatin, collagen, fibrinogen, fibrin, fibronectin, fibroin, elastin, laminin, basic albumins from plant, preferably napin, wherein preferably the protein component (B) is gelatine, more preferably gelatine, type A.

[0092] Preferably, the at least one protein of component (B) is chemically modified to promote crosslinking, wherein preferably the chemical modifications include acrylation, methacrylation, epoxidation, allylation, or a combination thereof.

[0093] According to a preferred embodiment, the at least one protein of component (B) is crosslinked, preferably by transglutaminase, peroxidase, laccase, tyrosinase, lysyl oxidase, glutaraldehyde, genipin, citric acid, photopolymerization or a combination thereof, preferably by transglutaminase is used, and wherein if transglutaminase is used as crosslinker, it is comprised in an amount of 0-50 U per g of protein, more preferably 0.1-10 U per g of protein.

[0094] Typically, the protein(s) of component (B) is present in a concentration in the paste in the range of 0.1-250 g per L of component (D).

[0095] According to yet another preferred embodiment, the cells of component (C) are present in a concentration in the paste in the range of 5-100 billion cells per L of component (D).

[0096] The additives of component (E) preferably include at least one crosslinking kinetic modifier to slow down the gelation kinetics of the crosslinkable polysaccharide by sequestering divalent or

polyvalent cations.
[0097] Preferably the additives of component (E) include at least one food-compatible compound selected from the group consisting of disodium phosphate, dipotassium phosphate, magnesium phosphate, ethylenediaminetetraacetic acid, ethylenediaminetetraacetic salts, or a combination thereof.
[0098] Typically the additives of component (E) are present in a concentration 1-100 mM.
[0099] The additives of component (E) May include at least one flow modifier to impart a shear thinning behaviour to the gel past so that the higher viscosity in resting condition mitigate cell sedimentation, whereas the lower viscosity during ejection allows processability, wherein preferably said flow modifiers are edible fillers in the form of micro/nanofibers, preferably insoluble in culturing conditions, and are preferably composed of protein and/or polysaccharide different from the other components of the paste, wherein such edible filler is preferably present at a concentration of 0-200 g per L of paste, and wherein further preferably edible polymers as flow modifier can be selected among gums, including gellan gums, guar gums, xanthan gums), PEGs, or combinations thereof, and wherein preferably edible polymers are present at a concentration of 0-50 g per L of gel paste.
[0100] Preferably, such a paste is further supplemented with a micro-structuring agent, for unfolding of protein structure, preferably selected from the group consisting of bicarbonate, including sodium bicarbonate, potassium bicarbonate, ammonium bicarbonate, magnesium bicarbonate, or a combination thereof. Preferably the micro-structuring agent is added to the paste in an amount to lead to at a concentration ranging from 0.1-300 g per L of paste, preferably, of 1-200 g per L of paste. The micro-structuring agent is preferably added in powder form. The micro-structuring agent and the paste may also take the form of a kit-of parts, which is joined right before carrying out the method for making the microfibril structure as detailed below.
[0101] Preferably, the hardening bath comprises Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+} or a combination thereof in water, preferably at a concentration in the range of 1-500 mM, more preferably between 20-100 mM.
[0102] The hardening bath of step (c) can be buffered at a pH ranging from 6 to 8, preferably by using HEPES buffer, wherein further preferably the HEPES buffer is used in a concentration comprised between 1-100 mM.
[0103] Preferably, the paste is extruded from a nozzle preferably with a circular geometry at the ejection point.
[0104] According to yet another preferred embodiment, either the hardening bath comprises a protein cross-linking agent or subsequent to step (b) and/or during step (c) the fibres are immersed in a protein cross-linking bath with such a cross-linking agent.
[0105] Preferably such a cross-linking agent is selected from the group consisting of transglutaminase, peroxidase, laccase, tyrosinase, lysyl oxidase, glutaraldehyde, genipin, citric acid, or a combination thereof, preferably transglutaminase, and wherein if transglutaminase is used, it is comprised in an amount of 1-2000 U per mL of crosslinking bath.
[0106] Further preferably cross-linking is performed at a temperature in the range of 20-45° C., preferably for a time span in the range of 10-120 min, more preferably 30-90 min.
[0107] Muscle bundles can be prepared by cultivation of cell-laden microfibrillated hydrogel fibers in an environment promoting myogenesis.
[0108] Preferably, the resulting fibres, preferably having an individual diameter in the range of 0.1-50 μm, preferably aligned in the same axis ±40° with respect of the fiber axis, are converted into muscle tissue by culturing them, e.g. in a step (e) as defined above, in differentiation media designed for the used cell type, wherein if the fibres have been produced from a paste without cells, before culturing the fibres are seeded with cells. Preferably the fibers, e.g. in the form of bundles, are cross-linked together to obtain a solid edible structure, wherein cross-linking of the fibres can be effected by one or several cross-linking agents selected from the group consisting of

transglutaminase, peroxidase, laccase, tyrosinase, lysyl oxidase, glutaraldehyde, genipin, citric acid, or a combination thereof, preferably transglutaminase

[0109] Normally, the resulting muscle tissue or assembly of is mixed with further constituent to form a consumer cultured meat product.

[0110] According to yet another preferred embodiment, the extrusion in step (c) takes place with a drawing factor, defined as the ratio of the ejection speed to the distancing speed of the second nozzle plate relative to the first nozzle plate during step (c), of at least 1.1, preferably at least 1.5 or at least 2.

[0111] According to yet another aspect, the present invention relates to a reactor for carrying out the method according as described above, wherein this reactor, which preferably allows to work under sterile conditions, comprises a closable reactor container of elongated shape along a main axis with constant cross-section, preferably of cylindrical or rectangular/square or hexagonal shape, a (preferably stationary) extrusion plate (having the corresponding cross-sectional shape of the reactor container) at one end of the reactor and at least one attachment plate (also having the corresponding cross-sectional shape of the reactor container), preferably movably mounted in said reactor container, elements for controlled supply of paste for extrusion thereof upstream of said extrusion plate and elements for controllably moving said attachment plate along the main axis, as well as means for supplying at least one of culturing media, hardening media, cross-linking media to and from the inside of the reactor container, preferably also to a space upstream of said (stationary) extrusion plate.

[0112] According to a preferred embodiment of this reactor, the extrusion plate is located at a bottom of said closable reactor container, and said attachment plate is located above of the extrusion plate.

[0113] According to yet another preferred embodiment, the extrusion plate can be moved by said elements in a contactless manner, preferably by magnetic attraction, wherein preferably to this end the extrusion plate is mounted in and/or on a mounting structure with at least one magnet and outside of the reactor container there is a movable magnetic element, preferably in the form of a ring with at least one magnet, which movable magnetic element can be moved automatically, preferably by motor.

[0114] Said (stationary) extrusion plate and/or said attachment plate preferably each comprise a plurality of nozzle openings, preferably at least 20, more preferably at least 100, most preferably at least 200 or in the range of 250-1000 nozzle openings, and/or the nozzle openings cover 5-90% or between 5% and 95% of the plate cross-section area, more preferably between 20% and 90% or 20-80%, most preferably between 40% and 70%, and/or the nozzle openings are provided in said extrusion plate and/or said attachment plate with a nozzle density of 1-5000 nozzles/cm2, preferably in the range of 5-400 nozzles/cm2.

[0115] Preferably the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, more preferably 100-1500 μm, in particular the above nozzle specifications in terms of numbers, % and/or densities are given in combination with nozzle sizes with nozzle openings having a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, more preferably 100-1500 μm.

[0116] The nozzle openings can be of circular cross section, oval, rectangular, square-shaped opening, and most preferably all with the same circular diameter, which are preferably arranged in an overlapping manner in the two plates, if also the attachment plate is provided with nozzle openings.

