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RECENT ADVANCES IN *IN-VITRO* MEAT PRODUCTION – A REVIEW

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Abstract

***In-vitro* meat production has entered into the phase of pilot-commercial scale production from the conceptual-laboratory phase. The main challenge for *in-vitro* meat production on a commercial scale is the very high cost of its production, mainly due to the cost of cell culture media, growth regulators, and the requirement of highly skilled manpower. The development of serum-free and animal-free culture media with plant, microbial, and fungi-derived compounds through recombinant technology and media recycling is crucial for scaling up *in-vitro* meat production and reducing the price of the end products. The proper design of bioreactors specific to *in-vitro* meat production, their automation, utilization of natural and edible scaffolds, and microcarriers made up of edible materials are the present focus of researchers. The co-culturing by proliferating various cells such as adipocytes, chondrocytes, fibroblasts, and endothelial cells are applied for imparting textural and organoleptic attributes to developed products similar to conventional meat. The industrial process to produce *in-vitro* meat needs a clear synergy between the biological, chemical, technical, and industrial fields because at the moment the main research focus is on the development and improvement of cell lines available to set up cell culture and culture media, bioreactors, cell lines, scaffolding, and biofabrication. The research on *in-vitro* meat is limited by the fact that from the industry the protocols are not properly divulgated.**

Key words: stem cells, culture medium, scaffolds, microcarriers, bioreactors, 3D bioprinting

Conventional meat production is considered an inefficient process of converting vegetable proteins into animal proteins by rearing and slaughtering meat animals. With the ever-increasing human population crossing the 8 billion mark recently (on 15 November 2022), it becomes quite imperative to ensure a proper meat supply in the future in a sustainable manner with the already dwindling natural resources (Kumar et al., 2022 a, 2021, 2019, 2017). To fulfill the future demand for meat for the burgeoning population, the meat production system needs a major transformation and technological breakthrough as incremental gains by improving the available technologies or scaling up the production of conventional meat production would not be sufficient (Chen et al., 2022). Thus, the present focus of the food industry is to ensure a sustainable supply of meat by harnessing the available resources and improving the technology for the production of meat alternatives such as plant-based meat analogs, cultured meat, insect meat, and hybrid mead products (Kumar et al., 2022 b, c; Sharma et al., 2022). Among these alternatives, cultured meat/*in-vitro* meat has attracted significant attention from researchers and policymakers worldwide. As per Reiss et al. (2021) and Lanzoni et al. (2022) estimations, *in-vitro* meat pro-

duction requires 89% less water, 99% less land, and 96% lower GHGs (greenhouse gases) as compared to intensive livestock farming.

In-vitro meat as the term indicates refers to producing edible biomass from stem cells by applying cell culturing technology (Stephens et al., 2018). This technology has the potential to supply high-quality animal proteins at low environmental impact with minimal use of animals, later restricted to the harvesting of stem cells through muscle biopsy (Post et al., 2020). The *in-vitro* meat production process is completed in the 4 basic steps viz., 1) harvesting stem cells from embryos or biopsy, 2) proliferation of these stem cells in the presence of growth factors, and nutrients followed by cell growth on scaffolds/microcarriers, 3) induced maturation of cells into myofibers, adipocytes, and other mature cells and 4) processing this edible tissue into meat products (Guan et al., 2021). To get desired texture and sensory attributes, co-culturing of multiple cells or 3D/4D bioprinting are applied (Zhang and Wang, 2019). There are three approaches for *in-vitro* myogenesis viz., muscle stem cells, differentiated from pluripotent stem cells and direct reprogramming applying transgenesis (Choi et al., 2019, 2020, 2021; Ding et al., 2018; Genovese et al., 2017). During the harvesting of efficient stem cells, animal species, age (age-induced loss of proliferation could be controlled by IGF 1 factor) and sex (effect of sex hormones, males have more satellite cells than females) affect the overall collection of the satellite cells (Choi et al., 2020). For harvesting stem cells from donor animals, tissue biopsy (by using a needle, quick process to collect up to 0.5 g sample, little stress to animals) and a small incision (more invasive, up to 15 g sample) could be used with a frequency of 4 samples collection in every session with one session in every three months to facilitate proper recovery to animals (Melzener et al., 2021). Figure 1 depicts various steps in the production of *in-vitro* meat.

Due to its minimal involvement of animals and potential sustainability, *in-vitro* meat is also known as "cell-based", "cultivated", "clean", "slaughter-free", "lab-grown", and "nano-pastured" meat (Pakseresht et al., 2022). The production techniques involve the collection and purification of cells and storage, transportation, standardization, quality control, and food processing technology are employed on it for the creation of meat products based on *in-vitro* meat (Post et al., 2020). The *in-vitro* meat is created under sterile circumstances, thus associated with the additional merits of being a safe, long-lasting meat, with lower food loss (Furuhashi et al., 2021), provided the whole production process follows high-quality GMP (good manufacturing practices), hygiene and sanitation as per the globally recognized standards such as European Union and FDA standards.

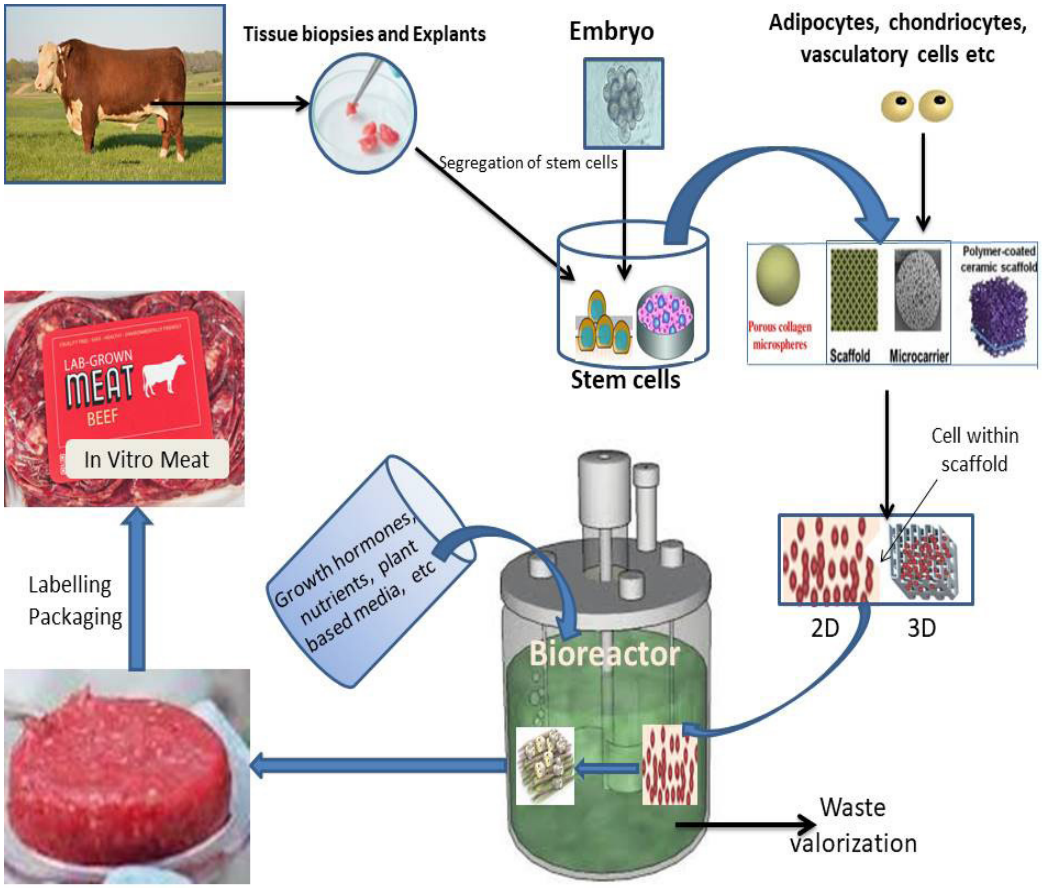

Figure 1. Production of *in-vitro* meat (adopted from Kumar et al., 2021 a)

