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REVIEW

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Enhancing recombinant growth factor and serum protein production for cultivated meat manufacturing

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Abstract

The commercial growth factors (GFs) and serum proteins (SPs) contribute to the high cost associated with the serum-free media for cultivated meat production. Producing recombinant GFs and SPs in scale from microbial cell factories can reduce the cost of culture media. *Escherichia coli* is a frequently employed host in the expression recombinant GFs and SPs. This review explores critical strategies for cost reduction in GFs and SPs production, focusing on yield enhancement, product improvement, purification innovation, and process innovation. Firstly, the review discusses the use of fusion tags to increase the solubility and yield of GFs & SPs, highlighting various studies that have successfully employed these tags for yield enhancement. We then explore how tagging strategies can streamline and economize the purification process, further reducing production costs. Additionally, we address the challenge of low half-life in GFs and SPs and propose potential strategies that can enhance their stability. Furthermore, improvements in the *E. coli* chassis and cell engineering strategies are also described, with an emphasis on the key areas that can improve yield and identify areas for cost minimization. Finally, we discuss key bioprocessing areas which can facilitate easier scale-up, enhance yield, titer, and productivity, and ultimately lower long-term production costs. It is crucial to recognize that not all suggested approaches can be applied simultaneously, as their relevance varies with different GFs and SPs. However, integrating of multiple strategies is anticipated to yield a cumulative effect, significantly reducing production costs. This collective effort is expected to substantially decrease the price of cultivated meat, contributing to the broader goal of developing sustainable and affordable meat.

Keywords Cultivated meat, *Escherichia coli*, Growth factor (GF) production, Serum protein (SP) production, Cell line engineering, Bioprocess optimization

Introduction

Cultivated meat, also known as lab-grown or cultured meat, is produced by cultivating animal cells in a controlled bioreactor environment, without raising and slaughtering animals [1, 2]. This cruelty-free alternative has the potential to reduce the environmental impact of traditional meat production by consuming fewer natural resources and emitting fewer greenhouse gases [3]. It is estimated that producing meat in bioreactors at scale could use 80% less water and requiring 35–67% less land compared to conventional approaches [1]. However, the primary challenge lies in the economic viability of

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making cultivated meat cost-competitive with traditional meat. The techno-economic analyses conducted by various groups, based on the manufacturing processes used in the pharmaceutical industry, indicate that the culture media is the major contributor to production cost of cultivated meat [4–7]. To achieve commercial viability, recent studies suggest that the cost of the culture media must be steeply reduced, with target costs falling below US$1 per liter [7].

Currently, Fetal Bovine Serum (FBS), a complex mixture containing Growth Factors (GFs), hormones, lipids and serum proteins (SPs), is obtained from blood drawn from unborn calves, and it has often been used as a growth supplement to sustain cells cultured *in-vitro* [8]. However, the use of FBS in cultivated meat production has been strongly discouraged for the following reasons. First, FBS is costly, and its quality can vary largely between batches, resulting in loss of reproducibility during cell culture. Second, the fact that FBS is sourced from neonatal animal calves nullifies the ethical advantage of cultivating meat from cells as opposed to procuring meat from traditional farming which involves animal slaughter [9]. While efforts have been directed to explore the feasibility of using lower cost food-grade media components such as hydrolysates derived from plants and non-animal [10] as alternatives to support cell culture. However, these alternatives have not yet been widely adopted. Hence, there is a need for a more economical method in the production of GFs and SPs for serum-free cultivated meat media.

GFs are signaling proteins that bind to cell surface receptors and activate several downstream pathways, resulting in cell migration, proliferation and differentiation. In cell culture applications, including cultivated meat production, recombinant GFs are used as serum replacements [11], negating the need for fetal bovine serum (FBS). For cultivated meat media, essential growth factors typically include Fibroblast Growth Factor 2 (FGF2), Epidermal Growth Factor (EGF), Insulin-like Growth Factor 1 (IGF1), Neuregulin 1 (NRG1), Transforming Growth Factor Beta (TGFβ1), and Platelet-Derived Growth Factor Subunit B (PDGFB). Key serum protein components such as albumin, insulin, and transferrin are also required. Albumin, insulin, and transferrin collectively support cell growth, survival, and differentiation in cultivated meat media by stabilizing growth factors, facilitating nutrient uptake, regulating metabolic activity, and maintaining essential iron homeostasis for tissue development. However, the high cost of recombinant GFs and SPs poses a significant challenge to the cultivated meat industry. According to a report by the Good Food Institute (GFI), the combined cost of recombinant GFs and SPs must remain below 10% of the total cost per kilogram of meat to ensure commercial viability [12]. The report recommends that, under various projection scenarios, the average production costs should be approximately $10/kg for albumin, $1,000/kg for insulin and transferrin, and $100,000/kg for growth factors [4]. Achieving these cost reductions is essential for the widespread adoption of cultivated meat.

To address the challenge of cost-effective production, GFs and SPs should be produced using engineered microbes through precision fermentation. This review focuses on *Escherichia coli* as the microbial chassis for this purpose. *E. coli* is the most extensively studied organism and is already widely used for large-scale production of therapeutic proteins in the pharmaceutical industry. Its rapid growth, cost-effectiveness, and high-yield protein expression make *E. coli* one of the most employed hosts for recombinant protein production, including mammalian growth factors [13]. The production of recombinant proteins in *E. coli* is well-established, dating back to the successful production of recombinant insulin in 1978 [14]. However, despite the technological maturity, achieving cost-effective production still remains a significant challenge. Current applications of recombinant GFs are primarily in the biopharmaceutical industry and for academic research, where smaller quantities suffice, and cost constraints are less stringent. Overcoming this hurdle is crucial for the broader application of these proteins in industrial scale cultivated meat manufacturing. In this light, this review will explore several strategies to enhance the cost-effective production of recombinant GFs and SPs, which are essential for developing serum-free culture media for cultivated meat manufacturing. This strategy focuses on four key objectives: yield enhancement, product improvement, purification innovation, and process innovation. The first section examines the literature already available on producing GFs for cultivated meat media using various tags to enhance yield, offering a foundation of optimism that *E. coli* can be an effective production platform. Following this, the review consolidates and categorizes current knowledge on recombinant protein production in *E. coli*, highlighting approaches that, while not traditionally applied to GFs and SPs for cultivated meat, have the potential to reduce production costs. Although some of these methods may not yet be employed in GFs and SPs production, the technology is adaptable, and future research can be directed towards their implementation (Fig. 1).