[0117] Preferably the nozzle openings are of circular form and most preferably all with the same circular diameter, which are arranged in an overlapping manner in the two nozzle plates.

[0118] Further preferably, said (stationary) extrusion plate and/or said attachment plate further comprises at least one opening for controlled supply of liquids through the respective plate.

According to yet another preferred embodiment of the reactor, it is equipped with a control, in particular for controlling at least one of the extrusion speed of the paste, the distancing speed of the attachment plate, the supply of and removal of at least one of culturing media, cross-linking media, hardening media.

[0119] According to another aspect of the present invention, it relates to the use of a method as described above for the manufacturing of a consumer cultured meat product. Preferably this entails using the corresponding elongated structures, preferably after cultivation in the form of bundles, in combination with [0120] (i) fat and or oil-based components including cultured fat, vegetable fat or animal fat based components and its derivatives, in particular in combination with separately cultured fat cells and/or separately cultured fat cell aggregates, [0121] (ii) and/or structuring agents including hydrocolloids, and/or cellulose and its derivatives, and/or proteins derived from plant, animal, recombinant technology, cell cultivation; [0122] (iii) and/or connective tissue components including animal derived connective tissue, cultured connective tissue, in particular in combination with cultured fibroblast and/or chondrocytes and/or separately cultured fibroblast and/or chondrocytes aggregates.

[0123] Said components can be assembled to form fibre bundles compact bundle, like meat pieces.

[0124] Furthermore, the present invention relates to a cultured meat obtained using a method as described above, and/or obtained using a reactor as described above.

[0125] Further embodiments of the invention are laid down in the dependent claims.

Description

BRIEF DESCRIPTION OF THE DRAWINGS

[0126] Preferred embodiments of the invention are described in the following with reference to the drawings, which are for the purpose of illustrating the present preferred embodiments of the invention and not for the purpose of limiting the same. In the drawings,

[0127] FIG. **1**A-FIG. **1**C shows a bioreactor according to a first embodiment with a low dead volume, wherein in FIG. **1**A a perspective representation with the upper extrusion plate in a medium lower position is shown, in FIG. **1**B a first axial cut is shown in a plane through the closure mechanism and in FIG. **1**C a second axial cut in a plane perpendicular to the ones given in FIG. **1**B;

[0128] FIG. **2**(A)-FIG. **2**C shows a bioreactor according to a second embodiment with a high dead volume, wherein in FIG. **2**(A) a perspective representation with the upper extrusion plate in a medium lower position is shown, in FIG. **2**B a first axial cut is shown in a plane through the closure mechanism and in FIG. **2**C a second axial cut in a plane perpendicular to the ones given in FIG. **2**B;

[0129] FIG. **3** shows an axial cut through the bioreactor according to the first embodiment in a position in which the upper extrusion plate is in the uppermost position and including the mounting of the reactor;

[0130] FIG. **4** shows an axial cut through the bioreactor according to the second embodiment in a position in which the upper extrusion plate is in the lowermost position;

[0131] FIG. **5** shows a perspective view onto a bioreactor according to the first embodiment in a perspective representation including the mounting of the reactor and further elements of the automatic system, wherein the upper extrusion plate is in an medium position;

[0132] FIG. **6**(A)-FIG. **6**C shows the bottom extrusion plate wherein in FIG. **6**(A) a perspective representation is given, in FIG. **6**B the cut along A-A in FIG. **6**C and in FIG. **6**C a top view;

[0133] FIG. **7**(A)-FIG. **7**D shows the upper extrusion plate, wherein in FIG. **7**(A) a perspective view from the top is given, in FIG. **7**B a cut along A-A in FIG. **7**D, in FIG. **7**C a perspective view from the bottom and in FIG. **7**D a top view is given;

[0134] FIG. **8**(A)-FIG. **8**I the sequence of operation of the proposed bioreactor, wherein in FIG. **8**(A) the step of pre-filling the gel is illustrated, in FIG. **8**B the upper clamping is illustrated, in FIG. **8**C the extrusion is illustrated, in FIG. **8**D the lower clamping is illustrated, in FIG. **8**E the cross-linking fill-up is illustrated, in FIG. **8**F the actual cross-linking is illustrated, in FIG. **8**G the filling up with media and the cross-linker drain is illustrated, in FIG. **8**(*h*) the media fill-up is illustrated and in FIG. **8**I the cultivation is illustrated;

[0135] FIG. **9**A-FIG. **9**C materials and results of E.1. Perforated attachment plate FIG. **9**A, 3D model of attachment grid FIG. **9**B, transferred extruded fiber bundles within a petri-dish (diameter 9 cm) FIG. **9**C;

[0136] FIG. **10** attachment plates used for E.2. Attachment plates with a density of 11% (left), 22% (middle), and 45% (right);

[0137] FIG. **11**A-FIG. **11**B results of E.3. Microscopy images of extruded fibers encapsulating 20 million cells per mL (**2***a*), and 60 million cells per mL (**2***b*). The top images were taken from fiber sections right underneath the attachment plate, the bottom images were taken from fiber sections right above the extrusion plate;

[0138] FIG. **12**A-FIG. **12**C results of E.4. Extruded fibers clamped between the bottom extrusion plate and the top attachment plate of the differentiation bioreactor in cell culture medium after 4 days in culture FIG. **12**A; light microscopy image of the extruded fibers after harvesting, showing microfibrillar texture FIG. **12**B; Calcein-AM images of the harvested muscle fibers after 5 days in cell culture medium, showing cell spreading and alignment along the fiber direction FIG. **12**C;

[0139] FIG. **13**A-FIG. **13**C results of E.5. Extruded and fixed fibers between extrusion and attachment plate in muscle differentiation bioreactor maintained in saline crosslinking solution FIG. **13**A, draining of saline crosslinking solution after extrusion, showing the stability of the clamped fibers FIG. **13**B, extruded fiber bundle after harvesting FIG. **13**C.

DESCRIPTION OF PREFERRED EMBODIMENTS

[0140] FIGS. **1**A-**1**C show a bioreactor **1** according to a first embodiment with an insert **17** providing for a low dead volume, i.e. for producing bundles having a high length.

[0141] FIGS. **2**A-**1**C on the other hand shows a corresponding bioreactor **1** with a high dead volume, i.e. with an insert **17** to avoid use of too much processing liquids if one desires to produce shorter microfibrillar bundles.

[0142] The embodiments according to FIGS. **1**A-**1**C and **2**A-**1**C are apart from that essentially the same, and correspondingly the reference numerals in those two figures and in the other ones indicate the same or equivalent elements, in as far as this is not specifically stated otherwise.

[0143] As one can see from FIG. **1**A, a perspective representation from the top, the reactor **1** comprises a cylindrical circumferential wall **2** which, along its main axis, is supported in the middle section by an axial reinforcement structure **3**.

[0144] The reactor **1** is covered on the top by a top cover **4**, which is provided with a central first inlet/outlet **5**, and a second lateral inlet/outlet **6**. The top portion of the reactor is based on an upper frame portion **15**, which is circumferential and one piece with the axial part **3**. That is followed by an upper circumferential closure extension **11** (see FIG. **1**B), which is attached to the upper frame portion **15** by way of a lower top closure bracket **8**, which hinges around hinge **10** and which is closed by the closure mechanism **13**.

[0145] Between the upper frame portion **15** and the upper closure extension **11** there is provided a circumferential seal **16**. To the top this upper closure extension is followed by the above mentioned top cover **4**, which is attached by way of another upper top closure bracket **7**, hinged around axis **9**, and fixed by closure mechanism **12**. Also here there is provided a circumferential sealing **14** between the upper closure extension **11** and the top cover **4**.