The fundamental advantage of "*in-vitro* meat" is that it potentially allows for mass production from a small number of animal cells, derived either from farm animals or from cell lines that multiply in bioreactors. As a result, this method utilizes fewer/minimum animals to produce a large quantity of meat, hence termed "victimless meat", "slaughter-free meat" or "cruelty-free meat" (Chriki and Hocquette, 2020). However, while producing *in-vitro* meat, suitable technological innovations such as co-culturing should be applied for matching the nutritive value of *in-vitro* meat to conventional meat such as fatty acid profile, vitamin B_{12}, and trace minerals. It is also desired to produce *in-vitro* meat owing to organoleptic attributes such as color, appearance, flavor, and texture similar to conventional meat. Cultured meat production is thought to be safer for customers, healthier, and disease-free as compared to traditional meat production (Arshad et al., 2017). The other associated merits acclaimed to *in-vitro* meat are a production method complying with animal welfare due to slaughter-free production, better compositional control and nutritive quality of the developed product, and better food safety and lower public health risk (zoonoses) (Post et al., 2020). Based on the life cycle assessment (LCA), *in-vitro* meat had a 7–45% lower energy requirement, 78–96% lower greenhouse gas emission, and 82–96% lower land requirement as compared to conventional meat (Tuomisto and de Mattos, 2011). However, the gains in energy and greenhouse gas (GHG) emissions depend upon the specific production process and utilization of clean energy in the production of *in-vitro* meat (Lynch and Pierrehumbert, 2019). The increased CO_2 would likely to affect agricultural production and food quality with comparatively less effect on photosynthesis in C4 plants. In general, increased CO_2 results in increased growth, higher photosynthesis, lower stomatal conductance, decreased water consumption, and lowering tissue protein contents in plants (Taub, 2010; Gamage et al., 2018). In the context of cell culturing, CO_2 via bicarbonate buffer plays a crucial role in pH maintenance and cellular integrity; where CO_2 inhibition causes acidification of medium (Dubey et al., 2021). Further, the CO_2 concentration in the bioreactor also affects the utilization of glucose and production of lactate as up to 40–50% lower glucose utilization at 140 mmHg of partial CO_2 (DeZengotita et al., 1998). In addition to avian and mammalian cell culture for *in-vitro* meat production, fish cells are regarded as potentially better suitable for tissue culture due to their physiological properties such as tolerance to hypoxia, salt tolerance, buffering capacity, ability to grow at low temperatures (Rubio et al., 2019 a).

The production process of *in-vitro* meat has three basic requirements: cell, media, and scaffold (Allan et al., 2019). To realize the acclaimed merits of *in-vitro* meat, it should be popularized and produced at a mass scale. For example, for the production of 1 ton of *in-vitro* meat, 10^{14} cells need to be produced, which would need a bioreactor of 10,000 L with 10^7 cells/mL density in the bioreactor (Guan et al., 2021). Similarly, for producing 1 kg protein from muscle cells, approx. 2.9×10^{11} to 8×10^{12} cells are required (Rosser and Thomas-Vazquez, 2018). Thus, for a perceivable contribution to conventional meat production, huge investment and technological breakthrough are needed. As per a report by CE Delft, the availability and cost of cell culture media are the main driver affecting the mass-scale production of *in-vitro* meat (CE Delft, 2021), with growth factors and recombinant proteins accounting up to 99% of the cost of cell culture media.

Based on information gathered from industry leaders and published literature, Garrison et al. (2022) analyzed the cost of production of *in-vitro* meat production on a large scale with an annual production capacity of 540,000 kg in a scenario assuming several technological advancements/innovations leading to marked cost reduction in the growth hormones and culture media. The authors reported the cost of 1 kg of *in-vitro* meat to $63 in the proposed model with cell-culture medium, bioreactors, and labor charges accounting for $55/kg (more than 80%) of the overall cost of production. Thus, there is an urgent need to develop low-cost culture media, large-scale bioreactors, and automation to make this novel product affordable to the common consumer. Thus, to make *in-vitro* meat affordable and competitive with conventional meat, ready and cheap availability of these ingredients is crucial. According to one estimate, the reduction of the cost of cell culture media has the potential to decrease the cost of *in-vitro* meat to $15/kg (Chen et al., 2022). Other important factors are a trained and skilled workforce and the utilization of efficient and clean energy. This warrants the upgradation and scaling up of the supply chain and ensures a proper supply of inputs such as media, stem cells, etc.

This technology has undergone a significant overhauling in the last decade with technological advancements in producing and maintaining more stable and efficient cell culture lines, novel and sustainable media ingredients, microcarriers for improved delivery of nutrients ensuring improved cell growth, scaffolds materials and designs for structuring the edible tissue and bioreactors. In this context, the present review summarized the recent advancements in production and scaling up the *in-vitro* meat.

Current market scenario

The first breakthrough in the production of *in-vitro* meat has been in August 2013 with the launching of the *in-vitro* meat burger for sensory evaluation at a press conference in London, UK prepared by Mark Post from Maastricht University, The Netherlands. At present, there are more than 33 start-ups/companies working on better technological inventions to produce *in-vitro* meat on an industrial scale and at an affordable cost (Kumar et al., 2021 a). JUST, UPSIDE Foods, Aleph Farms, Mosa Meat, New Harvest, Memphis Meats, Modern Meadow, and Meatable are major start-ups related to *in-vitro* meat production. In December 2020, Eat Just, a US startup formerly known as Beyond Egg or Hampton Creek, launched chicken nuggets/bites prepared from tissue

grown in the bioreactor in Singapore at its 1880 restaurant chains after getting due approval from Singapore Food Agency. For this production, the company used a bioreactor of 1200 liters capacity using plant nutrients in fetal bovine serum as the growth medium. The price of this product was very competitive ($23) as compared to $325,000 for the product developed by Mark Post.

Similarly, Ivy Farm Technologies established a pilot plant having 600 liters bioreactor with the help of Oxford University. The company is expecting to get approval for the sale of cultured pork in 2023 with the production of 12,000 tons of pork in a year, equivalent to pork obtained after slaughtering 170,000 pigs (Mridul, 2021). The US has also given safety approval to the *in-vitro* meat as safe for consumption and approved Upside Foods (previously Memphis Meats) to harvest cells from chicken and culture these cells in the laboratory to produce meat products without slaughter. Thus, the current trend in the *in-vitro* meat industry is to develop products on a commercial scale and focus on ways to get a sustainable and inexpensive source of various ingredients. Like any other production technology to reach commercialization, *in-vitro* meat technology needs technological advancements, financial investment in research and development, financial support and incentives, a supportive regulatory framework, and conducive market dynamics.

Advances in *in-vitro* meat production

For scaling up the production of *in-vitro* meat, the present research is focused on the optimization of the harvesting of stem cells with good stemness and pluripotency, and sustainable supply of animal-free, serum-free culture media at an inexpensive price. The proper development of suitable scaffolds, microcarriers, and aggregates, designing large-scale bioreactors with better process control and automation, co-culturing and bioprinting, and protocol for producing suitable thicknesses of the edible tissues have important roles in scaling up the technology of *in-vitro* meat production.

Stem cells

For *in-vitro* meat production, it is desirable to have stem cells (ESC – embryonic stem cells obtained from the inner mass of blastocysts, adult stem cells mostly from bone marrow as well as skeletal muscle, and induced pluripotent stem cells (iPSC) by inducing gene responsible for pluripotency in somatic cells) with high Hayflick limit and have high proliferation and differentiation potential in scaffolds in a bioreactor or suspensions. Further, the cell lines should have high resistance to sheer pressure, tolerate and grow in a nutrient starvation state and withstand metabolic wastes. The mesenchymal stem cells (MSCs), fibro-adipogenic progenitors (FAPs), adipose-derived stem cells (ADSc) and resident muscle stem cells/muscle satellite cells/myosatellite cells are adult stem cells (Knežić et al., 2022). The cells should maintain their proliferative potential without any spontaneous differentiation. For the production of 10–100 kg of edible tissue, approx. 10^{12}–10^{13} cells are needed (Bellani et al., 2020). The self-renewing capacity varies with cell lines such as an adult stem cell has the capacity of 50–60 multiplications whereas iPSCs and ESCs have theoretical immortality with indefinite self-renewal capacity (Kadim et al., 2015).

Recently pluripotent bovine embryonic stem cells (pESC) having consistency in pluripotency marker gene expression, morphology attributes, karyotype, transcriptome, and epigenetic attributes were successfully produced from bovine blastocysts by using fibroblast growth factor (FGF) and an inhibitor of the canonical Wnt-signaling pathway (Bogliotti et al., 2018). Further, adult stem cells present in skeletal muscle such as myosatellite cells are the most widely applied *in-vitro* beef patties (Mosa Meat, 2021).

The pluripotent cell sources should be differentiated into suitable progenitor and mature cell types. The protocols related to the use of skeletal myocytes (Jiwlawat et al., 2018, 2017), myosatellite cells (Al Tanoury et al., 2020), adipocytes (Guénantin et al., 2017), and mesenchymal cells have been established for mouse and human cells. There is a need to design suitable protocols for pluripotent stem cells (PSC) for livestock species and their culture protocol for the production of *in-vitro* meat at a commercial scale (Reiss et al., 2021). This can be achieved by genetic engineering to control gene expression, optimize cell culturing conditions, and proper supply of nutrients and growth regulators. By direct reprogramming of gene expression and molecule exposure, these cells could be harvested from skeletal cells (Bar-Nur et al., 2018).