Fusion tags for improved GF expression

Production of recombinant proteins in *E. coli* is often fraught with low solubility issues and incorrect folding resulting in low final titers [15]. Genetically engineered protein fusion tags confer advantages of enhancing

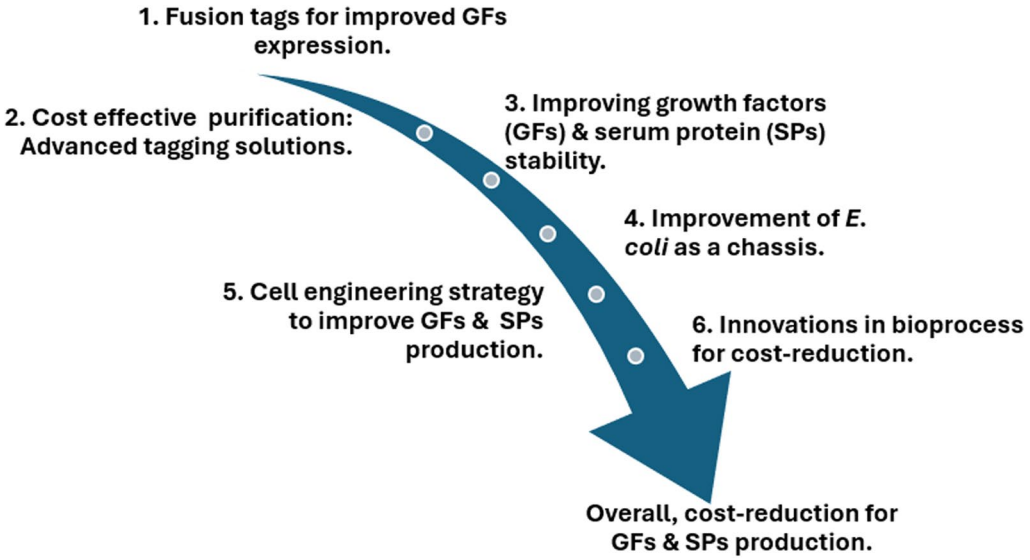

Fig. 1 Approaches to decrease the cost associated with production of Recombinant Growth Factors (GFs) and Serum Proteins (SPs) for serum free media

protein folding by providing stability and preventing aggregation. Hence, fusion tags can be used to overcome incorrect protein folding issues and improve GFs production. These fusion tags have been shown to act as chaperones to aid correct folding and prevent the formation of non-functional aggregates. The enhanced solubility of the target protein resulting from the fusion tag also facilitates subsequent downstream processing. Table 1 summarizes the application of various tags to expression of selected GFs. In general, the addition of solubility-enhancing fusion tags like maltose-binding protein (MBP) [16], glutathione S-transferase (GST) [17], N-utilization substance (NusA) or Superfolder green fluorescent protein (sfGFP) [18] are widely-used to promote proper folding and solubility of the target protein. Several other fusion tags have also been developed in the past few years to address specific challenges or provide unique features to facilitate GF expression.

Disulfide bond oxidoreductase A (DsbA), a disulfide bond isomerase has been shown to vastly improve the expression of soluble proteins containing disulfide bonds in *E. coli* by correcting disulfide bond formation via intra- and intermolecular catalysis [19]. Emamipour has demonstrated that fusing DsbA to N-terminus of mammalian IGF1 and expression in Shuffle *E. coli* T7 strain significantly increase the concentration of soluble protein by ninefold [20]. This finding was further verified by Venkatesan's group, which also presented that the use of DsbA can aid in a higher yield of PDGF & TGFβ1 [21] It was also shown that the retention of DsbA does not affect the bioactivity of GFs in cell culture media. However, Venkastesan did show that DsbA could be cleaved with the aid of Tobacco Etch Virus (TEV) protease cleavage. *Fascioloa hepatica* 8-kDa antigen (Fh8) is a small antigen that is secreted by the parasite *F. hepatica* in the early stages of infection [22]. First discovered by Conceicao Fh8 is just one of the few fusion tags that functions both as a solubility enhancer partner and robust purification handle, with its low molecular weight having an advantage over larger fusion tags in the production of

Table 1 Application of fusion tags in the expression of recombinant growth factors

Tag	Fusion tag	Size (aa)	Organism	Growth Factor	References
DsbA	Thiol disulfide oxidoreductase	208	E. coli	IGF, PDGF, TGFβ1	[20] [21]
Fh8	Fasciola hepatica 8-kDa antigen	69	F. hepatica	FGF1, FGF2, EGF, hGH, IGF1, VEGF165, KGF1, PGF	[15] [23]
MBP	Maltose-binding protein	396	E. coli	FGF21, VEGF165	[25] [26]
Trx	Thioredoxin	109	E. coli	EGF, FGF2, PDGF	[20] [28] [33]
SUMO	Small ubiquitin modifying protein	~100	Homo sapiens	EGF, FGF21, FGF23, IGF	[20] [29] [30] [31]
sfGFP	Superfolder green fluorescent protein	237	E. coli	TGFβ3	[18]

recombinant protein in *E. coli* [15]. The results published by Kim demonstrates that utilizing Fh8 as a fusion partner result in an enhanced production of a wide variety of GFs in recombinant *E. coli*, including FGF, hGH, IGF, VEGF and PGF at an industrial scale of g/L [23].

Maltose-binding protein (MBP) is a relatively large cysteine-less protein, utilized as a periplasmic fusion tag for enhanced secretion and purification of soluble recombinant protein [19, 24]. Nguyen reported that with the attachment of MBP to the N terminus of hFGF21, there was an enhanced solubility of the GF being produced, with its bioactivity matching those of commercial hFGF21 [25]. Similarly, MBP was first shown to aid in producing soluble bioactive human VEGF165 by acting as a chaperon to promote proper folding in *E. coli* [26]. Nguyen further demonstrated that even with the cleavage of MBP using TEV, hVEGF can remain soluble and bioactive, with its bioactivity comparable to commercial hVEGF [26]. Thioredoxin (Trx) was first developed as a gene fusion partner in 1993 by LaVallie to aid in the production of soluble and bioactive mammalian cytokines and growth factors [27] Much recently, Ferreira et. al (2022) have specifically shown the requirement of Trx as a fusion partner for the proper folding and production of EGF in *E. coli*, obtaining 20mg of Trx recombinant growth factor after purification [28]. Venkatesan further expanded the list of recombinant GFs that Trx when added as a fusion tag further enhanced the production of FGF and PDGF [20].