[0146] As one can see in particular from the axial cut in FIGS. **1**B and C, in the top portion there is provided an insert **17**, which does not have the same but a lower outer diameter than the circumferential wall **2**, such that there is an interspace between the insert and the wall, providing

for a passage **20** around this insert **17**. The insert **17** encloses a dead volume **18**, which is not accessible to any liquid. This insert **17** is penetrated by an axial central vertical pipe **19** or channel, which is attached to the first inlet/outlet **5** on top, and sealed relative to that, if needed, by way of a protruding portion **21** in contact with the lower opening of the first inlet **5**. On the other hand, the second inlet/outlet **6** is connected with the above-mentioned passage **20** and allows for circulation of liquid through the passage **20** in to the space **58** below the insert.

[0147] The reactor as illustrated in FIGS. **1**A-**1**C has an attachment upper plate **22** shown in a lower medium position. This attachment plate **22** is mounted in a circular mounting structure **23**, and has a central opening **32**, e.g. suitable and adapted to be penetrated or connected/touched with the vertical pipe **19** at its lower end, if the attachment plate **22** has reached the uppermost position. Also there are provided lateral openings **40** in that attachment plate **22**. These openings **40** are provided for, if needed, allowing fluid to be supplied and/or removed through the corresponding plate **22**. Importantly, this attachment plate **22** is provided with a multitude of nozzle openings or perforations **42**, as will be detailed further below.

[0148] Below that attachment plate **22**, there is located, in a stationary manner, the extrusion plate **24**. Also this extrusion plate **24** is provided with a large number of perforations **44**, and in fact the perforations in the two plates **22**,**24** are arranged and the plates are mounted in a way that if the two plates **22**,**24** are located adjacent to and in flat contact with each other, the perforations in the two plates **22**,**24** align and allow the extrusion material to pass both plates **22**,**24**, so the nozzle openings in individual plates **22**,**24** combine to a double plate nozzle opening.

[0149] This extrusion plate **24** is provided with surface areas without nozzle openings **41** and **33** aligned with the openings **40** and **32**, respectively, in the attachment plate **22**. As one can see in particular from FIGS. **1** B and C, there can be a material layer **31** around the mounting structure **23** for the attachment plate **22**, e.g. to hold the magnets mentioned further below. Also, one can see that in the bottom construction, there is a bottom plate **34**, on which there is provided the bottom part **37**, which is attached to an upper circumferential bottom part **38**, these two elements are attached to each other by way of a lower bottom closure bracket **26**, with a corresponding closure mechanism **28**. This is followed in the upper direction by the lower frame portion **43**, which is attached to the upper bottom part **38** by way of the upper bottom closure bracket **27**, with a corresponding closure mechanism **29**. Between these elements there is again provided circumferential sealing elements **39**.

[0150] There is a bottom inlet **25**, which allows to supply extrusion material, the above mentioned paste, first to horizontal contiguous space **92** below the first lower extrusion plate **24**, this space **92** is also provided with a side inlet/outlet **35**. For supply to the space above the extrusion plate **24** and below the attachment plate **22**, that is the space **57**, there is provided a lateral bottom inlet/outlet **30**. The extrusion plate **24** is sealed by way of one or several circumferential sealings **36**.

[0151] As one can see from the representations in FIGS. **2**A-**2**C, here the insert **17** is much larger in an axial direction and occupies a large fraction of the void volume of the reactor inside the circumferential wall **2**. In this sense the space below the insert, that is the space **58**, combined with the space **57** between the second upper extrusion plate and the extrusion plate **24**, is much smaller. As indicated above, using this insert **17** is suitable and adapted for production processes where short fibrous bundles are to be produced.

[0152] FIG. **3** shows an axial cut through the bioreactor according the first embodiment (FIGS. **1**A-**1**C) in a position in which the attachment plate **22** is in the uppermost position, and the figure in addition to that shows the mounting of the reactor **1** as well as the means provided for shifting the attachment plate **22** during the extrusion process.

[0153] The reactor **1** is mounted on a bottom mounting structure **48**, which stands on a number of corresponding vertical legs **52**. There is provided a housing **50** which may house or comprise control elements for the movement of the plate **22** or for the supply of the liquid, and a corresponding carrier structure **51**. This carrier structure **51** in particular is there to mount a vertical

rail **46**, on which there is moveably mounted mounting structure **49**, on which a shifting bracket **45** is attached. On this shifting bracket **45** there is provided a circumferential shifting ring **53**, which controls the axial position of the mounting structure **23** for the attachment plate **22**. There is provided a motor **47**, and this motor **47**, by way of a corresponding belt or chain, allows to vertically move the mounting structure **49** and correspondingly the shifting ring **53** depending on the process.

[0154] How this is done in a contactless way to allow for sterile conditions in the inside of the reactor **1** is best seen in FIG. **4**. Here, a corresponding reactor **1** with a large insert, also having a slightly different top cover geometry, so similar to the embodiment in FIGS. **2**A-**2**C, is illustrated in a position where the attachment plate **22** is in the lowermost position, i.e. is adjacent and essentially in contact with the extrusion plate **24**. Here one can see, that the shifting ring **53** is provided with magnets **54**. Also the carrier structure **23** for the attachment plate **22** is provided, in corresponding axial extensions, with counter magnets **55**. Due to the corresponding magnetic attraction, the shifting ring **53** and the mounting structure **23** and the corresponding attachment plate **22** are fixed relative to each other, and if the motor **47** starts pulling the mounting structure **49** in an upwards direction it will correspondingly move the shifting ring **53** upwards and due to the magnetic attraction this will draw the mounting structure **23** and the corresponding attachment plate **22** also in a vertical upwards direction.

[0155] Yet another perspective illustration of the reactor setup with the surrounding elements is illustrated in FIG. **5**. Here one can see that around the actual reactor there can be provided a glass housing **56**, and in the bottom part various control and/or supply handling elements **93** can be provided.

[0156] FIGS. **6**A-**6**C illustrate the extrusion plate **24**. Here one can see that this is actually a nozzle plate with a very large number of perforations, specifically in this case, there is provided **6700** perforations in this plate. As already indicated above, there is also a first area without openings **33** on the axis of the plate **24**, as well as two laterally offset areas without openings **42**. The vast majority of the surface of this plate **24** is covered by these openings/nozzles/perforations **42**. These perforations have a diameter of 0.7 mm.

[0157] FIGS. **7**A-**7**D show corresponding representations of the second upper extrusion plate. In this case, the plate **22** is provided with a circumferential rim **59**, which is provided with an attachment rib **60** for attaching it to the mounting structure **23**.

[0158] Also, in this case, the central opening **32** is provided with a circumferential rim protruding upwards, and so are the lateral openings **40** as mentioned above.

[0159] Again the vast majority of the surface of this plate **24** is covered by the perforations, and the perforations have the same distribution and geometric arrangement as well as the same size as the perforations in the extrusion plate **24** illustrated in FIGS. **6**A-**6**C. This is why, if the two plates **22**,**24** are put adjacent and in flat contact to each other, these openings will align and form the extrusion nozzles in the first phase of the making process.

[0160] The sequence illustrated in FIG. **8**A-J is used to explain the manufacturing method which is possible with a reactor **1** as described above.

[0161] In the context of the FIG. **8**A the individual reference numerals are described, the same reference numerals are used in the following figures b)-i) and designate the same elements, but in the context of the following figures only the respective method step description is given.

[0162] FIGS. **8**A-**8**J schematically illustrate on the left side the reactor **1**, and in this reactor **1** the mounting structure **23** and the attachment plate **22** is in each case illustrated in three different positions, the bottom position **88**, the middle position **89**, and the uppermost position **90**. Depending on the corresponding process situation, only one of these positions is assumed.

[0163] There are three media containers, an actual growth medium container **61** (M), a container for calcium chloride **62** (CC) which acts as the hardening agent, and a container **63** (CS) for a cross-linking solution.