Alternatively, iPSCs are produced by inducing the various pluripotent transcription factors (Sox2, Lin 28, octamer-binding factors Oct3/4, Krüppel-like factors Klf4, and Nanog) (Takahashi and Yamanaka, 2016). The production of iPSC is relatively easier. These cells are extensively characterized but gene editing requirements, comparatively low yield, and inability to completely mimic the primary stem cells are some challenges in their production at the commercial scale (Specht et al., 2018). The first iPSC cells were derived from mouse embryonic fibroblast cells and are readily accessible and differentiated into myofiber under suitable conditions (Scarfone et al., 2020; Takahashi and Yamanaka, 2006), followed by bovine fetal fibroblast cells by using 6 bovine transcription factors. These alkaline phosphatase-positive cells express pluripotent cell markers such as Nanog, SOX2, and SSEA1 (Han et al., 2011). These iPSC cells were also produced by using embryonic fibroblasts in goats (Sandmaier et al., 2015) and pigs (Wu et al., 2009). These cells have self-renewing and immortality owing to the up-regulation of the enzyme telomerase and epigenetic alterations (Hochedlinger and Jaenisch, 2015).

Genetic editing for the production of iPSC involving viral vectors in *in-vitro* meat production is replaced by poly-promoter vectors and nanomaterials due to food safety and regulatory provisions. Various nanomaterials

such as carbon nanotubes, inorganic nanoparticles, nanoscale polymeric material, peptide-based nanoparticles, and inorganic nanoparticles are applied in gene delivery. The application of nanomaterials has advantages over viral vectors in terms of lower immune response, design flexibility, more specificity, scalability, cost, and lower cytotoxicity in biological systems (Riley and Vermerris, 2017). Alternatively, microfluidic cell stretching technology was explored for gene delivery. It utilizes the intrinsic inertial flow present in a T-junction microchannel and stretching of cells due to flows creates perforation on cell membranes resulting in the internalization of biological nanomaterials such as mRNA, siRNA, DNA (Hur et al., 2020).

Various small molecules are used for maintaining cell pluripotency and overcoming growth limitations such as ascorbic acid, GSK3-β inhibitor CHIR-99021, GSK3-β inhibitor, histone deacetylase inhibitors, and DNA methyltransferase inhibitors (Chen et al., 2020 a). By inhibiting the signaling pathway p38-MAPK, bovine satellite cells maintain the differentiating capacity, thereby enabling mass cell production (Ding et al., 2018). A selection of suitable cell lines with inherent properties of suspension growth, tolerating nutrient starvation, and immortalization *in-vitro* could be a viable option for cultured meat production such as obtained from insect tissue engineering and cell culture (Rubio et al., 2019 b).

For scaling up *in-vitro* meat production, a 30–50 doubling of stem cells is needed (Thorrez and Vandenburgh, 2019). However, this high rate of cell multiplication on an industrial scale could lead to the accumulation of genetic changes in the cells such as observed in long-term ESC and mesenchymal stem cells (MSC), consequently resulting in apoptosis or cell aging/senescence (Bonab et al., 2006). The telomerase activation or inactivation of tumor suppressor genes could be applied for extending cell proliferation, but it has the risk of developing unwanted cells in this process which mandate proper monitoring for genetic stability by using advanced technologies such as RNA-seq analysis, transcriptomics, single-cell genomics, and epigenomic sequencing (Jo et al., 2020 a).

At present livestock cell lines are not well developed yet as these cell lines have not been explored for research in biotherapy and genetic engineering, thus there is a need to understand the cell surface markers and other characteristics. This results in the lack of development of stem cell lines from livestock and their purification and maintenance which is required for establishing a high-quality cell line required for large-scale *in-vitro* meat production. Chen et al. (2022) proposed storing cryopreserve-validated cell lines in master cell banks to avoid the potential risk of genetic instability and inconsistencies in final products. These master cell banks store working cell banks and supply to culture in a bioreactor. There is a high possibility that some *in-vitro* manufacturers could use genetic engineering to optimize their production. There is a need for advanced and inexpensive sequencing technologies to characterize the function of specific genes in cell functioning and develop suitable cell lines for industrial *in-vitro* meat production. Figure 2 depicts various types of stem cells to be used in the *in-vitro* meat preparation.

Co-culturing

Co-culturing is adopted to impart the typical taste and texture of meat to the *in-vitro* meat by culturing adipocytes (fat), endothelium cells, and extracellular matrix-secreting cells. Aleph Farms co-cultured multiple cell types consisting of myosatellite cells, endothelial cells, osteoblasts, chondrocytes, and extracellular secreting cells in a 3D porous scaffold to produce a meat steak (Yablonka-Reuveni, 2011). For the commercialization of *in-vitro* meat, there is a need for the development of low-cost, rapid, and massive production of adipocyte stem cells. This warrants the development of suitable bioprocessing systems by considering the buoyancy of differentiating adipocytes and other challenges associated with the proliferation and differentiation of adipocytes (Allan et al., 2019; Fish et al., 2020).

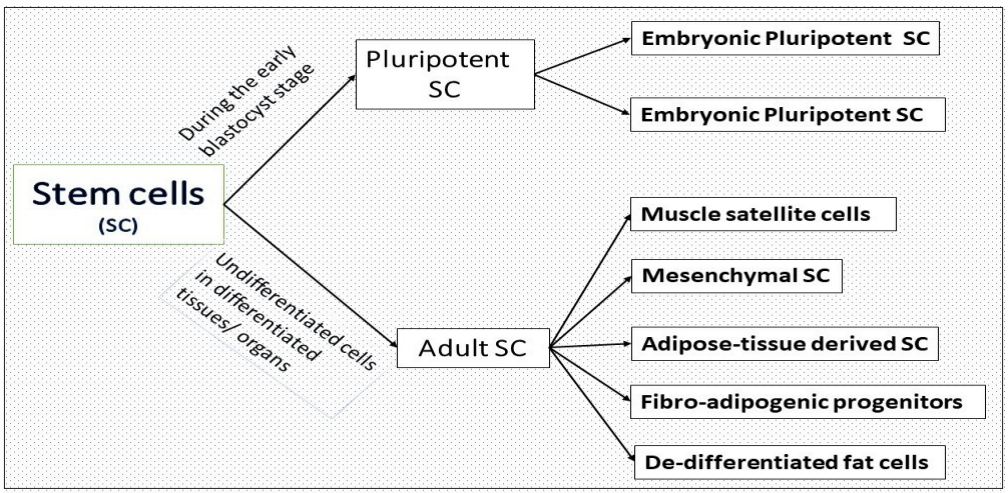

Figure 2. Various types of stem cells used in the *in-vitro* meat production

Various sources for adipose tissue could be applied in cell culturing such as dedifferentiated fat (DFAT) cells harvested from mature adipocytes, adipose-derived stem cells, preadipocytes, mesenchymal stem cells, fibroadipogenic progenitors, bovine expanded potential stem cells and engineered cell lines (Fish et al., 2020; Yuen Jr et al., 2022). DFAT cells are multipotent and could maintain proliferation and adipogenicity for longer durations, even after 50 passages (Peng et al., 2015). Immortalized chicken preadipocytes were developed by overexpression of telomerase reverse transcriptase (TERT) alone or in combination with telomerase RNA (Wang et al., 2017).

The smooth muscle cells when co-cultured in collagen during *in-vitro* meat production were observed to significantly increase ($P<0.05$) the collagen content while reducing the pressure loss (from 98.5% in control to 54% in the co-cultured meat) (Zheng et al., 2021). The hydrogels in the system were observed to be more tightly compact resulting in improving textural attributes such as hardness, chewiness, and springiness. This could be due to the secretion of extracellular matrix compounds from the smooth muscle cells (Zheng et al., 2021).

A peanut wire-drawing plant biomaterial was used to produce a 3D porous scaffold for culturing porcine smooth muscle cells under low serum conditions and higher extracellular matrix protein for cell differentiation and adhesion (Zheng et al., 2022). The authors observed improved quality of cultured meat by the extracellular protein and materials secreted by smooth muscle cells; resulting in the production of *in-vitro* meat with better quality attributes (Zheng et al., 2022).

Culture media

Culture media supply the essential nutrients and various growth factors required for the proliferation and differentiation of the cells. Its requirement depends upon the minimum working volume of bioreactors to maximize the achievable cell density and for nutrition to the proliferating cells. Currently, animal-sourced serum such as fetal bovine serum (derived from fetuses in pregnant cows during slaughter) is the most commonly used animal cell medium used. Its exorbitant cost, large variations in composition, batch variations, intricate production process, contamination risk, extraction of transferrin, albumin, and matrigel from animals to be added into this, and ethical issues are major issues in their application. Thus, it is very crucial for the development of a sustainable, serum-free, animal component-free, and low-cost cell culture medium to ensure food safety, accuracy, controllability, and sustainability is crucial in the large-scale production of *in-vitro* meat (Fang et al., 2017; Guan et al., 2021).

The culture media along with growth hormones are the major factors behind the high cost of *in-vitro* meat. It is imperative to reduce the cost of these compounds to get the price parity of *in-vitro* meat with conventional meat. The animal serum also has issues such as inconsistency in components, batch-to-batch variations, contamination (bacterial, viral, or prion), and ethical issues during its production. Thus, cellular agriculture technology is working on the development of a cost-effective serum-free media and growth factors for the large-scale production of *in-vitro* meat at a lower cost (O'Neill et al., 2021). In serum-free media, the majority of costs (88–96%) are due to cytokine and transferrin owing to the technical challenges in the production of these proteins on a large scale due to issues of purity, endotoxins, and other impurities (Choudhury et al., 2020). Alternatively, the production of these proteins on food grade could reduce the production cost significantly.