Small Ubiquitin-related Modifier Protein (SUMO) has also been known to be an effective fusion system in recent years in enhancing the expression of soluble GFs such as IGF[20], FGF [29, 30] and EGF [31] Wang and team demonstrated that with the attachment of SUMO, high level expression and purification of FGF21, an upcoming treatment that is used to fight metabolic diseases can be produced [29]. Similarly, Su reported that by fusing the SUMO tag to hEGF, expression level of the soluble protein increased from 39 to 98% [31]. The fusion tag has also been reported to be easily cleaved off using SUMO proteases, without affecting its bioactivity. Superfolder green fluorescent protein (sfGFP) is a fluorescent protein was first found to contribute greatly to the expression of TEV protease [25] and anti-influenza PB2 single-chain variable fragment (scFv) [32] in *E. coli* when incorporated as a fusion tag. Bilgin more recently was able to be the first to successfully produce the GF TGF-β3 when fused with sfGFP [18]. Using *E. coli* BL21, a high yield of 20mg of GF and purity of 98% was achieved with the incorporation of sfGFP [18].

After expression of the GFs, fusion tags are usually cleaved off during the downstream operations. The size of fusion tags can impact protein structure, function, and interactions. In some cases, the presence of a fusion tag may interfere with protein–protein interactions or alter the target protein's biological activity [20]. This becomes particularly important when studying protein–protein interactions or designing therapeutically relevant proteins. For instance, fusion tags derived from non-human sources may introduce immunogenic epitopes that trigger unwanted immune responses *in-vivo* and reduce the efficacy of the therapeutic protein [34]. Therefore, removing the fusion tag becomes crucial to preserve the native form of the recombinant protein. Enzymatic cleavage mediates precise and selective removal of fusion tags. Proteases, such as enterokinase, thrombin, and factor Xa, are commonly used for fusion tag removal [35]. Enterokinase specifically cleaves fusion tags at a specific recognition sequence—DDDDK—which facilitates tag removal. Thrombin and factor Xa proteases cleave at specific recognition sites, allowing the removal of fusion tags with high specificity and efficiency. Cyanogen bromide (CNBr) is a traditional chemical agent that selectively cleaves polypeptide chains at methionine residues and is commonly employed to remove fusion tags containing such residues [36]. However, selectivity of CNBr cleavage also restricts its application to tags bearing specific amino acid sequences. On the other hand, there are fusion tags that enable self-cleavage. Likewise, CASPON platform i.e., circularly permuted caspase-2 is promising for tag removal and has been used to remove tag from Fibroblast Growth Factor (FGF) [37]. Inteins, for instance allows the removal of the fusion tag without the need for external proteases [38]. Tag removal often involve additional purification steps, such as chromatography or enzymatic treatments, which require additional time and labour, leading to an increased cost of recombinant protein production. By eliminating these tag removal steps, the overall production process becomes more streamlined, labour efficient and cost-effective. In our hands, we have found that fusion protein-tagged GFs maintain the similar level of biological activity in cell-based proliferation assays (unpublished data). These data suggest that fusion tags do not always interfere with the biological activity of GFs and may therefore be retained. Moreover, fusion tags can elevate the stability of target proteins in serum-free media applications.

Cost effective purification: advanced tagging solutions.

The purification of recombinant GFs and SPs is a major contributor to the overall production costs, which in turn drive up the expense of cultivated meat media. Reducing purification expenses is a promising approach to lower the cost of cultivated meat media [4]. While the previous section discussed the role of fusion tags in

yield enhancement, their utility also extends to simplifying the purification process, which will be discussed here (Table 2). Employing fusion tags in purification processes offers economic benefits by, reducing reliance on expensive chromatographic columns [39] or utilizing cost-effective columns made from economical materials [40]. It is important to note that while these tags may not yield recombinant GFs and SPs of extremely high purity, they effectively concentrate proteins to a reasonable purity level. Conventional polishing steps can then be applied to achieve the desired final purity.

Elastin-Like Polypeptides (ELP) is a well-studied aggregating tag, comprising repeated amino acids. ELPs demonstrate a reversible phase transition phenomenon, forming aggregates at temperatures exceeding their transition temperature and dissolving at lower temperatures [41, 42]. The sequence and length of ELPs can be engineered to modulate the phase transition temperature, facilitating efficient protein purification through temperature adjustments [43]. The research conducted by Wood's group [42, 44] and Chilkoti's group [39, 43] among many others has extensively explored ELP, showcasing its efficacy in purifying target proteins. Nevertheless, a notable drawback lies in the considerable length of ELP, which, for achieving phase transition behaviour at 37 °C, necessitates a minimum of 180 amino acids [43]. This extended length may pose challenges when fused with the protein of interest, potentially straining cells and impeding high-yield production.

Minimizing the size of aggregating tags holds advantages, offering a competitive edge. The β-Roll Tag (BRT17), constructed from 17 repetitions of a 9-amino acid monomer, undergoes reversible self-assembly and becomes insoluble in low concentrations of calcium ions [44]. Likewise, ELK16, 16 amino acids aggregating tag, has also been used for protein purification. When ELK16 was fused to a protein at C-terminal, purification of protein aggregates with straightforward two-step wash method was possible and was reported to be more efficient than traditional affinity chromatography [45]. Recently, HlyA60, a novel 60-amino acid aggregation peptide, was identified from the hemolysin A secretion system. This peptide also could induce aggregate formation within cells. When fused with acetyl xylan esterase and lipase A, HlyA60 achieved a column-free purification process with a 98.8% recovery rate [46]. Lately, CspB, a part of the cell surface protein from *Corynebacterium glutamicum*, was also used as a novel pH-responsive tag, exhibiting reversible precipitation–redissolution behavior in fusion proteins, with a distinct pH response on neutral pH, rendering it a promising candidate for protein purification [47].