[0164] Also there is provided a container or a reservoir together with a pump for the actual cell paste, this is illustrated by reference numeral **64**. This reservoir **64** is attached by way of valve **67** and via line **65** to the bottom inlet **25** of the reactor as illustrated above. To allow for complete filling of the space **92** below the attachment plate **22** in the first step of the manufacturing, which is illustrated in FIG. **8**A, there is also provided an outlet **66** which can be opened and closed by way of valve **68** allowing air and/or paste to escape from the space **92** (see FIG. **1**B).
[0165] The media container **61** is connected to the reactor by way of the line **70** controlled by way of the valve **71** leading to the collecting line **72**. In this connecting line **72** there is a pump **69** and a valve **73** for control supply and/or removal from bottom inlet/outlet **30**. Also there is a crossline **91** connecting this collecting line **72** with the upper branch of tubing, also here there is provided a corresponding valve **94**.
[0166] Also connected to the collecting line **72** is the calcium chloride container **62** and this by way of line **74** which is controlled by valve **75**. Also the container with the further crosslinking solution **63** is connected to collecting line by way of line **76** and controlled by valve **77**. In the upper branch there is an upper collecting line **79**, which is attached to the second inlet/outlet **6** provided in the top cover **4** of the reactor. This upper collecting line **79** is connected by valve **80** and to the growth media container **61** by way recirculation line **81** controlled by way of valve **82**, to the calcium chloride container **62** by way of circulation line **83** and to the further crosslinking solution container **63** by way of line **85** controlled by valve **86**. The lines are also provided with means for outflow to collection means or waste means **78**.
[0167] In FIG. **8**A the step of prefilling with gel from the reservoir **64** is illustrated. In this step, the cell paste is pumped into the bioreactor. From reservoir **64**, the paste is pumped through valve **67** and pipe **65** to the space **92** below the extrusion plate **24** and in this case the second upper extrusion plate is in the bottom position **88**. The extrusion is carried on until the cell paste passes through both adjacent extrusion plates **22**,**24** or rather through the perforations in these extrusion plates **22**,**24** and forms a layer of paste above the attachment plate **22** or forms at least some widening portions. To allow a complete filling of the space **92** the line **66** is opened until the space is completely filled and outflow is then controlled by way of valve **68**.
[0168] Once a layer (or widening portions) of gel above the attachment plate **22** is achieved, the next step as illustrated in FIG. **8** B is initiated, the step of upper clamping. Now the container for calcium chloride **62** is connected, i.e. valves **75**, **73** are opened and the pump **69** is activated such that the reactor volume is now filled with calcium chloride solution. This leads to a hardening of the cell paste layer above the second, upper extrusion plate and leads to the automatic adherence of individual strings of paste located in and above the nozzle openings. During this phase, the pumping of the cell paste is interrupted.
[0169] In the next step, schematically illustrated in FIG. **8** C, the paste is extruded from container **64** and in parallel the mounting structure **23** is successively moved upwards until reaching the position as illustrated in this figure, i.e. at the end of the process the attachment plate **22** is in the position **90**, that is the uppermost position. So in that phase the pumping of the cell paste is turned on again, so extrusion takes place, and the clamping plate, i.e. the attachment plate **22** with the attached uppermost parts of the paste above the plate **22** moves upwards, forming multiple individual fibres from each or the holes of the attachment plate **22**. During extrusion, these nascent fibres are in the hardening bath, so they are successfully hardened concomitant to extrusion. The speed of extrusion is chosen to be somewhat lower than the speed of upwards motion of the attachment plate **22** so there is certain degree of stretching during extrusion.
[0170] Then follows the step as illustrated in FIG. **8** D. In this step, the pump of unit **64** is stopped, and also of the motion of attachment plate **22**, but now the hardening solution is supplied by way of lines **74**, **72** and **66** so across valves **75**, **73** and **68** to the space **92**, so that paste located in that interspace is also cross-linked.
[0171] So this leads to a situation that the bottom part of the fibres is also clamped by way of this

contiguous patch of paste located below the extrusion plate **24**. So at the end of this process phase there is a bundle of microfibrillar fibres clamped by two contiguous patches of cross-linked or hardened paste, one above the attachment plate **22**, and a second one below the extrusion plate **24**.
[0172] This is then followed by what is illustrated in FIG. **8** E, here a second crosslinking solution CS is introduced into the reaction cavity from container **63**, for example an actual crosslinking solution acting on the protein contents of the paste. The calcium chloride solution is drained from the container during that step.
[0173] As illustrated in FIG. **8** F, now the complete reaction container volume **94** is filled with the crosslinking solution from container **63**, leading to a final crosslinking of the microfibrillar structure.
[0174] As illustrated in FIG. **8** G the crosslinking is then drained from the reactor by opening valve **30** and allowing the crosslinking solution to drain by way of line **87**, while at the same time by opening valves **71** and **94** and activating pump **69**, culturing medium M is pumped from media container **61** by way of lines **70**, **72**, **91** and **79** into the container from the top.
[0175] This leads to the situation as illustrated in FIG. **8** H, now the volume **94** of the reactor is filled with growth medium leading to growth of the cells in or on the fibrillary structures. If the paste does not already contain cells, during this step or before media can be supplied with cells, and corresponding seeding of the microfibrillar structure can take place.
[0176] This is then followed as illustrated in FIG. **8** I by the actual cultivation process, so the reactor is kept at the appropriate temperature and cultivation conditions to lead to corresponding growth of muscle cells in an/or on the microfibrils. Optionally, the attachment plate **22** can move up and down in this phase to create a cyclic mechanical stimulation of the fibres. After this process is finished, the media can be allowed to flow out of the container and the microfibrillar structure can be taken out and can be further processed to lead to a cultivated meat product.
[0177] Experiments E1 and E2 describe experimental evidence for the generation of fiber bundles. In both examples, the same method and the same extrusion prototype were used but altering the nozzle density of the extrusion plate or the geometry of the attachment plate.
Equipment
[0178] Syringe pump (Harvard Apparatus), generic peristaltic pumps, disposable syringes (Omnifix®), small fiber-extrusion prototype (manufactured by JAG Jakob AG, consisting of a bottom part containing the inlets for the hydrogel/paste, the extrusion chamber, and exchangeable extrusion plates, and a cylindrical glass vessel (20 cm{circumflex over ( )}2×10 cm)), perforated attachment plates (JAG Jakob AG), 3D printed attachment grid (polyinylidene fluoride (PVDF), MIRAI Foods AG).
Reagents, Chemicals, and Solutions
[0179] Bovine acid bone gelatine (Gelita®), Sodium Alginate (Kimica), micro-structuring agent (MSA) (MIRAI Foods AG), Calcium Chloride (Sigma Aldrich, C1016), MilliQ water.
Experiment 1 (E.1.)—Fiber Bundle Extrusion with Different Attachment Plate Geometries
SUMMARY
[0180] In E.1., fiber bundles were extruded through extrusion plates with a total area of 15 cm{circumflex over ( )}2, containing 330 circular perforations with a diameter of 0.7 mm. Two different attachment plates were tested: [0181] 1. Attachment plate with 330 circular perforations matching the perforations of the extrusion plate [0182] 2. 3D printed attachment grid with a mesh size of 1 mm{circumflex over ( )}2
Methods
Step 1: Solution Preparation:
[0183] Saline crosslinking solution: [0184] 100 mM calcium chloride was dissolved in milliQ water. [0185] The pH was adjusted to 7.2. [0186] Solution was stored at RT. [0187] Hydrogel solution: [0188] 40 mg/ml gelatine and 25 mg/mL sodium alginate were completely dissolved in milliQ water under vigorous stirring at 58° C. [0189] Hydrogel solution was let cool down to 37°