To date, commercially available serum-free additives and medium are B27™ (Thermo Scientific) and XerumFree™ (TNC Bio) additives, as well as the Essential 8™ (Life Technologies) and mTeSR1™ (Stemcell Technologies) medium. These media proved effective in supporting the *ex-vivo* growth and stemness maintenance of various cell types. The 'Clear Meat', an animal-free growth medium, was claimed to save up to 80% as compared to serum-based media (Mishra, 2022). Recently, two media are developed and evaluated by Stout et al. (2022) as inexpensive serum-free culture media for bovine satellite cells (BSC) (Beefy-9 and Beefy-9+). The serum-free media are available for biotherapeutic and medical research for experimental cell lines (such as Essential 8™, FBM™, TesR™) but these are very expensive and contain animal-originated ingredients such as growth factors. However, it should be noted that some serum-free culture media contain human origin components and thus may not be ethically used for the production of *in-vitro* meat such as human platelet lysates, though proliferate adipose tissue but are not suitable for human consumption (Chelladurai et al., 2021). Further serum-free media were observed to have a lower effect on the expansion of myoblasts, hence needing proper technological interventions (Kolkmann et al., 2020). Table 1 details the commonly available serum-free media for cell culture.

A chemically-defined medium STK2 having EGF (epidermal growth factor), cytokines, basic FGF, glucose, and other nutrients was observed to promote a higher growth proliferation rate of adipocyte-derived mesenchymal satellite cells than fetal bovine serum-containing media such as DMES (Lee et al., 2017 b). STKs (serine/threonine kinases) play an important role in different stages of antiviral defense. In eukaryotes, these control metabolism, regulate cell-cycle control, cell division, cell wall synthesis, and exit of dormancy (Pereira et al., 2011). These findings could be useful in the development of media for producing *in-vitro* meat on an industrial scale.

The plant-derived ingredients such as soy extract and yeast could be a promising source for the supply of nutrients in the culture media for cell growth. Plant protein hydrolysates (wheat and cotton peptones) have been explored as a potential substitute for animal protein (bovine serum albumin) in embryo culture media (George

et al., 2009). The culture media prepared with plant or fungi-derived ingredients have the advantage of marked cost reduction, food safety as easy integration into the final product, absence of religious and ethical issues, and improving flavor profile of the developed product. However, wide variations and impurities/toxic compounds in these ingredients and adaptation and growth of cell lines in these media need further improvement in the current technologies.

By genetically engineered plants, fungi, and microbes, recombinant growth factors could be produced at an affordable cost and could be sustainable alternatives to animal-originated growth factors. Various novel technologies are applied in plant biotechnology to produce transgenic plants such as genetic engineering, genome analysis, informatics, and omics (proteomics, lipidomics, and metabolomics). Transgenic plants developed by genetic engineering by modifying the expression of gene/genes of interest or inserting appropriate genes into metabolic pathways could produce valuable metabolites and recombinant proteins on a large scale for cell culturing (Matsuura et al., 2018; Kowalczyk et al., 2022). These plants have higher yield, disease resistance, drought and salt resistance, and higher production of valuable secondary metabolites. The transgenic tomato fruits were recorded to have a higher amount of squalene, phytosterols, tocopherols, and carotenoids (Liao et al., 2018) and higher beta-carotene in golden rice and golden banana (Paul et al., 2017; Zhang et al., 2016). The risk of transfer of antibiotic resistance to animals/food chain, loss of biodiversity, and risk of emergence of super-pest if used in an uncontrolled manner, higher cost of production, and lack of biosynthetic pathways of producing useful metabolites remain major roadblocks for this sector (Tabei and Muranaka, 2020; Wheeler et al., 2014). Further, the production of these valuable metabolites and recombinant proteins involves complex process and warrants an interdisciplinary approach (Tripathi and Shrivastava, 2019).

The utilization of growth factors produced by genetically engineered *E. coli* (expressed with neuregulin 1, FGF2, and TGFβ3) was observed to reduce the cost (3%) of commercial media (B8) along with growth and maintain pluripotency over 100 passages (Kuo et al., 2020). The protein hydrolysates from cowpea (*Vigna unguiculata*) were observed to stimulate insulin-associated cell signaling pathways (Akt phosphorylation) in the cell culture (Barnes et al., 2015). Thus, proper identification and screening of these biomolecules present in plant or fungi resources could be useful in designing and producing growth factors for *in-vitro* meat production.

Various plant protein hydrolysates were used for the cell culture such as whey protein isolates prepared by using alcalase and protamex reported to induce proliferation and differentiation of MC3T3-E1 osteoblasts (Jo et al., 2020 b). Similarly, yeast extracts were observed to induce cell growth and monoclonal antibody production in Chinese hamster ovary (CHO) cells (Hu et al., 2017). The application of algal extract was observed to substitute glucose and amino acids in basal cell media used for growing C2C12 mouse myoblasts (Okamoto et al., 2020).

Similarly, natural flavonoids such as luteolin were observed to improve the migration (migration index) and differentiation (fusion index) of porcine myoblasts with a comparatively weak effect on cell proliferation (Guan et al., 2022). The luteolin was reported to upregulate the expression of myogenin and MyHC by increasing the phosphorylation of PI3k/Akt/mTOR signaling pathway (Guan et al., 2022). The flavonoids (10 μM 3,2-dihydroxyflavone, 50 nM quercetin, and 7.5 nM icariin) were observed to induce the proliferation and differentiation of porcine muscle stem cells by upregulating the expression of paired box transcription factor 7, and myosin heavy chain (Guo et al., 2022).

Table 1. Serum-free culture media for the growth of different cells

Cell types	Composition	Features	Trade name	References
Bovine satellite cells	Recombinant human albumin addition in B8 media	Growth rate 39 h per population doubling time	Beefy-9	(Stout et al., 2022)
Bovine myoblasts	–	Good proliferation, need improvement in cell attachment and cell survivability	FBM	(Kolkmann et al., 2020)
	–		TesR™	
	DMEM/F12, TGF-β, insulin, minerals, PGF-2		Essential 8™	
Pig embryonic stem cells	MEM, glutamax, NEAA, antimicrobials, beta-mercaptoethanol	Able to form teratoma	Knockout DMEM	(Choi et al., 2019)
Pig myosatellite cells	Basal media, dexamethasone, EGF,	Expressed pluripotency marker and form teratoma into nude mice upon subcutaneous injection	SkGM-2	(Choi et al., 2020)
PSC and iPSC from various species	Amino acids, vitamins, insulin, minerals, transferrin, lipid-rich albumin		Knockout™ serum replacement	(Paul et al., 1999)

MEM – minimum essential medium, NEAA – nonessential amino acids, EGF – epidermal growth factor, DMEM – Dulbecco's modified eagle medium.

The cell culture media should promote cell adhesion and growth. The development of cell-specific culture media under various conditions would further improve the efficiency of the whole process (Burton et al., 2000). By considering the wide variations in cell types, media ingredients, and production process, further research is needed in this aspect. Several factors in culture media and extracellular environment affect overall cell proliferation and differentiation of cells. This signaling could be stimulated by electrical, mechanical, topographic flow, co-culturing, growth factors, and chemical factors (Ramani et al., 2021) and have an important role in the physiological establishment during cell culturing (Maleiner et al., 2018; Verbruggen et al., 2018).

Reuse/recycling of cell culture medium is an important aspect in the overall cost reduction and saving of the environment by reducing the environmental footprint of *in-vitro* meat production. There is a need to develop suitable media recycling technology to harness this. The recycling of culture media was proposed by applying size-exclusion dialysis filters or dialyzers that remove waste but recycle water and other non-metabolized media (Moritz et al., 2015). Media recycling technologies are in the development phase and there is a need to develop feasible, highly efficient recycling technologies that could be suitable for large-scale bioreactors to be used in future *in-vitro* meat production.

Scaffolds

The scaffold, a spongy, 3D porous network structure acting as an extracellular matrix for cell attachment and growth, is developed for providing an optimum microenvironment for the regeneration and growth of tissues in medical tissue engineering (Jiao et al., 2020; Handral et al., 2022). It constitutes a major portion of cultured meat. The porous network helps in supplying nutrients and gases and removal of metabolic wastes. After attaining the desired growth, cells are transferred to bioreactors to grow and differentiate into edible tissue. Scaffolds facilitate cell attachment and assemble to take three-dimensional structures resembling the natural structure of conventional meat.

The scaffolds should have a large surface area for cell attachment, cell compatibility, cell affinity, flexibility, optimum porosity, and physical strength, thereby allowing proper media perfusion and tissue maturation (Campuzano and Pelling, 2019; Zidarič et al., 2020). Low cost, food safety, scalability, digestibility, and sustainability are other desired attributes of a scaffold to be used in *in-vitro* meat production (Browe and Freeman, 2019). Further, to produce cultured meat with a 3D structure resembling conventional meat, the scaffold should support tissue maturation of a minimum of 1 cm thickness (Chen et al., 2022).