Exploring approaches to utilize cost-effective columns in protein separation can be another approach to reduce purification cost. EctP1, a 10 amino acid short tags, offer a cost-effective purification solution by non-covalently binding to unmodified silica and titanium [48]. This tag can leverage the cost effective non-modified adsorbents to reduce downstream cost. Likewise, the Spy-Tag and Spy-Catcher system, known for their strong interactions, presents another avenue for efficient separation. Spy-Catcher, which can easily be immobilized on solid support, when combined with the fusion of the protein of interest to Spy-Tag, facilitates effective capture of the protein of interest on the solid support [49]. Another exciting realm of research involves the fusion of the protein of interest with Cellulose Binding Domain (CBD). CBD, rich in proline and hydroxy amino acids, exhibits a strong interaction with cellulose. In nature, these domains play a crucial role in binding cellulase enzyme complexes to cellulose. CBD can be leveraged for affinity chromatography applications. CBD is inexpensive and abundant, its application in downstream processes is promising in reducing the costs associated with purifying

Table 2 Fusion tags for cost effective protein purification

Tag	Size (aa)	Description	References
Elastin Like Polypeptide (ELP)	> 180	Temperature-dependent reversible aggregating tag	[39] [42] [43]
β-Roll Tag (BRT17)	153	Calcium-dependent reversible aggregating tag	[44]
ELK16	16	Self-induced aggregation via the intermolecular beta structure	[45]
Hemolysin A (HlyA60)	60	Self-induced aggregation through electrostatic and hydrophobic interactions	[46]
Cell surface protein B (CspB)	50	pH dependent reversible aggregating tag	[47]
EctP1	10	Ability to bind to unmodified silica	[48]
Spy-Tag & Spy-Catcher	13 & 138	A protein with a Spy-Tag can bind to an immobilized Spy-Catcher	[49]
Cellulose Binding Domain (CBD)	Variable	Capability to bind to cellulose, enabling its use as column material	[50] [51]

GFs and SPs for cultured meat and ultimately lowering the costs of cultivated meat production [50, 51].

Improving GF and SP stability

Enhancing the thermostability of recombinant GFs and SPs is crucial for cultivated meat media, as it prolongs their activity, ultimately reducing production costs by minimizing protein degradation. Ensuring protein stability is essential to address the rising expenses linked to protein degradation. This section explores both traditional methods and emerging approaches, such as machine learning, and discusses their potential for improving the thermostability of GFs and SPs (Fig. 2).

Conventional approaches

Increasing thermostability of proteins could be achieved using different rational approaches. Phylogenetic analysis of protein of interest with homologous proteins, if possible, with thermophiles is one of the methods to prepare scaffold for thermostable protein [52]. Thermophilic proteins have more hydrophobic and charged residues compared to mesophilic proteins. A rational approach to engineering thermostable protein involves designing scaffolds that minimize uncommon residues and favor common ones [53]. Likewise, replacing residues and loops showing unfavorable Ramachandran angles and high B-factors can also be used to make protein thermostable [54]. Furthermore, disulfide bridges have been incorporated to make protein thermostable [55]. A recently engineered human Fibroblast Growth Factor (FGF) with a disulfide bridge showed improved stability, as indicated by an increased melting temperature, without affecting its activity. The authors demonstrated that the engineered FGF also enhanced cell proliferation, stemness, and differentiation of human pluripotent stem cell cultures (hESCs and iPSCs) compared to the wild-type FGF [56]. Another approach is to introduce salt bridges in the protein [57]. Salt bridges occur when oppositely charged amino acids, such as positively charged cationic ammonium (RNH_3^+) of lysine or arginine residues (basic amino acids), and negatively charged anionic carboxylate ($RCOO^-$) of aspartic acid or glutamic acid residues (acidic amino acids), come into proximity and form ionic bonds [53]. It is found that thermophilic protein has isolated charges and polar residues in the core that helps form stable salt-bridges [58]. Similarly, cyclization of proteins has also been used to increase the thermostability of proteins. The SpyCatcher and SpyTag

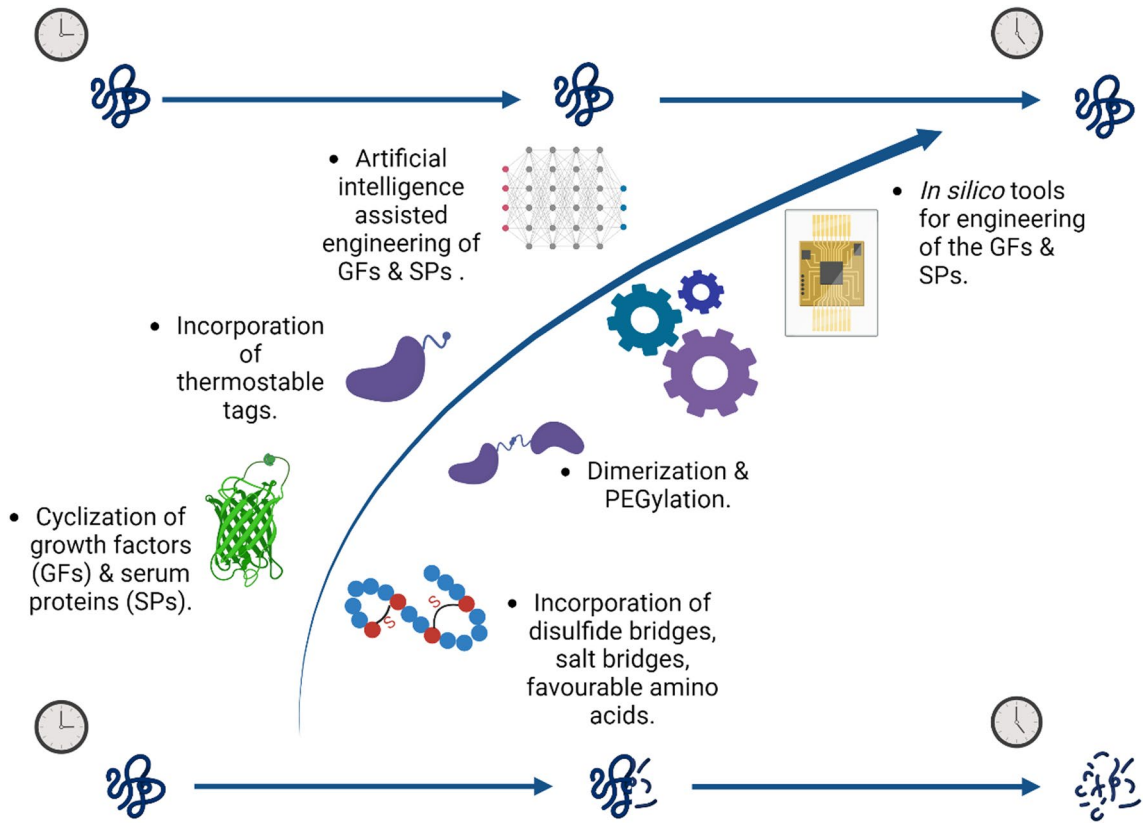

Fig. 2 Approaches to improve the Recombinant Growth Factors (GFs) and Serum Proteins (SPs) stability. (Created with www.biorender.com)