C. [0190] 100 mg/mL MSA was added to the hydrogel and homogenously mixed by vortexing.
Step 2: Fiber Extrusion:
[0191] 1) The prototype was assembled according to the manufacturer's instructions. The assembled prototype had a small reactor volume of approximately 200 cm{circumflex over ( )}2. A tube was connected to the bottom inlet with a luer lock. [0192] 1. The attachment plate was attached to a metal rod through a magnet and placed on top of the extrusion plate, so that the perforations of the extrusion and attachment plate were overlapping. [0193] 2. A 3D printed attachment grid was mounted onto a threaded rod and placed on top of the extrusion plate. [0194] 2) 30 mL of the hydrogel was loaded into a disposable plastic syringe and placed onto the syringe pump. [0195] 3) The hydrogel was extruded with a speed of 5 mL/min until a thin layer was formed on top of the attachment grid. [0196] 4) The extrusion of the hydrogel was interrupted. 200 mL of crosslinking solution was poured into the small reactor vessel. [0197] 5) The syringe pump was re-started. [0198] 6) The attachment plate/grid was slowly pulled upwards with a steady motion holding the rod until the end of the reactor vessel. [0199] 7) The extruded fiber bundle was transferred into a beaker containing crosslinking solution.
Results and Conclusion
[0200] Independent of the attachment plate used, perforated or grid, fibers can be efficiently attached to the attachment plate and extruded throughout the full length of the reactor vessel (FIGS. **9**A-**9**C). The hydrogel volume that is lost in the attachment process, is slightly higher using the grid compared to the perforated attachment plate.
Experiment 2 (E.2.)—Fiber Bundle Extrusion with Different Fiber Densities Summary
[0201] In E.2., fiber bundles were extruded through extrusion plates with a total area of 15 cm{circumflex over ( )}2, containing circular perforations with a diameter of 0.7 mm. The extrusion of fiber bundles with variable fiber density was assessed using a density of 11%, 22%, and 45% perforated area respective to the total extrusion area (see FIG. **10**).
Methods
Step 1: Solution Preparation:
[0202] Saline crosslinking solution: [0203] 100 mM calcium chloride was dissolved in milliQ water. [0204] The pH was adjusted to 7.2. [0205] The solution was stored at RT. [0206] Hydrogel solution: [0207] 40 mg/mL gelatine and 25 mg/mL sodium alginate were completely dissolved in milliQ water under vigorous stirring at 58° C. [0208] Hydrogel solution was let cool down to 37° C. [0209] 100 mg/mL MSA was added to the hydrogel and homogenously mixed by vortexing.
Step 2: Fiber Extrusion:
[0210] 1) The prototype was assembled according to the manufacturers instructions. The assembled prototype provided a small reactor with a volume of approximately 200 cm{circumflex over ( )}2. A tube was connected to the bottom inlet with a luer lock. A 3D printed attachment grid was mounted onto a threaded rod and placed on top of the extrusion plate. [0211] 2) 30 mL of the hydrogel was loaded into a disposable plastic syringe and placed onto the syringe pump. [0212] 3) The hydrogel was extruded with a speed of 5 mL/min until a thin layer was formed on top of the attachment grid. [0213] 4) The extrusion of the hydrogel was interrupted. 200 mL of crosslinking solution was poured into the small reactor vessel. [0214] 5) The syringe pump was started again with an extrusion speed of: [0215] a. 5 mL/min for the extrusion plate with 11% fiber density [0216] b. 10 mL/min for the extrusion plate with 22% fiber density [0217] c. 20 mL/min for the extrusion plate with 45% fiber density [0218] 6) The attachment grid was slowly pulled upwards with a steady motion holding the threaded rod until the end of the reactor. [0219] 7) The extruded fiber bundle was transferred into a beaker containing crosslinking solution.
Results and Conclusion
[0220] Homogenous fiber bundles could be extruded independent of the fiber density with a length of approximately 10 cm. All fibers were crosslinked efficiently, even with the highest fiber density. With increasing fiber density, however, it is required to replace the crosslinking solution after the

fiber extrusion to fully crosslink the hydrogel. This demonstrates that the method of fiber extrusion can be easily upscaled by increasing the fiber density.

Overview of Experiments E3, E4, and E5

[0221] Experiments E3, E4, and E5 describe experimental evidence for the generation and cultivation of fiber bundles. In all three examples, the same fiber extrusion method and the same 9 L differentiation bioreactor were used.

Equipment

[0222] Syringe pump (Harvard Apparatus), generic peristaltic pumps, disposable syringes (Omnifix®), 9 L differentiation bioreactor (manufactured by JAG Jakob AG according to FIGS. **1**A-**1**C and FIG. **5**, with exchangeable extrusion and attachment plates), 2 L bench-top bioreactor as medium reservoir.

Reagents, Chemicals, and Solutions

[0223] Bovine acid bone gelatine (Gelita®), Sodium Alginate (Kimica), micro-structuring agent (MSA) (MIRAI Foods AG), Calcium Chloride (Sigma Aldrich, C1016), MilliQ water, MIRAI Muscle Growth Medium (MIRAI Foods AG), Microbial Transglutaminase concentrate (mTGase) —2000 U/g (BDF Ingredients), Chinese hamster ovary (CHO) cells (provided by Zurich University of Applied Sciences (ZHAW)), MIRAI Muscle Cells (MIRAI Foods AG), Calcein-AM (C3100MP, Fisher Scientific).

Experiment 3 (E.3.)—Fiber Bundle Extrusion in 9 L Differentiation Bioreactor with Variable Cell Density

SUMMARY

[0224] In E.3., fiber bundles are extruded encapsulating 20-60×10{circumflex over ( )}6 cells per mL of hydrogel solution. Cell distribution along the fiber length is observed underneath the microscope.

Methods

Step 1: Solution Preparation:

[0225] Saline crosslinking solution: [0226] 100 mM calcium chloride was dissolved in milliQ water. [0227] The pH was adjusted to 7.2. [0228] The solution was sterile filtered and stored at RT. [0229] Hydrogel solution: [0230] 80 mg/mL gelatine was completely dissolved in milliQ water under vigorous stirring at 58° C., then sterile filtered. [0231] 50 mg/mL sodium alginate was completely dissolved in milliQ water under vigorous stirring at 58° C., then autoclaved at 110° C. for 30 minutes. [0232] The gelatine and alginate solutions were mixed 1:1 under sterile conditions using magnetic stirring. [0233] Hydrogel solution was let cool down to 37° C. [0234] 100 mg/mL MSA was sterilized using UV irradiation (Spectronics Operation, XL-1000 UV crosslinker) and added to the hydrogel solution.

Step 2: Cell Encapsulation.

[0235] An aliquot of Chinese hamster ovary (CHO) cells in suspension was transferred into a 50 mL Falcon tube to have a cell number of: [0236] 1) 400 million cells [0237] 2) 1.2 billion cells and centrifuged at 350 g for 10 minutes. [0238] the supernatant was removed and the cells were resuspended in 20 mL of hydrogel solution, resulting in a cell density of: [0239] 1) 20 million cells per mL [0240] 2) 60 million cells per mL

Step 2: Fiber Extrusion:

[0241] 1) The bioreactor was assembled according to the manufacturer's instructions and autoclaved at 120° C. for 20 minutes. The attachment plate was attached to a metal rod through a magnet and placed on top of the extrusion plate, so that the perforations of the extrusion and attachment plate were overlapping. [0242] 2) The following steps were conducted under sterile conditions under laminar flow. [0243] 3) 30 mL of the hydrogel was loaded into a disposable plastic syringe and placed onto the syringe pump. [0244] 4) The hydrogel was extruded with a speed of 5 mL/min until a thin layer was formed on top of the attachment grid. [0245] 5) The extrusion of the hydrogel was interrupted. 200 ml of crosslinking solution was poured into the

small reactor vessel. [0246] 6) The syringe pump was re-started. [0247] 7) The attachment plate/grid was slowly pulled upwards with a steady motion holding the rod until the end of the reactor vessel. [0248] 8) The extruded fiber bundles were transferred into a petri-dish containing crosslinking solution, and imaged using light microscopy.