Scaffolds are produced from natural materials or synthetic materials and can be categorized as edible, non-edible, and non-edible but degradable (Bodiou et al., 2020). A range of natural biomaterials such as decellularized plant, cellulose, alginate, collagen, hyaluronic acid or fibrin, chitosan (derived from fungi, crustaceans, and yeast), and fungal mycelium is used to prepare scaffolds (Campuzano and Pelling, 2019). These scaffolds are easily degraded in the environment and are biocompatible. The scaffold derived from animal-origin biomaterials such as gelatine has been used in cultured meat production due to its higher functionality and better cell attachment. Various synthetic materials such as polyethylene, polycarbonate, polycaprolactone as well as natural polymers are used for producing scaffolds with desired attributes viz., degradation, mechanical strength, and biocompatibility (Langelaan et al., 2010).

Edible scaffolds of various sizes and dimensions are developed by using bacterial nanocellulose. These are having high porosity, biocompatibility, and large surface-to-volume ratio as desired for cell culturing of mammalian cells (BNC, n.d.). The plant proteins and polysaccharides derived from plants or microbes could be a potentially sustainable and low-cost material for producing scaffolds for *in-vitro* meat production (Campuzano and Pelling, 2019). Decellularized plant tissue materials impart vascular networks in scaffolds; that could be used for providing various structures in cultured tissue (Jones et al., 2021). The bovine satellite cells maintained 99% viability and 25% expression of myosin-heavy chains on decellularized spinach leaves-based scaffold after 14 days and performed similarly to gelatin-coated glass (Jones et al., 2021). An edible scaffold derived by extrusion of gelatin microfibers was reported to support cell attachment, proliferation, and maturation of cow and rabbit myosatellite cells (MacQueen et al., 2019).

Rolled scaffolds offer high-density adherent cell culture and lower the shear stress in large-scale bioreactors along with the quick and easier movement of gases and nutrients through their unique microstructure causing unidirectional laminar flow (YekrangSafakar et al., 2020). These are polymer films with spacers rolled into a cylinder by leaving a predetermined gap in each turn. The cells are anchored at the inner surface while media flow through the gaps; the whole design markedly improves the volumetric productivity (100 cm^2/mL) (YekrangSafakar et al., 2020).

It is desirable that scaffold should be derived from natural sustainable resources, be edible, nutritive and impart texture to the end product. Ben-Arye et al. (2020) developed a scaffold derived from texturized soy protein and myogenic-related growth factors for bovine satellite cell attachment and proliferation for culturing muscle tissue. Further, these scaffolds support co-culturing of bovine satellite cells, smooth muscle, and endothelial cells. The developed products were noted with flavor and organoleptic attributes similar to meat. Scaffolds derived from natural materials such as plant materials could have flavor compounds and thus impart flavor to the cultured tissue. The application of algal-based scaffolds could improve the flavor of cultured fish tissue. However, this flavor should be compatible with the cultured tissue as the

algal flavor may prove undesirable in cultured beef production (Grahl et al., 2018; Choudhury and Sen Sarkar, 2017).

A range of synthetic polymers and polyester (polyethylene and polyamide) compounds are also explored for the development of scaffolds after chemical modification, making them safe and degradable. The polylactic and polylactic-co-glycolic acids derived from these polymers are safe and effective polymers used for nano- and micro-particles (Elmowafy et al., 2019). Physical or chemical links by cross-linking in polymers are helpful to alter biological, mechanical, and degradation attributes of hydrogels (Oryan et al., 2018). However, there is a need for cost reduction, food safety issues, modifying the surface characters of these scaffolds for cell adhesion sites, and changes in solubility.

Scaffolds derived from non-animal origin are preferred due to the availability of a range of variations, and safety compliance with ethical/social concerns. Cellulose is a linear polysaccharide that is thought to be the most sustainable compound because it is a never-ending source of biopolymers from plant cell walls (Trache et al., 2017). Recombinant plants and microbes are used to produce collagen and fibrin with high compatibility such as transgenic tobacco plants (Willard et al., 2013). However, these technologies should be made more feasible and economically viable for the production of cheap and large-scale industrial production of *in-vitro* meat production.

Chitosan is derived from chitin through alkaline deacetylation, naturally present in the exoskeleton of crustaceans. It can be used as material for scaffold preparation used for tissue engineering due to good biocompatibility, and antimicrobial effects (Rodríguez-Vázquez et al., 2015). The conventional scaffolding with cells could produce a thin layer about 100–200 μm thick due to poor diffusion, and media perfusion (Warner, 2019). To increase the thickness of edible tissue, the cells can be mixed with scaffolding biomaterials or three-dimensional (3D) bioprinting can be applied (Zhang and Wang, 2019). Further, for co-culturing, scaffolds should support the differentiation of various cells and spatial heterogeneity in the developed product (Chen et al., 2022). To improve the differentiation, by nano-scale incorporation of additional materials within hydrogel scaffolds can also induce differentiation without exogenous growth factors.

Edible hydrolyzed collagen scaffolds were prepared by 3D printing for *in-vitro* meat production by using post-printing freeze-drying to impart proper shape and porosity to these scaffolds (Koranne et al., 2022). The hydrogel bio-ink: 4% GelMA (sodium alginate-gelatin and gelatin-methacrylate) –20% silk fibroin was observed to improve the performance of 3D bioprinting (powder-based 3D printing, fused deposition modeling, and stereolithography) and constructed good network (15 mm^2 size, 1000 μm porosity in 4-, 6-, and 8-layered structure) of porcine skeletal muscle satellite cells (Li et al., 2021). The 4- and 6-layered grid was observed to form a compact muscle fiber structure of multinucleated myotubes after 16 days of culture suggesting great potential of scaffold material (4% GelMA and 20% silk fibroin) hydrogel in *in-vitro* meat production. Fish gelatin powder consisting of edible gelatin microsphere and bonding activities was observed to improve the proliferation of myoblasts (C2C12; 10 passage) in a serum-reduced medium, thus leading to the development of meat-like cell sheets rapidly at low cost (Park et al., 2021).

Scaffold fabrication

While designing scaffolds, various processing technologies are used to impart desirable mechanical, biological, surface, and architectural properties, so as to facilitate cell attachment, multiplication, differentiation, spread, and maturation during *in-vitro* meat production (Seah et al., 2022).

Conventional porous scaffold fabrication technologies can be categorized as:

1. SCPL-solvent casting and porogen leaching: this method is traditionally used for film preparation. In this method, polymer solution in organic solvent with insoluble particulate/porogen cast on mold followed by solvent evaporation and immersion in an aqueous solution to leach particulates (Prasad et al., 2017). It is very rapid, inexpensive, maintains uniformity, and can attain desirable attributes (such as swelling behavior, reorientation of crystals on the surface, and surface heterogeneity) by types of solvents, the pore size of porogen, and processing conditions (Ghosal et al., 2018; Hulko et al., 2019). The limited porosity (up to 90%) and wide range of micropores (5–600 μm) with irregular dimensions and lethal properties of organic solvents are some disadvantages associated with this method (Page et al., 2013; Sola et al., 2019).

2. Physical crosslinking: scaffolds are prepared by using physical crosslinking in terms of ionic interactions, temperature interactions, and dehydrothermal (high temperature under vacuum) techniques (Nonoyama et al., 2020; Chen et al., 2020 b) by applying suitable crosslinking agents such as crosslinking hydrogels.

3. Chemical crosslinking: by applying suitable chemical/covalent crosslinking agents such as genipin, dopamine, glutaraldehyde (toxic nature hence restricted usages), and tannic acid able to form crosslinking under enzymatic and photothermal effect (Oryan et al., 2018; Xue et al., 2022). The scaffolds produced by chemical crosslinking are more stable with good mechanical properties.

4. Miscellaneous:

a) Phase separation: by separating the mixture into two phases viz., polymer-rich and polymer-poor phase by thermodynamically unstable conditions (Akbarzadeh et al., 2014).

b) Gas foaming: by generating gas inside the material

c) Sintering: by bonding ceramic fibers or particles with polymer (Pilliar et al., 2001).

Scaffold fabrication technologies: 3D printing, freeze-drying, electrospinning, self-assembly, micromolding and decellularization are some novel technologies used for scaffold fabrication.

1. Freeze-drying: it is used to produce porous scaffolds with various processing factors viz., temperature, polymer concentration, and pressure having an effect on the size and distribution of pores and mechanical properties of scaffolds (Lv and Feng, 2006).