system was incorporated at the N and C terminals of the Polyethylene terephthalate (PET) hydrolase for covalent cyclization of PETase, leading to enhanced thermostability [59]. The cyclization strategy has also been applied in the context of growth factor necessary for cultivated meat. Notably, a dimerized and cyclized Fibroblast Growth Factor (FGF) has demonstrated enhanced thermal stability [60]. Furthermore, introduction of thermostable tags can be used to make protein stable. In a study, a novel multifunctional tag named S1v1 increased thermostability and/or activity of three proteins. The S1v1 tag used in the study was developed by modifying the self-assembling amphipathic peptide found in the Zuotin protein sequence, wherein lysine residues were substituted with histidine residues [61]. Additionally, two newly identified thermostable tags from *Thermotoga neapolitana* and *Pyrococcus furiosus*, demonstrated an enhanced catalytic activity and stability [62]. Also, dimerization, which involves connecting two protein monomers with a linker, and PEGylation, the covalent attachment of polyethylene glycol (PEG) chains to proteins, are strategies used to enhance protein stability and bioactivity. These approaches have also been applied to Fibroblast Growth Factor 2 (FGF-2) [63].

In silico approaches

Structure-based computational design can be employed to enhance protein thermostability. In silico design methods are expected to propose stabilizing mutations that increase stability while minimizing changes to the backbone conformation and active site, thereby preserving the protein's catalytic activity. One of the earliest instances showcasing rational in silico design for protein thermostabilization was done by Baker and his colleagues using Rosetta, their in-house software [64]. Rosetta is designed to predict protein structures from amino acid sequences, protein folding, and protein–protein interactions. It has been used for protein stabilization and identifying mutations that enhance protein robustness against changes in temperature, and pH. For instance, Rosetta was employed to enhance the stability of cytosine deaminase, which converts a non-toxic prodrug into the toxic compound with applications in cancer therapy [64]. Furthermore, the Janssen group at the University of Groningen developed a framework called "Framework for Rapid Enzyme Stabilization by Computational libraries" (FRESCO), which utilizes Rosetta along with molecular dynamics software [65]. This framework generates point mutations using Rosetta and FoldX, followed by Molecular dynamics-based screening. Thereafter, experimental testing of surviving mutations is done to confirm an actual increase in melting temperature while maintaining catalytic activity. Another tool for protein stabilization is the "Protein Repair One Stop Shop" (PROSS), developed by the Fleishman Lab at the Weizmann Institute of Science. PROSS also employs Rosetta to evaluate potentially stabilizing mutations, selecting only those that result in an energy decrease compared to the wild type [66]. More detailed review for improving the thermostability of proteins using computational rational approach [67] and approach based on phylogenetic analysis can be found elsewhere [68].

The prediction of protein structure from sequence alone, long considered an insurmountable challenge, became achievable with the advent of AlphaFold [69]. This machine learning model represents a remarkable advancement in the field of biology with many practical applications. AlphaFold and other machine learning models like MUTCOMPUTE [70], TemStaPro [71], ProteinMPNN [72] has been utilized to improve the thermostability of proteins. MUTCOMPUTE, freely accessible online tool developed at the University of Illinois, offers a promising avenue for designing stable proteins. This tool employs a 3D convolutional neural network trained to identify novel gain-of-function mutations, which may not be anticipated by traditional energetics-based methods. The experimental validation of these in silico proposed mutations has demonstrated improvement in protein function [70]. In one application scenario, the algorithm evaluated the suitability of individual amino acids within their chemical microenvironments in a protein. This approach was utilized to predict sequence substitutions in Bst DNA polymerase. The algorithm predicted different variants with substantially enhanced thermotolerance and activity. Combining these mutations of different variants led to additive thermostability, with denaturation temperatures up to 2.5 °C higher than the original Bst DNA polymerase [73]. In another instance, the same algorithm was utilized to engineer PETase, that can degrade plastic but is hindered by its slow catalytic activity. The algorithm generated a new PETase sequence with five mutations distinct from the wild-type PETase. This engineered PETase exhibited superior PET-hydrolytic activity across a temperature range of 30 to 50 °C and various pH levels, demonstrating the effectiveness of AI-assisted protein engineering [74]. Recently, ProteinMPNN, a deep learning-based model for protein sequence design, was utilized to enhance the stability of myoglobin and tobacco etch virus (TEV) protease [75]. These developments suggest that AI models are becoming integral tools that will continue to assist researchers in improving protein stability.

In 2005, researchers employed a homology-based approach to identify two point mutations that significantly extended the half-life of fibroblast growth factor (FGF), a critical and costly component in cultivated meat

media [76]. In 2018, wild-type FGF was further engineered using various in silico techniques to introduce nine additional mutations, resulting in a novel FGF variant with a markedly enhanced half-life compared to the original protein. This engineered FGF is now widely produced by multiple growth factor suppliers for use in cell culture media [77]. Similarly, in another study, bioinformatics tools such as multiple sequence alignment, evolutionary conservation analysis, and molecular dynamics (MD) simulation were utilized to identify mutations that could improve the thermal stability of FGF [78]. The application of in silico tools to enhance FGF stability is a promising indicator of future advances. These approaches could be further refined to improve FGF stability even more and adapted to enhance the stability of other GFs and SPs needed for cultivated meat media in the future. While the use of machine learning to improve the thermostability of growth factors and recombinant proteins for cultivated meat is yet to be explored, its application in this field is likely in the future.

With the availability of various in silico tools, many of which require no coding expertise [79], even scientists without formal computational training can readily use them. These resources assist experimentalists to design thermostable GFs and SPs.