Results and Conclusion

[0249] The extruded fibers can be seen on FIGS. **11**A-**11**B with 20 million per mL (FIG. **11**A), and 60 million per mL (FIG. **11**B). Cell distribution is homogeneous along the fiber length. This experiment demonstrates that fiber bundles can be efficiently extruded up to 60 million cells per mL.

Experiment 4 (E.4.)—Muscle Fiber Cultivation in 9 L Differentiation Bioreactor

SUMMARY

[0250] Using the 9 L differentiation bioreactor with a reactor volume of 9 L (FIGS. **2**A-**2**C), muscle fibers were extruded and maintained throughout 5 days in cell culture medium. The aim of the experiment was to demonstrate that muscle fibers can be extruded, crosslinked, and cultivated under sterile conditions within a single muscle differentiation bioreactor.

Methods

Step 1: Preparation of Bioreactor:

[0251] 1) The 9 L differentiation bioreactor was assembled according to the manufacturer's instructions and sterilized by autoclaving. The extrusion and attachment plate had a matching pattern of perforations consisting of 390 circular holes with a diameter of 0.7 mm. The reactor was fully assembled with the attachment plate positioned at the bottom end. An insert (FIG. **2**A, #17) as a volume blocker was integrated into the reactor vessel to reduce the volume to 4 liters. All in- and outlets for the hydrogel (FIG. **1**C, #25), and media perfusion (FIG. **1**C, #5 and #30), were equipped with weldable tubings. [0252] 2) For the medium reservoir, a bench-top bioreactor was autoclaved and equipped with weldable tubings.

Step 2: Solution Preparation:

[0253] Saline crosslinking solution: [0254] 100 mM calcium chloride was dissolved in milliQ water. [0255] The pH was adjusted to 7.2. [0256] The solution was sterile filtered and connected through a peristaltic pump to the bottom inlet of the differentiation bioreactor (FIG. **1**C, #30). [0257] Enzymatic crosslinking solution: [0258] 6.25 mg/ml mTGase powder was dissolved in MIRAI Muscle Growth Medium, then sterile filtered and pre-warmed to 37° C. [0259] Hydrogel solution: [0260] 160 mg/mL gelatine was completely dissolved in milliQ water under vigorous stirring at 58° C., then sterile filtered. [0261] 100 mg/mL sodium alginate was completely dissolved in milliQ water under vigorous stirring at 58° C., then autoclaved at 110° C. for 30 minutes. [0262] the gelatine and alginate solutions were mixed 1:1 under sterile conditions using magnetic stirring. [0263] Hydrogel solution was let cool down to 37° C. [0264] 15 mL of the hydrogel solution was transferred into a 50 mL Falcon tube under sterile conditions. [0265] 3 g of MSA was sterilized using UV irradiation (Spectronics Operation, XL-1000 UV crosslinker) and added to the hydrogel solution, and mixed by vortexing. [0266] The hydrogel/MSA mix was kept at 37° C.

Step 3: Cell Encapsulation.

[0267] 200 million MIRAI Muscle Cells were aliquoted into a 50 mL Falcon tube and centrifuged at 350 g for 10 minutes. [0268] The supernatant was removed and the cells were resuspended in 15 mL MIRAI Muscle Growth Medium. [0269] The resuspended cells were added to the hydrogel/MSA, and mixed by vortexing until a homogenous paste was visible.

Step 4: Fiber Extrusion:

[0270] 1) 30 mL of hydrogel/cell mix were equally loaded into two disposable syringes under sterile conditions and connected to the differentiation bioreactor (FIGS. **1**A-**1**C, #35), and mounted onto a syringe pump. [0271] 2) The hydrogel/cell mix was extruded until a thin layer was formed on top of the attachment grid, then interrupted. [0272] 3) The saline crosslinking solution was

pumped into the bottom of the reactor vessel. [0273] 4) Once the hydrogel/cell layer was fully covered by saline crosslinking solution, the syringe pump was re-started and the attachment plate was lifted up 10 cm with a speed of 2 mm/s. The syringe pump was stopped at 18 cm.

Step 4: Fiber Crosslinking

[0274] 1) The fibers were crosslinked for 10 minutes in saline crosslinking solution. The crosslinking solution was drained from the bottom of the bioreactor, and replaced from the top inlet by the enzymatic crosslinking solution. [0275] 2) The fibers were enzymatically crosslinked for 1.5 h.

Step 5: Muscle Fiber Cultivation

[0276] 1) After enzymatic crosslinking, the crosslinking solution was drained from the bottom of the bioreactor, and replaced from the top inlet by fresh MIRAI Muscle Growth Medium. [0277] 2) 9 liters of MIRAI Muscle Growth Medium (3 L in medium reservoid, 4 L in differentiation bioreactor) were perfused through the reactor for 5 days. [0278] 3) After 4 days in culture, the extruded fibers were stretched 10% by moving up the attachment plate by 10 mm.

Step 6: Fiber Harvesting

[0279] 1) On day 5, the medium was fully drained and the fibers were harvested. [0280] 2) Cell viability, cell spreading, and cell distribution was assessed by a Calcein-AM imaging.

Results and Conclusion

[0281] 30 mL of muscle fibers encapsulating approximately 6 million cells per mL could be successfully extruded for 10 cm and maintained in culture for 5 days (FIG. **12**A). Due to some clogging of the extrusion plate, the extruded fibers showed some defects and bulges, but could nevertheless, be clamped in between the extrusion and attachment plate for 5 days without rupture, and withstand 10% of stretching. The exchange of two crosslinking solutions and the perfusion of media within a single bioreactor could be managed without damaging the fibers, demonstrating the possibility of maintaining and differentiating muscle fibers within a single bioreactor. As can be seen on FIG. **12**B, the muscle fibers have a microfibrillar structure. Cell viability was good and the cells aligned along the fiber direction (FIG. **12**B).

Experiment 5 (E.5.)—High Density, Full Length Muscle Fiber Extrusion in 9 L Differentiation Bioreactor

SUMMARY

[0282] Using the 9 L differentiation bioreactor (FIG. **5**), up to 6′700 cell-free fibers with a length of 40 cm were successfully extruded and fixed in place.

Methods

Step 1: Preparation of Bioreactor:

[0283] The 9 liter differentiation bioreactor was assembled according to the manufacturer's instructions. The extrusion and attachment plate had a matching pattern of perforations consisting of 6′700 circular holes with a diameter of 0.7 mm (FIGS. **6**A-**6**C). The reactor was fully assembled with the attachment plate positioned at the bottom end. A peristaltic pump was connected to inlets for the saline crosslinking (FIGS. **1**A-**1**C, #30) and the hydrogel injection (FIGS. **1**A-**1**C, #25).

Step 2: Solution Preparation:

[0284] Saline crosslinking solution: [0285] 100 mM calcium chloride was dissolved in milliQ water. [0286] The pH was adjusted to 7.2. [0287] The solution was connected through a peristaltic pump to the bottom inlet of the differentiation bioreactor (FIGS. **1**A-**1**C, #30). [0288] Hydrogel solution: [0289] 40 mg/mL gelatine and 25 mg/mL sodium alginate was completely dissolved in milliQ water under vigorous stirring at 58° C. [0290] Hydrogel solution was let cool down to 37° C. [0291] 60 g of MSA was added to 0.6 liter of hydrogel solution and mixed by magnetic stirring until homogenously distributed. [0292] The hydrogel/MSA mix was kept at 37° C.