2. Electrospinning: it is preferred for scaffolds owing to its fibrous structure with a fiber diameter ranging from 10 nm to microns (Li and Xia, 2004). The produced fiber-based material resembles the extracellular matrix (ECM). These fibrous scaffolds have the advantages of a high surface-to-volume ratio and can support high cell density, modulation of cell behavior, and pore size (Brown et al., 2018; Das et al., 2020; Chen et al., 2019). The nanofibers with desirable attributes could be achieved by proper control of setup parameters (such as electric field, diameter of needle, flow rate, position of needle and collector), and polymer attributes (concentration, viscosity, and surface tension) (Mo et al., 2019; Nemati et al., 2019; Rahmati et al., 2021).

3. Three-dimensional printing (3D printing): it creates channelled structures and permits perfusion and co-cultures for producing *in-vitro* meat closely mimicking conventional meat (Jandyal et al., 2021; Kumar et al., 2022, 2021 b). Thus cellular cultures are able to sustain on these for longer duration due to perfusion/vascularization such as up to 6 weeks observed by Kolesky et al. (2016). This technology requires precise control of parameters and a top-down approach is used for *in-vitro* meat. Based on the process used, this can be laser-assisted printing, stereolithography, ink-jet-based and extrusion-based 3D printing (Kumar et al., 2023).

4. Decellularization: by removal of nucleic acids and cells from the plant, fungal or animal tissue by using physical (impulse freezing, freeze-thawing), enzymatic or chemical methods, a decellularized material similar to extracellular matrix (ECM) with native 3D microenvironment could be obtained (Kumar et al., 2023). Under chemical methods detergents such as Triton-X-100 and sodium dodecyl sulfate are applied to dissolve cell membranes and disrupt structural arrangement (Mendibil et al., 2020). Under the enzymatic method, trypsin is commonly applied with EDTA. EDTA helps in breaking cellular integrity and trypsin acts on C-side bonds of lysine and arginine amino acids (Rieder et al., 2004). Figure 3 presents the process of preparation of decellularized scaffolds from plant materials.

5. Self-assembly: a tissue engineering technology mimicking the development of native tissue based on the differential adhesion hypothesis (Raghothaman et al., 2014) or differential interfacial tension (Lee et al., 2017 a; Kang et al., 2021).

6. Micromolding: provides small dimensions of molded structures with a high aspect ratio at very high precision for growing muscle fibers for *in-vitro* meat production (Orellana et al., 2020).

By applying suitable surface treatment such as improving wettability or incorporating charged functional groups, and biofunctionalization by adding bioactive molecules in the scaffold, a desirable cell attachment by cell-material interaction could be achieved (Tallawi et al., 2015). A gas plasma treatment on well plates was observed to improve the hydrophobicity and net surface charge during cell culture on polystyrene scaffolds (Lerman et al., 2018). Further suitable modifications in the biophysical properties of scaffolds are desirable to mimic systemic vascularization in cultured tissue. As it is challenging to have a single polymer or biomaterial with all desirable attributes, so a novel approach to fabricate composite or hybrid scaffolds at inexpensive/minimal cost is recommended (Kumar et al., 2023).

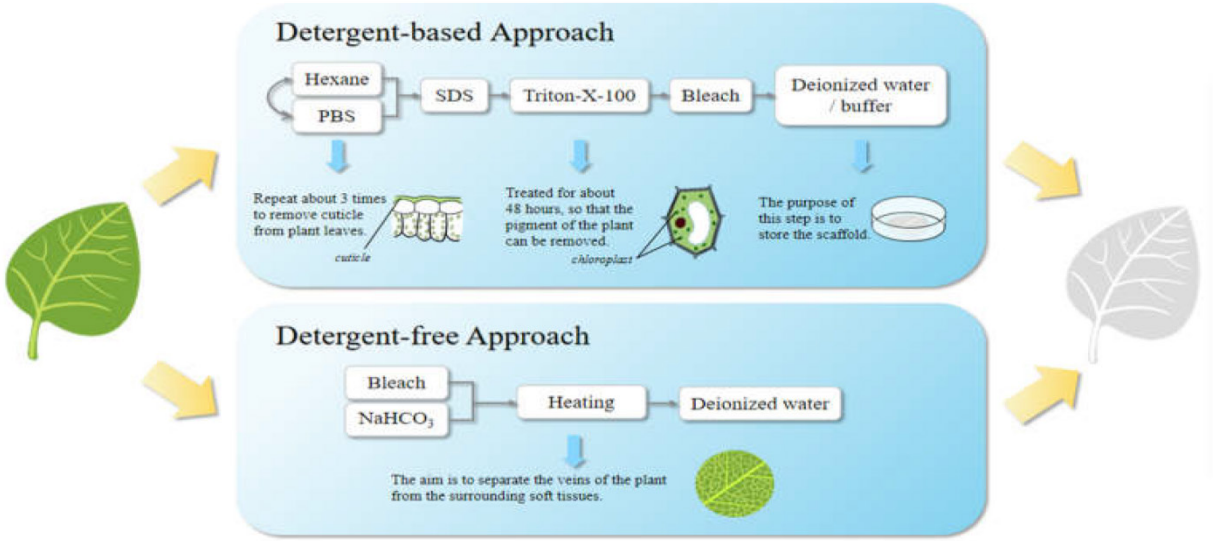

Figure 3. Method for preparation of plant-derived decellularized scaffolds (adopted from Lu et al., 2022)

Microcarriers and aggregates

Most mammalian cells are anchor-dependent cells by nature and grow in aggregates or by attaching to microcarriers in bioreactors. The culture in aggregates grows by the formation of clumps of cells in a three-dimensional shape, which provides a surface for the attachment of other nearby cells (Moritz et al., 2015). Theoretically, aggregates help in achieving very high cell density by providing a native environment to grow but it is difficult to control the size of aggregates and lack of perfusion of media into the inner area resulting in necrotic areas (Egger et al., 2018; McKee and Chaudhry, 2017). Thus, aggregates are mostly applied for maintaining *in-vitro* cell cultures rather than their proliferation. Further, the slow multiplication also limits its uses in the production of *in-vitro* meat (Aguanno et al., 2019).

Microcarriers are beads of various materials with varying porosity and topography, have a large surface/volume ratio, and provide attachment to the growing cells. These are extensively used in the medical field for stem cells and cell lines but specific microcarriers dedicated to *in-vitro* meat production are still in the developmental phase (Bodiou et al., 2020). Further, by proper bead surface curvature, the cells can have a 3D structure (Werner et al., 2019). Process control and monitoring are easy in cell culturing by using microcarriers as compared to fixed bed bioreactors, thereby improving the quality of end products at low cost. Based on usage, the microcarriers can be categorized under three categories as per Bodiou et al. (2020) viz.:

1. Microcarriers serve as a temporary attachment for cell growth and are later detached from cells after the process, preferably by mechanical and thermal processes. These should have a high detachment yield and easy removal from culture mass. After detachment, separation is performed by filtration, centrifugation, inertia, and magnetism.

2. Microcarriers (natural or synthetic) serving as an attachment for cell growth and later degraded or dissolved after bioprocess. This degradation can be achieved by various processes viz., chemical, photoreduction, thermal, mechanical, and biological processes.

3. Microcarriers made up of edible materials and embedded in the cultured mass after the process. These edible materials are made by using polysaccharides (cellulose, starch, chitosan, alginate, pectin, carrageenan, and carboxymethyl cellulose), lipids (paraffin, shellac, etc.), polypeptides (gluten, collagen, gelatin, etc.), and composite or synthetic polymers.

Food-grade microcarriers derived from sustainable raw materials that do not need dissociation from the final meat product could be a good strategy for commercial cultured meat production. Andreassen et al. (2022) developed food-grade microcarriers having interconnected porous structures from turkey collagen and eggshell membrane and observed high adhesion, proliferation, and low cell cytotoxicity of bovine skeletal muscle satellite cells in a spinner flask system for 8 days. The proliferated cells had lower PAX7 and increased MYF5 markers, resulting in the induction of proliferation marker MKI67 (Andreassen et al., 2022).

The production capacity can be improved by adding microcarriers as cells can migrate to new beads and proliferate (Verbruggen et al., 2018). This 'bead to bead transfer' happens via detaching cells from confluent microcarriers followed by reattaching onto other microcarriers or forming bridges between microcarriers upon collision (Leber et al., 2017). Adding a small number of microcarriers with adapted agitation could result in a decrease in lag phase and higher yield during cell culturing even potential risk of the formation of cell-loaded microcarriers aggregates (Jossen et al., 2016; Takahashi et al., 2017). Microcarriers can be used for controlled nutrients delivery to growing cells, thereby reducing the risk of contamination such as sol-gel derived bioactive glass microcarriers loaded with cytochrome C protein and fibroblast growth factor facilitated the controlled release of these compounds for several weeks (Perez et al., 2014). Other technologies are explored for the potential use of these compounds such as microencapsulation and controlling environmental conditions such as temperature and pH (Matsumoto et al., 2019; Zhou et al., 2018).