In conclusion, enhancing the thermostability of GFs and SPs is vital for reducing production costs in cultivated meat media by mitigating protein degradation. Both traditional and emerging methods, including machine learning and in silico approaches, hold significant promise in improving protein stability. The integration of these technologies into the design of thermostable GFs and SPs offers an exciting path forward, enabling more cost-efficient and sustainable production processes for cultivated meat. As machine learning continues to evolve, its application in protein thermostability promises to further accelerate advancements in the field.

Improvement of *E. coli* as a chassis

The optimization of *E. coli* strain is essential for enhancing the overall yield of recombinant proteins which shall result in reducing the cost of recombinant GFs and SPs production. This involves different gene deletion and addition strategies to improve the overall yield of GFs and SPs, improving the processes, or addressing areas where *E. coli* as a chassis needs to be modified.

Engineering *E. coli* for glycosylation

Transferrin, a component of serum-free media, is a glycol-protein and has to undergo post translational modification. Human transferrin has two N-glycosylation sites—at asparagine 432 and asparagine 630. However, glycosylation in *E. coli* is rare. Only two glycoproteins have been recognized in *E. coli* [82, 83]. Therefore, to use *E. coli* to produce glyco-protein, strain engineering is required. Genetic engineering of *E. coli* with specific glycotransferase encoded in the genome is necessary to make this happen. For example, *N*-glycosyltransferase from *A. pleuropneumoniae* was co-expressed in *E. coli* expression system and glycosylation was observed in Human interferon α-2b (IFNα). Importantly, IFNα retained biologically activity and displayed proteolytic stability proving that *E. coli* can be utilized to produce glyoprotein required for serum free media [84] (Fig. 3).

Reducing acetate accumulation in *E. coli*

Higher acetate accumulation has been correlated to recombinant protein production [85], and high acetate concentration inhibits the cell growth and metabolism [86]. Thus, strains that produce less acetate is desirable for recombinant protein production. Knock out strains with deletion of genes of the acetate metabolism can be an avenue to explore for low acetate producing strains [87]. In a recent work, a single gene knock-out strain wherein arcA deletion, a global transcriptional regulator, decreased acetate under both transient and prolonged oxygen limitation [87]. In another work, *E. coli* (MEC697) strain with triple gene deletion i.e., nadR, nudC, and mazG genes exhibited decrease in acetate production and two-fold increase in β-galactosidase output during growth on glucose. nadR, nudC, and mazG genes are involved in the degradation pathway of NADH which subsequently results in a reduced NADH/NAD + ratio [88]. Identifying genes that can be selectively deleted to reduce acetate production without compromising other aspects of growth and metabolism enhances *E. coli's* suitability for recombinant protein production and simplifies bioprocess development.

Another strategy for reduced acetate production would be to engineer *E. coli* that has reduced glucose uptake [89, 90]. This way the slow glucose uptake not only reduce the effect of high acetate concentration but also mimic fed batch growth dynamics in batch mode. In one of the work, *E. coli* strains had targeted deletions in genes involved in glucose transport. One mutant strain (ΔptsHIcrr, ΔmglABC) outperformed the wild-type strain, producing up to 14 times more GFP in batch mode and yielding approximately 450 mg/L GFP compared to 220 mg/L for the wild type under equivalent fed-batch conditions. This approach reduces the time and resources typically required for process optimization and also provides a platform for early-stage screening, allowing for rapid identification of high-performing strains. The fed-batch process in a batch mode accelerates bioprocess development and enhances the scalability of recombinant GFs and SPs production in *E. coli* [90].

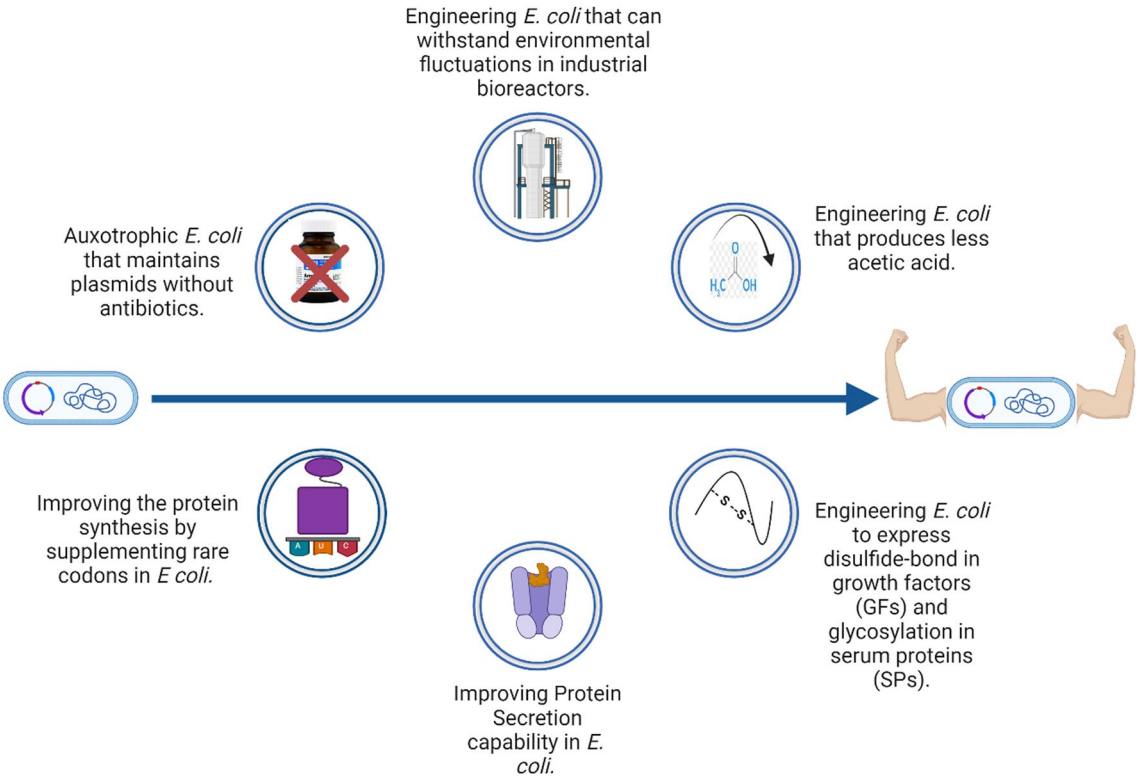

Fig. 3 Schematic highlighting key avenues in improving *E. coli* chassis for improving recombinant Growth Factors (GFs) and Serum Proteins (SPs) production for cultivated meat. (Created with www.biorender.com)