Step 4: Fiber Extrusion:

[0293] The hydrogel solution was connected to the hydrogel injection points (FIGS. **1**A-**1**C, #30) through a peristaltic pump. [0294] The hydrogel mix was pumped in until a thin layer was formed

on top of the attachment grid, then interrupted. [0295] The saline crosslinking solution was pumped into the bottom of the reactor vessel. [0296] Once the hydrogel layer was fully covered by saline crosslinking solution, the syringe pump was re-started and the attachment plate was lifted up 40 cm with a speed of 2 mm/s. The injection of the hydrogel was stopped at 38 cm. [0297] The fibers were crosslinked for 10 minutes in saline crosslinking solution. Then the solution was drained and the fibers were collected.

Results and Conclusion

[0298] This experiment demonstrated the ability to extrude up to 6′700 muscle fibers with a length of 40 cm in less than four minutes time. The fibers were successfully fixed between the extrusion and attachment plate (FIG. **13**A). The fiber bundle could be successfully harvested, resulting in a thick bundle with a weight of approximately 300 g (FIG. **13**C).

TABLE-US-00001 LIST OF REFERENCE SIGNS 1 reactor 2 circumferential wall of 1 3 reinforcement structure 4 top cover 5 first inlet/outlet in 4 6 second inlet/outlet in 4 7 upper top closure bracket 8 lower top closure bracket 9 hinge of 7 10 hinge of 8 11 upper closure extension 12 closure mechanism of 7 13 closure mechanism of 8 14 sealing between 4 and 11 15 upper frame portion 16 sealing between 11 and 15 17 insert 18 dead volume in 17 19 vertical pipe through 17 20 passage around 17 21 protruding portion of 19 22 attachment plate 23 mounting structure for 22 24 extrusion plate 25 bottom inlet 26 Lower bottom closure bracket 27 upper bottom closure bracket 28 closure mechanism of 26 29 closure mechanism of 27 30 bottom inlet/outlet 31 sealing around 23 32 central opening in 22 33 surface area of extrusion plate without nozzle openings 34 bottom plate 35 side inlet in 25 36 sealing around 24 37 bottom part 38 upper bottom part 39 sealing 40 lateral opening in 22 41 surface area of extrusion plate without nozzle openings 42 perforations in 22 43 lower frame portion 44 perforations in 24 45 shifting bracket 46 rail 47 motor 48 bottom mounting structure 49 mounting structure for 45 50 housing 51 carrier structure for 46 52 leg 53 shifting ring 54 magnet of 53 55 magnet of 23 56 glass housing 57 space between 22 and 24 58 space above 22 59 circumferential rim 60 attachment rib 61 media container 62 container for calcium chloride 63 container for cross-linking solution 64 reservoir and pump for cell paste 65 pipe from 64 to 1 66 outlet from bottom area 67 valve in 65 68 valve in 66 69 pump 70 line from media container 71 valve and 70 72 collecting line 73 valve and 72 74 line from container for calcium chloride 75 valve and 74 76 line from container for cross- linking solution 77 valve in 76 78 collection 79 upper collecting line 80 valve in 79 81 recirculation line to media container 82 valve in 81 83 recirculation line to container for calcium chloride 84 valve in 83 85 recirculation line to container for cross-linking solution 86 valve in 85 87 outlet line from 72 88 bottom position of 22 89 middle position of 22 90 uppermost position of 22 91 cross-line 92 space below 24 93 supply/control elements 94 valve 95 reactor volume M media CC calcium chloride CS further cross linking solution

Claims

1. A method for the manufacturing of an elongated structure for the fabrication of cultivated meat, wherein (a) a paste is extruded through an extrusion plate with at least one nozzle opening and at least partly through an adjacent attachment plate, (b) paste located in said attachment plate and/or in said space is at least partly hardened, (c) said paste is continued to be extruded through said extrusion plate while increasing the distance between said extrusion plate and said attachment plate, under formation of said elongated structure between said plates.

2. The method according to claim 1, wherein the extrusion takes place at an ejection speed under the generation of a speed gradient downstream of the respective nozzle opening in the extrusion plate by way of a distancing speed between extrusion plate and said attachment plate larger than the ejection speed to form said elongated fibrous structure.

3. The method according to to claim 1, wherein in at least one of step (b), step (c) and after step (c),

the paste in said space and/or in the interspace between the plates is immersed in a hardening bath.

4. The method according to claim 1, wherein said extrusion plate and/or said attachment plate comprises at least 20 nozzle openings, and/or the nozzle openings cover 5%-90% of the plate cross-section area, and/or the nozzle openings are provided in said extrusion plate and/or said attachment plate with a nozzle density of 1-500 nozzles/cm2, and/or the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, and/or wherein the attachment plate is a mesh, a porous plate, or a plate with nozzle openings.

5. The method according to to claim 1, wherein extrusion in steps (b) and (c) takes place into a reaction container, and subsequent to step (c), if needed followed by a step of further hardening of the extruded elongated structure, the reaction container is filled with culturing growth media and the elongated structures are used for growing meat cells seeded onto said structures and/or already contained in said paste.

6. The method according to to claim 1, wherein (a) said paste is extruded through said extrusion plate and an adjacent attachment plate into a space downstream of the attachment plate, (b) stopping or slowing down said extrusion and hardening paste located in said space by flooding said space downstream of the attachment plate with a hardening bath, (c) followed by continued extrusion of said paste through said extrusion plate while increasing the distance between said extrusion plate and said attachment plate, under formation of said elongated structure between said nozzle openings, (d) stopping said distancing and extrusion, and hardening the paste in the form of elongated structures, in the space between the two nozzle plates, by flooding said space downstream of the attachment plate with a hardening bath, (e) replacing what is in the space between the plates with culturing media and using the elongated structures, for growing meat cells seeded onto said elongated structures, and/or already contained in said paste.

7. The method according to to claim 1, wherein said paste is a paste for the fabrication of cultivated meat, comprising of consisting of the following components: (A) at least one polysaccharide that can form a solidified gel by the action of divalent or polyvalent cations, thermal gelling, light-induced addition reaction or light induced condensation reaction, or a combination thereof, in a concentration in the paste in the range of 0.01 g per L of component (D); (B) optionally one or more proteins, in a concentration in the paste in the range of 0-500 g per L of component (D), (C) cells selected from mammalian cells, fish cells, crustaceous cells or a combination thereof, in a concentration in the paste in the range of 0-300 billion cells per L of component (D); (D) water or a water-based culturing medium; (E) additives different from (A)-(D), selected from the group consisting of crosslinking kinetic modifier in a concentration in the paste in the range of 0-500 mM, flow modifier in a concentration in the paste in the range of 0-200 g per L of component (D), or a combination thereof, and/or wherein said hardening bath comprises Ca2+, Mg2+, Fe2+ and Fe3+ or a combination thereof in water, and/or wherein the hardening bath is buffered at a pH ranging from 6 to 8, and/or wherein either the hardening bath comprises a protein cross-linking agent or subsequent to step (b) the fibres are immersed in a protein cross-linking bath with such a cross-linking agent.

8. The method according to to claim 1, wherein during culturing the two nozzle plates are mechanically and/or electrically oscillated relative to each other.

9. The method according to to claim 1, wherein the extrusion in step (c) takes place with a drawing factor, defined as the ratio of the ejection speed to the distancing speed, of at least 1.1.