The microcarriers surface can be modified to make it better compatible with cell attachment such as by modifying (increasing) the hydrophilic properties and surface charge (more positively charged cells for attaching negatively charged cells) by incorporating chemical treatment by adding amino or carboxyl groups (Derakhti et al., 2019; Meng et al., 2017). Other approaches to improve the attachment alter the shape, size, roughness, stiffness, flexibility, and topography of microcarrier beads and optimize the seeding conditions (Bodiou et al., 2020). Recently, edible microcarriers with grooved topology were developed by embossing technology applying water-in-oil emulsions as templates for gelatin microparticles (Norris et al., 2022). These microcarriers with smooth surface/spherical and grooved surfaces in suspension facilitated the proliferation and differentiation of mouse myogenic C2C12 cells, with grooved surface microcarriers performing better (Norris et al., 2022). Further, the cultured cells harvested by centrifugation had shape and browning during cooking similar to meat patty (Norris et al., 2022).

The iPSC and ESC obtained from humans and mice were observed to show proper growth in aggregates of small molecules and cytokines (Burrell et al., 2019; Lipsitz et al., 2018). Cell proliferation in proliferates is easier and costs less but it is difficult to control the aggregate size, resulting in unpredictive yield results (Tsai et al., 2017). Alternatively, microcarriers are used in stirred bioreactors to provide attachment to the mammalian cells for stem cell growth (Li et al., 2015). These are small beads (up to 500 μm in diameter) having a large surface/volume ratio and can be easily scaled up by increasing the number of beads (Bodiou et al., 2020). The application of edible microcarriers would further improve the scalability and ease the process of preparation of cultured meat production by alleviating dissociation/degradation/

separation steps. Verbruggen et al. (2018) successfully grew bovine myoblasts on microcarriers (CellBIND®, Synthemax® and Cytodex 1 MC®) and reported bead-to-bead transfer by these cells and similar growth attributes to human mesenchymal stem cells. The wide variations in the microcarrier's attributes such as size, shape, and materials result in variations in the surface areas per weight, swelling properties, and volumetric productivity (surface area of microcarrier/mL of media), later very crucial factors to be considered while scaling up the process (Bodiou et al., 2020).

Bioreactors

The multiplication and maturation of stem cells take place in the bioreactor under controlled conditions such as pH, dissolved oxygen, temperature, and carbon dioxide. The temperature of the incubator is 36.5–37.5ºC and at 5–10% CO_2 for pH control. The development of intelligent bioreactors that provide proper growth and maturation conditions to cells *ex-vivo* is very crucial. For the production of 1 ton of *in-vitro* meat containing approx.. 10^{14} cells required with an optimum cell density of $1–3\times10^7$, the bioreactor configuration should be 10 m^3 or using 10 bioreactors of 1 m^3 dimensions would be needed (Post et al., 2020).

During *in-vitro* meat production, cell expansion occurs on microcarriers or in aggregates in the expansion bioreactor facilitating the exponential growth of cells without their differentiation by maintaining a proper supply of oxygen, nutrients, and growth factors. After proper proliferation, cells are then transferred to tissue perfusion bioreactors for differentiation by following proper media flow through 3D structured tissue resembling the vascular system or attached to scaffolds (Chen et al., 2022).

The bioreactor used in the development/pilot scale production for *in-vitro* meat is usually smaller in volume. Scaling up the bioreactor requires proper design and suitable technologies. An inappropriate bioreactor could cause reduced cell growth, higher energy and nutrients requirement, and accumulation of metabolic by-products leading to growth inhibition, slower cell metabolism, higher shear stress damaging cells, lower cell density, and limiting bioreactor volume (Allan et al., 2019; Chen et al., 2022). Alternatively, stem cells are suspended using microcarriers or through cell aggregation for large-scale production with high-density cultivation (Moritz et al., 2015).

Based on the design of bioreactors used in cell culturing, it could be stirred tank bioreactor, airlift, rocking platform (wavelike) bioreactor, hollow fiber bioreactor, and packed bed/fluidized bed bioreactor (Post et al., 2020). The stirred tank bioreactor consists of a cylindrical culture vessel equipped with a central impeller to ensure homogenous distribution of nutrients, oxygen, and growth regulators for desirable cell proliferation. However, the central impeller movements may induce shear on cells resulting in exploring the use of rocking platform bioreactors with a wave-like fluid motion for lowering stress (Panchalingam et al., 2015). Alternatively, for large-scale cell cultures of >20,000 L, airlift bioreactors could be a potential solution for limiting shear stress, homogeneity, and lower energy requirements. The culturing parameters were reported to have a 36 l/h mass transfer coefficient, 46 W/m^3 energy dissipation rate, and 103 s mixing time, and a single bioreactor with a size of 300 m^3 could cater to the demand for meat for 75,000 (Li et al., 2020).

The rocking platform and stirred tank bioreactors can be used as single-use bioreactors (SUB) with disposal bags. These bags are prepared with food-safe polymers, polyethylene, polypropylene, polystyrene, and polytetrafluoroethylene. These are having merits of lower energy consumption, lower contamination risk, and easier sterilization but need to reduce the price of production to make their production economically feasible (Djisalov et al., 2021). Hollow-fiber bioreactors are used for perfusion bioreactors made up of cylindrical cartridges containing parallel porous capillaries facilitating the growth of cells with low-shear stress and larger surface area (Djisalov et al., 2021). The hollow-fiber bioreactors were observed to support the proliferation of adipose-derived stem cells and the formation of adipose tissue (Gerlach et al., 2012).

Table 2. Various types of bioreactors developed for large-scale production of *in-vitro* meat production process

Bioreactor type	Max capacity and cell density	Merits	Constraints
Stirred tank with microcarriers	2,000 L/m^3, 2×10^8 c ells/mL	Flexible system and easy to scale up Suitable for high-volume bioprocess Enable real-time monitoring	Need large volume Higher shear stress Need optimization with cell lines and microcarriers for proper attachment
Wave bioreactor with microcarriers	20 L/0.02 m^3, 2×10^6 cells/mL	Easier scale-up with higher productivity Reduced shear stress Batch-wise operations	Suitable for scaling up to <100 L Need more space
Hollow fiber bioreactor	150 cm^2/mL, 1×10^9 cells/mL	Higher surface-to-volume ratio Better media perfusion similar to vascular system	Harvesting cells difficult Concentration of gradients
Packed beds	500 m^2/0.03 m^3, 3×10^6 cells/mL	Large surface area leading to achieve high cell density Batch-wise production Less frequent cell passage	Packaging material is difficult to harvest

Adopted and modified from Bellani et al. (2020).

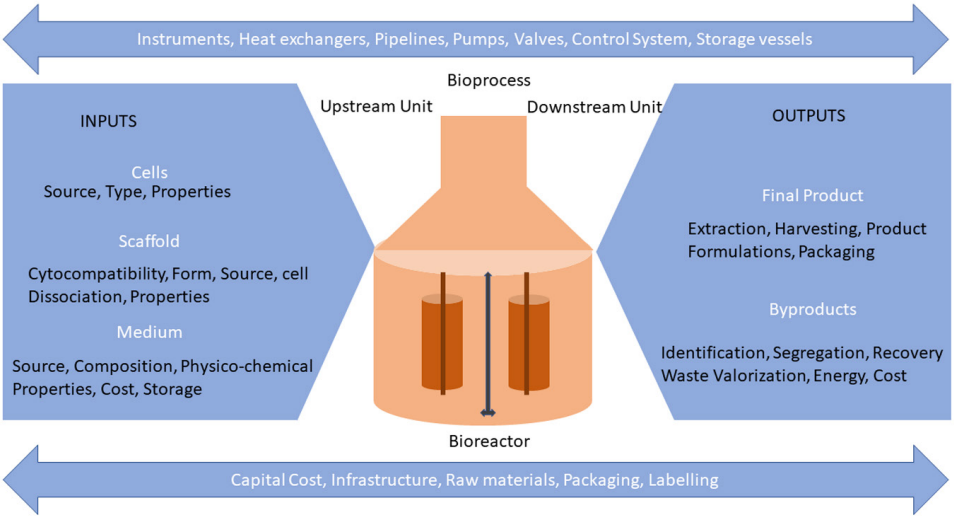

Figure 4. Summary of the key areas of consideration for cultured meat bioprocess design

There is a need for an automated, closed, and continuous system without any contamination of cell culture growth equipped with a real-time monitoring system for monitoring media conditions, cell viability, and productivity (by optimization of media and nutrient usages, recycling of media) (Djisalov et al., 2021; Specht et al., 2018). The bioreactor monitoring indicators placed at the appropriate site should be designed to assess dissolved gases, pH, temperature, lactic acid concentration, glucose, cell density, protein expression, morphology, and characterized genes (Xu et al., 2018). Recycling media after the removal of toxic compounds and supplementing the nutrients can reduce the cost and maximize the use of media. Table 2 summarizes the various types of bioreactors to be used for large-scale production of *in-vitro* meat.

There is a need to develop suitable technologies for cell culturing on a continuous process to increase efficiency, reproducibility, product quality, and reduce the overall cost of production. It is possible to generate 1 million cells per day in a medium size tissue culture flask of 155 cm^2 by following suitable continuous processing (Miotto et al., 2017). Various prospects for scaling up *in-vitro* meat production are summarized in Figure 4.