Optimizing *E. coli* strains for enhanced protein expression

Enhancing existing *E. coli* strains holds immense potential for optimizing protein yield. Codon-supplemented strains offer a solution to improve translation rates by alleviating bottlenecks associated with rare codons. For instance: SixPack strain integrates six of the least abundant tRNA genes into the BL21(DE3) chromosome [91]. Other commercial strains like BL21-RP, BL21-RIL, BL21-RPIL, Rosetta, and Rosetta-gami, tailored with codon supplements, provide versatile options depending on the specific protein of interest. Similarly, strains engineered to express disulfide-bonded proteins, such as BL21 trxB, Origami™, Rosetta-gami™, and SHuffle® will be useful to express GFs that has disulfide bonds [92, 93]. *E. coli* cells are typically not ideal for expressing proteins with disulfide bonds due to their naturally reducing cytoplasmic environment. However, by inhibiting the natural reduction pathways, the cytoplasmic environment can be made more oxidative, promoting the formation of disulfide bonds. This is achieved by deleting genes responsible for maintaining the reducing environment, specifically the thioredoxin reductase (trxB) and glutathione reductase (gor) genes [94]. However, it is important to note that some engineered strain like Origami™ can be challenging to work with, as they are sensitive and may not support high cell density growth. Furthermore, the CyDisCo method, which involves the co-expression of the protein of interest along with a sulfhydryl oxidase and a disulfide bond isomerase, is a well-established and effective strategy for producing disulfide-bonded proteins in *E. coli* [95]. FGF, an important GF of cultivated meat media, contains disulfide bonds. Therefore, utilizing these strains provides an added advantage in using *E. coli* as a chassis for FGF production.

Engineering *E. coli* for protein secretion

E. coli strains capable of protein secretion would be highly beneficial. While *E. coli* is not typically recognized for its protein secretion abilities, recently, using *E. coli* to produce extracellular protein has gained momentum [96]. In the work by Hogyun et al. (2019), *E. coli* could secrete Polyethylene terephthalate (PET) hydrolase. The Sec-dependent translocation signal peptides was fused with PET hydrolase and protein could use sec dependent pathway for translocation [97]. In the Sec-dependent pathway, proteins are translocated across the membrane in an unfolded state, and they subsequently fold into their native structure on the trans-side of the membrane. In addition to using Sec-dependent translocation signal peptides, co-production of cytoplasmic chaperones [98].

GroEL and DnaK has also been associated with protein secretion in *E. coli*. Wild and colleagues (1992) demonstrated evidence of DnaK's function during protein export. They demonstrated that the processing of alkaline phosphatase (AP), a SecB-independent secretory protein, was notably hindered when functional DnaK was absent [99]. Similarly, GroEL also plays a role in protein secretion [100]. *E. coli* cells engineered to overexpress GroEL and DnaK exhibit the capability to shuttle proteins from the cytoplasmic membrane into the periplasmic space. Notably, recent advancements have demonstrated that combining the overexpression of GroEL with the fusion of a TAT signal peptide to the somatropin enables efficient secretion of the protein [101]. The Twin-Arginine Translocation (TAT) pathway facilitates the translocation of folded secretory proteins into the periplasmic space. Recently, this pathway has garnered significant interest due to its inherent "quality control" mechanism, which ensures the prioritization of correctly folded proteins. The "TatExpress" strain was developed and utilized to achieve milligram-level production of human growth hormone, demonstrating its significant potential. In the TatExpress strain, a strong inducible bacterial promoter, ptac, is positioned upstream of the chromosomal tatABCD operon to drive the expression of TAT pathway proteins [98]. Essentially, expressing proteins such as GroEL and DnaK aids in secretion, while overexpressing translocation pathway proteins facilitates the movement of proteins from the cytoplasm to the periplasmic space, with some proteins eventually leaking into the external environment. In short, expressing proteins like GRoEl, DnaK helps in secretion while overexpressing protein of the translocation pathway helps ferry proteins from cytoplasm to periplasmic space. Some proteins eventually leak from periplasmic space to outer environment. Deleting some genes in *E. coli* can assist in increasing the efficiency of this process. For instance, Wacker Biotech, has developed ESETEC, a modified strain of *E. coli* K-12 engineered to secrete correctly folded proteins into the growth medium. ESETEC strains are equipped with a modified outer membrane, due to a mutation in the lpp gene responsible for the major outer membrane lipoprotein. This mutation leads to a leaky outer membrane, allowing proteins in the periplasmic space to access the outer environment readily [102]. Similarly, a strain lacking genes encoding proteases like htr (DegP), ompT (OmpT), ptr3 (Protease III), and tsp (Prc) is utilized to minimize proteolytic degradation of recombinant proteins targeted to accumulate within the periplasm, thereby aiding in the enhancement of protein secretion [103]. In essence, protease-deficient strains that also overexpress certain chaperones and translocation proteins serve as an ideal secretory *E. coli* chassis. This secretory chassis is well-suited for producing recombinant GFs and SPs essential for cultured meat production. Since GFs and SPs are integral to the cultured meat media recipe, minimizing process-related contaminants like host cell DNA and endotoxins, which can be present in intracellular GFs and SPs, provides an additional advantage by facilitating compliance with regulatory requirements.

Optimizing *E. coli* for scalable bioprocessing

The ultimate objective of bioprocessing is to scale-up production. According to the consolidated report by the Good Food Institute, economies of scale are a major factor in reducing the production costs of recombinant GFs and SPs [4]. As per their projection, if cultivated meat becomes mainstream by 2030, it is estimated that the production volume for recombinant albumin could reach 100,000 tonnes [4], necessitating the use of much larger bioreactors than those currently employed in biopharmaceutical processes. However, growing cells in such large bioreactors presents significant challenges, including reduced process yield due to process heterogeneities inherent to large-scale operations [104]. Therefore, it becomes imperative to engineer strains capable of adapting to the inherent heterogeneity of industrial bioreactors. This necessitates the utilization of scale-down models to create strains optimized for conditions representative of large-scale growth and production. Such an approach enables the selection of strains that scale up more reliably [105].