10. A reactor for carrying out the method according to claim 1, wherein it comprises a closable reactor container of elongated shape along a main axis with constant cross-section, an extrusion plate and at least one attachment plate movably mounted in said reactor container, elements for controlled supply of paste for extrusion thereof upstream of said extrusion plate and elements for controllably moving said extrusion and/or attachment plates along the main axis, as well as means for supplying at least one of culturing media, hardening media, cross-linking media to and from the

inside of the reactor container.
11. The reactor according to claim 10, wherein the extrusion plate is located at one end of said closable reactor container, and said attachment plate is located adjacent to the extrusion plate.
12. The reactor according to claim 10, wherein said extrusion plate and/or said attachment plate comprise a plurality of nozzle openings, and/or the nozzle openings cover 5%-90% of the plate cross-section area, and/or the nozzle openings are provided in said extrusion plate and/or said attachment plate with a nozzle density of 1-5000 nozzles/cm2, and/or the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 10 to 5000 μm, and/or wherein said first extrusion plate and/or said attachment plate further comprises at least one opening for controlled supply of liquids through the respective plate.
13. The reactor according to claim 10, wherein it comprises a control for controlling at least one of the extrusion speed of the paste, the distancing speed between the extrusion and attachment plates, the supply of and removal of at least one of culturing media, cross-linking media, hardening media.
14. The method according to claim 1 used for the manufacturing of a consumer cultured meat product, using the corresponding elongated structures, in combination with (i) fat and or oil-based components including cultured fat, vegetable fat or animal fat based components and its derivatives, and/or (ii) structuring agents including hydrocolloids, and/or cellulose and its derivatives, and/or proteins derived from plant, animal, recombinant technology, cell cultivation; and/or (iii) connective tissue components including animal derived connective tissue, cultured connective tissue.
15. A cultured meat obtained using a method according to claim 1.
16. The method according to claim 1, wherein in step (a) a paste is extruded through an extrusion plate with at least one nozzle opening and at least partly through an adjacent attachment plate into a space downstream of the attachment plate.
17. The method according to claim 1, wherein in at least one of step (b), step (c) and after step (c), the paste in said space and/or in the interspace between the plates is immersed in a hardening bath comprising divalent or polyvalent cations or an acidic bath or a bath with a temperature different from the gel paste, including a hardening bath comprising divalent cations.
18. The method according to claim 1, wherein said extrusion plate and/or said attachment plate comprises at least 100, or at least 200 or in the range of 250-1000 nozzle openings, and/or the nozzle openings cover between 5% and 95% of the plate cross-section area, or between 20% and 90% or 20-80%, or between 40% and 70%, and/or the nozzle openings are provided in said extrusion plate and/or said attachment plate with a nozzle density in the range of 5-400 nozzles/cm2, and/or the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 100-1500 μm, and/or wherein the attachment plate is a plate with nozzle openings aligned to nozzle openings of the nozzle plate.
19. The method according to claim 1, wherein (a) said paste is extruded through said extrusion plate and an adjacent attachment plate into a space downstream of the attachment plate, (b) stopping or slowing down said extrusion and hardening paste located in said space by flooding said space downstream of the attachment plate with a hardening bath comprising divalent or polyvalent cations or an acidic bath or a bath with a temperature different from the gel paste, including a bath of calcium chloride, wherein the paste forms a hardened gel volume that is immobilized in and/or on the attachment plate through geometric constraints, (c) followed by continued extrusion of said paste through said extrusion plate while increasing the distance between said extrusion plate and said attachment plate, with a relative distancing speed larger than the extrusion speed from said extrusion plate under formation of said elongated, fibrous structure between said nozzle openings, (d) stopping said distancing and extrusion, and hardening the paste in the form of elongated fibers, in the space between the two nozzle plates, by flooding said space downstream of the attachment plate with a hardening bath comprising divalent or polyvalent cations or an acidic bath or a protein

cross-linking bath, including a bath of calcium chloride (CC), and, hardening the paste in a space upstream of the extrusion plate, (e) replacing what is in the space between the plates with culturing media and using the elongated fibers, for growing meat cells seeded onto said fibers, and/or already contained in said paste.

20. The method according to claim 1, wherein said paste is a paste for the fabrication of cultivated meat, comprising of consisting of the following components: (A) at least one polysaccharide that can form a solidified gel by the action of divalent or polyvalent cations, thermal gelling, light-induced addition reaction or light induced condensation reaction, or a combination thereof, in a concentration in the paste in the range of 0.01-200 g per L of component (D); (B) optionally one or more proteins, in a concentration in the paste in the range of 0-500 g per L of component (D), wherein said protein(s) assembles with the polysaccharide of component (A) via supramolecular or covalent interaction or a combination thereof (C) cells selected from mammalian cells, fish cells, crustaceous cells or a combination thereof, in a concentration in the paste in the range of 0-300 billion cells per L of component (D); (D) water or a water-based culturing medium; (E) additives different from (A)-(D), selected from the group consisting of crosslinking kinetic modifier in a concentration in the paste in the range of 0-500 mM, flow modifier in a concentration in the paste in the range of 0-200 g per L of component (D), or a combination thereof, and/or wherein said hardening bath comprises Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+} or a combination thereof in water, at a concentration in the range of 1-500 mM, or between 20-100 mM, and/or wherein the hardening bath is buffered at a pH ranging from 6 to 8, by using HEPES buffer, including where the HEPES buffer is used in a concentration comprised between 1-100 mM, and/or wherein either the hardening bath comprises a protein cross-linking agent or subsequent to step (b) the fibres are immersed in a protein cross-linking bath with such a cross-linking agent, wherein the cross-linking agent is selected from the group consisting of transglutaminase, peroxidase, laccase, tyrosinase, lysyl oxidase, glutaraldehyde, genipin, citric acid, or a combination thereof, and wherein if transglutaminase is used, it is comprised in an amount of 1-2000 U per mL of crosslinking bath, and wherein cross-linking can be performed at a temperature in the range of 20-45° C., for a time span in the range of 10-120 min, or 30-90 min.

21. The method according to claim 1, wherein during culturing the two nozzle plates are mechanically and/or electrically oscillated relative to each other for stimulating and influencing the growth process in the anisotropic microfibrillar structures.

22. The method according to claim 1, wherein the extrusion in step (c) takes place with a drawing factor, defined as the ratio of the ejection speed to the distancing speed, of at least 1.5, or at least 2.

23. The reactor according to claim 10, wherein it comprises said closable reactor container of elongated shape along a main axis with constant cross-section, of cylindrical shape, a stationary extrusion plate and said at least one attachment plate movably mounted in said reactor container, elements for controlled supply of paste for extrusion thereof upstream of said extrusion plate and elements for controllably moving said extrusion and/or attachment plates along the main axis, as well as means for supplying at least one of culturing media, hardening media, cross-linking media to and from the inside of the reactor container also to a space upstream of said extrusion plate.

24. The reactor according to claim 10, wherein the extrusion plate is located at one end of said closable reactor container, and said attachment plate is located adjacent to the extrusion plate, and wherein the attachment plate can be moved by said elements in a contactless manner, by magnetic attraction, wherein to this end the attachment plate is mounted in and/or on a mounting structure with at least one magnet and outside of the reactor container there is a movable magnetic element, including in the form of a ring with at least one magnet, which movable magnetic element can be moved automatically, including by motor.

25. The reactor according to claim 10, wherein said extrusion plate and/or said attachment plate comprise at least 20, or at least 100, or at least 200 or in the range of 250-1000 nozzle openings, and/or the nozzle openings cover between 5% and 95% of the plate cross-section area, or between

20% and 90% or 20-80%, or between 40% and 70%, and/or the nozzle openings are provided in said extrusion plate and/or said attachment plate with a nozzle density in the range of 5-400 nozzles/cm2, and/or the nozzle openings have a diameter or maximum extension in the lateral direction in case of non-circular openings in the range of between 100-1500 μm.

26. The method according to claim 14 for the manufacturing of a consumer cultured meat product, using the corresponding elongated structures, after cultivation in the form of bundles, in combination with (i) fat and or oil-based components including cultured fat, vegetable fat or animal fat based components and its derivatives, in combination with separately cultured fat cells and/or separately cultured fat cell aggregates, and/or (ii) structuring agents including hydrocolloids, and/or cellulose and its derivatives, and/or proteins derived from plant, animal, recombinant technology, cell cultivation; and/or (iii) connective tissue components including animal derived connective tissue, cultured connective tissue, in combination with cultured fibroblast and/or chondrocytes and/or separately cultured fibroblast and/or chondrocytes aggregates, to form fibre bundles compact bundle, like meat piece.