Organoleptic attributes and consumer acceptance

Sensory and nutritional attributes play a major role in determining the consumer acceptance of food products. Recently, various functional biomaterials, cells, physical and chemical factors are used in tissue engineering for producing *in-vitro* meat having similar sensory and nutritional attributes to conventional meat. The decellularized spinach scaffold having a vascular network was used for developing a meat-like system with sustaining very high viability (up to 99%). In the edible tissue, 25% of muscle cells exhibited myosin heavy-chain expression (Jones et al., 2021). 3D bioprinting by suitable biomaterials was used to create thick and complex three-dimensional structures facilitating large-scale tissue constructs. Li et al. (2021) used ion-cross-linked alginate-gelatin and light-crosslinked silk fibroin for creating 3D skeletal muscle during *in-vitro* meat production having structural and functional attributes similar to conventional meat.

For producing cultured edible tissue mimicking the taste, flavor, texture, and color of conventional meat, designing low-cost and edible scaffolds mimicking the extracellular matrix and supporting cell culture, are prerequisite. These scaffolds help in the differentiation of cells into myotubes and their proper organization to impart texture (Post et al., 2020; Ben-Arye et al., 2020). Jackfruit-based scaffolds were observed to impart marbling patterns in *in-vitro* meat due to the differential adsorption of polyphenols. The edible tissue constructs were observed to have high-density cultures of porcine myoblasts and develop brown color upon cooking, thus improving consumer acceptance of *in-vitro* meat by 8% (Ong et al., 2021).

The application of texturized soy protein added with myogenic-related growth factors was used in the muscle cell differentiation and development of 3D bovine muscle tissue. The co-culturing with bovine endothelial cells and smooth muscle cells in this structure resulted in the production of *in-vitro* meat which exhibited meaty texture, color, flavor, and appearance upon cooking as compared to monoculture tissue (Ben-Arye et al., 2020). Similarly, to get the complex texture of *in-vitro* meat, a photosensitive edible scaffold was fabricated by using soy-based polymers, and was observed to facilitate the production of cultured meat with a complex structure having thermomechanical characteristics not affected by cooking (Sealy et al., 2022). Kang et al. (2021) prepared artificial steak-like meat *in vitro* by assessing three types of fibers viz., muscle, fat, and vessel. The tendon-gel integrated bioprinting technology was applied for developing tendon-like structures in developed tissue as depicted in Figure 5 (Kang et al., 2021).

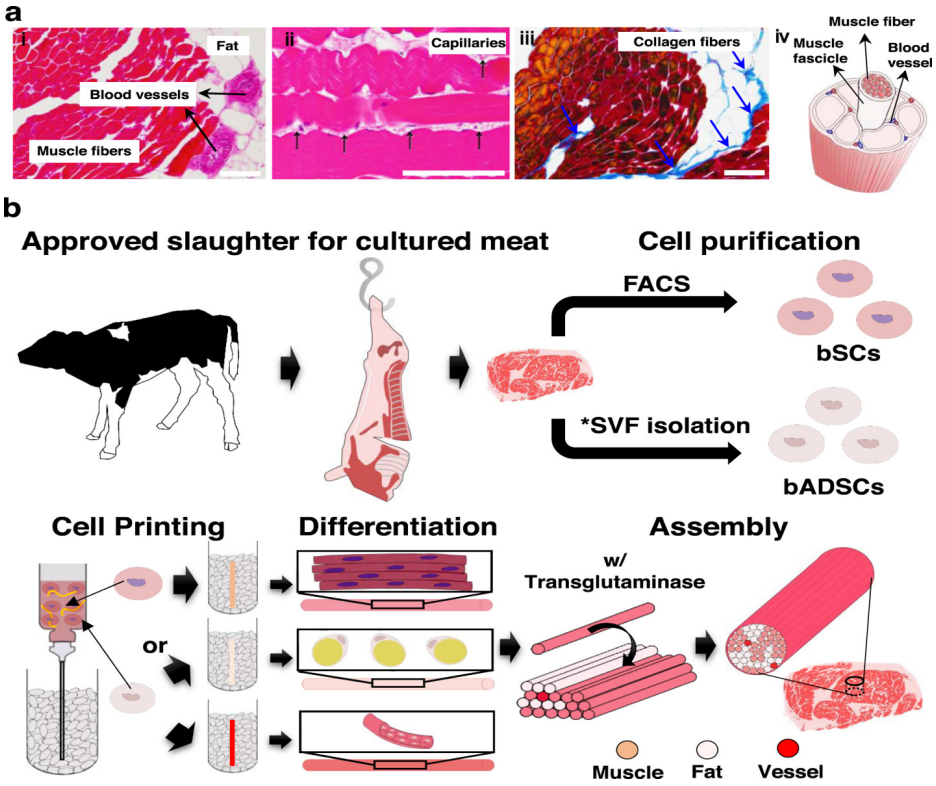

Figure 5. Structure of steak. **a** (i, ii) H&E- and (iii) Azan-stained images of a piece of steak. Representative images from three independent experiments are shown. All scale bars denote 100 µm (iv) Schematic of a hierarchical structure in muscle. **b** Schematic of the construction process for cultured steak. The first step is cell purification of tissue from cattle to obtain bovine satellite cells (bSCs) and bovine adipose-derived stem cells (bADSCs). The second is supporting bath-assisted printing (SBP) of bSCs and bADSCs to fabricate the muscle, fat, and vascular tissue with a fibrous structure. The third is the assembly of cell fibers to mimic the commercial steak's structure. *SVF – stromal vascular fraction. (Adopted from Kang et al., 2021)

A 3D scaffold developed from chitosan-sodium alginate-collagen and gelatin was observed to support adhesion and proliferation of porcine skeletal muscle, thereby resulting in structured *in-vitro* meat with textural attributes (chewiness, resilience, and springiness) and appearance similar to fresh pork (Li et al., 2022). Post et al. (2020) developed a cell sheet-based meat model made up of polysaccharide films of chitosan and carboxymethyl cellulose for *in-vitro* meat production by using C-phycocyanin, extracted from blue algae as an alternative to animal-derived serum.

Consumer acceptance is very crucial for the overall marketing, popularization, and development of the product. It is very difficult to forecast the consumer acceptance of this novel product due to the very limited presence of this product in the market (Post, 2014; Bryant and Barnett, 2018). The acceptance rate of *in-vitro* meat would likely be very high in some projections up to 63.5% (Wilks and Phillips, 2017) to very low up to 5–11% (Hocquette et al., 2015). The wide variations in various studies could be due to variations in population, questionnaires, sample collections, and regions (Hocquette et al., 2022). The main challenges in the consumer acceptance of *in-vitro* meat are food neophobia, the unnaturalness of the products, public misconception about safety and nutritive value, lack of awareness about the technology and process involved in the production, and exorbitant price (Kumar et al., 2021 a). Some strategies for improving consumer acceptance could be by emphasizing the naturalness of *in-vitro* meat and the unnaturalness of conventional meat (Bryant et al., 2019), perceived health benefits (Siegrist and Sütterlin, 2017), nutritional data (Dupont and Fiebelkorn, 2020) and emphasizing the final products rather than production process (Siegrist et al., 2018).

Prospects and conclusion

For perceiving the widely acclaimed merit of *in-vitro* meat in terms of sustainability, food safety, and ethical way of meat production, the scaling up of current technologies is required. There is a need for undertaking regulatory approvals and increasing awareness to consumers towards consumer acceptance of *in-vitro* meat. The *in-vitro* meat should mimic the textural and sensory attributes similar to conventional meat.

There is a need for developing suitable cell lines with gene editing for better stemness and maintaining pluripotency by modifying gene expression. The development of cell banks for a smooth supply of stem cells to various firms could maximize the gain, save time and mini-

mize the animal usage for harvesting these cells. There is a need to optimize the protocol for the production of fat cells, endothelial cells, fibroblasts, osteoblasts, chondrocytes, and other extracellular matrix-secreting cells for providing texture and flavor similar to conventional meat.

The price of culture media would be significantly reduced by using growth regulators, nutrients, and trace elements from inexpensive and sustainable sources such as plants, fungi, or bacteria by recombinant technology or gene editing. Application of plant hydrolysates and flavonoids has been used for inducing cell proliferation and maturation. There is a lot of investment of time and resources for the development of cell-specific culture media and thus better sharing among the scientists and firms needed for scaling up *in-vitro* meat production.

Suitable cell attachments such as scaffolds, microcarriers, and aggregates are required for the proper growth and proliferation of cells in the bioreactors. It is preferred that these materials should be edible, natural, and derived from sustainable sources, hence improving the overall process efficiency by avoiding the steps of cell detachment and separation. The commercial-scale bioreactor with proper control of environmental conditions (pH, temperature, oxygen, nutrient supply), media perfusion, and automation without causing shear stress is very essential for the scaling up of the *in-vitro* meat production.

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Conflict of interest

The authors declare no conflict of interest.

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