Industrial bioreactor conditions impose a considerable metabolic burden on cells, evidenced by a 40–50% increase in ATP maintenance demands. Strategic gene deletions can significantly reduce maintenance energy requirements while enhancing product yield. Researchers created an *E. coli* strain with targeted gene deletions in one example. These genes were identified through experiments that determined which genes increased maintenance energy demand when cells were grown in a scaled-down reactor mimicking industrial conditions. The resulting strain, with specific gene deletions, exhibited a notably lower maintenance coefficient compared to the wild-type *E. coli*. When grown in a scaled-down reactor simulating industrial bioreactor conditions, this deletion strain outperformed the wild-type, achieving a 44% higher eGFP yield after 28 h [106]. This study highlights the development of *E. coli* as a robust industrial chassis strain. A similar strategy was applied to enhance the production of industrially relevant octanoic acid. By deleting genes associated with energetically wasteful stress responses, researchers reduced basal maintenance energy requirements and improved productivity. The engineered *E. coli* strain significantly outperformed the wild-type in

a scale-down system, achieving higher yields of octanoic acid [107].

Looking forward, the use of well-studied proteome-reduced *E. coli* strains shows promise for industrial applications [107, 108]. Additionally, introducing beneficial genes to help cells adapt to process heterogeneities could further improve performance. For instance, the incorporation of the *Vitreoscilla* hemoglobin gene, which has high oxygen affinity, has been shown to improve *E. coli* growth under microaerobic conditions [109]. Combining these strategies may pave the way for more efficient and robust industrial microbial platforms.

Cell engineering strategy to improve recombinant GF and SP production

Gene addition and deletion alone may not always be sufficient to achieve the desired outcomes. In some cases, precise fine-tuning of genetic engineering is necessary to meet specific goals. In this section, we discuss strategies for engineering and optimizing genetic circuits within *E. coli* to strategically address key objectives, such as reducing production costs and improving yield.

Auxotrophic strain and autolysis

A promising avenue for early improvement lies in the development of strains capable of thriving in the absence of antibiotics without losing plasmid of interest. Auxotrophic *E. coli* strains are commonly used in the field of metabolic engineering, specifically substituting antibiotic selection pressure for plasmids with an auxotrophic approach. This involves the intentional removal of a vital survival gene from the chromosome, followed by its relocation onto a plasmid [80]. Creating antibiotic-independent strains can curtail the expenses associated with antibiotic use, presenting an approach for economical recombinant GFs and SPs production. Likewise, *E. coli* strains capable of autonomously producing lysozyme or other cell lysis proteins post-fermentation presents a promising approach to mitigate the expenses linked with lysis of the cells. Notably, XJ autolyzing strains commercially sold by Zymo Research present a proprietary solution to this challenge [81]. Chromosomally encoded bacteriophage lambda R gene, which encodes the lambda lysozyme, upon arabinose induction, can lyse cells and offers a cheaper way to burst open the cells to release intracellular protein.

Reducing cost associated with inducers

According to a techno-economic analysis based on producing industrially relevant enzymes using *E. coli*, Isopropyl β-D-1-thiogalactopyranoside accounts to a 10 percent cost in raw materials [110]. Therefore, leveraging well-characterized systems like the lac promoter in auto-induction media—comprising lactose and other sugars could be an effective cost mitigating strategy. Alternatively, model-based lactose addition strategies, along with fed-batch processes where high cAMP levels naturally induce lac promoter activity, could also reduce the need for IPTG while maintaining efficient induction [111].

Similarly, finding an alternative to IPTG induction can be another option. To start with, thermal induction emerges as a promising alternative. Current research has demonstrated various proof-of-concept studies supporting the efficacy of thermal induction [112]. Since protein induction is favorably influenced at lower temperatures and the bioreactor inherently integrates temperature controls, refining existing thermal induction methods emerges as a viable strategy for protein production while minimizing expenses related to IPTG. Additionally, alternative approaches such as quorum sensing, light-based induction [113], copper based induction [114], present possibilities for induction methods, further expanding the toolkit. The utilization of oxygen represents a promising strategy for induction in bioreactors [115], given the crucial need for precise oxygen control in such systems. Similarly, the adoption of urea, an inexpensive nitrogen source, for *E. coli* culture presents a viable alternative worthy of consideration for reducing induction costs [116].

Alleviating metabolic burden in *E. coli*

The metabolic burden associated with recombinant protein production in *E. coli* has been widely acknowledged [117, 118]. The burden adversely affects protein production efficiency over time, as the expression of heterologous proteins can overwhelm the cellular translation machinery. This in turn, impacts normal metabolism. As the cellular resources and energy are fixed, cells reorganize their metabolic pathway to adapt to recombinant protein production [119]. In the work by Basan et al., the expression of the gratuitous protein in *E. coli* resulted in a decrease of respiratory enzymes [120]. Thus, controlling the recombinant protein production so that it does not overwhelm the normal metabolism is helpful for healthy productive cells. Consequently, mitigating this metabolic burden is crucial to resolving this issue. Synthetic circuits that can sense metabolic burden in cells and readjust the translation of proteins accordingly can be another approach to alleviate the effect of metabolic burden. In one of the seminal works on burden-driven feedback control, a feedback-regulation system was developed to adjust the expression of a synthetic construct in response to cellular burden. This ensured the maintenance of the cell's capacity for basal gene expression, thereby guaranteeing robust growth. Consequently, cells equipped with

this regulatory system outperformed wild-types on their protein yield during batch production [121]. Subsequent studies have explored alternative genetic circuits [122] and different sensing mechanisms to detect metabolic burden within cells [123], building upon the foundational work.

Decoupling growth and production

An orthogonal system that decouples growth and recombinant protein production offers significant advantages in flexibility, control, and predictability. By allowing growth and production to occur independently, such systems can optimize resource allocation and enhance production yields. As demonstrated by the work of Michael Jewett [124] and Alexander Mankin's group [125], the ribosomes can be engineered to exclusively translate heterologous mRNA, leading to more predictable recombinant protein production and enhanced control over resource allocation. Customization of 16S rRNA in a ribosome creates an orthologous ribosome (o-ribosome). Notably, the o-ribosome retains the capability to translate endogenous mRNA in the absence of orthologous mRNA. However, as orthologous mRNA levels rise, the o-ribosome selectively recognizes and translates it, thereby preventing interference with normal host ribosome function and metabolism. Despite these advancements, it's important to note that orthogonal ribosomes translation still occurs at a slower rate compared to native ribosomes [126]. However, we can anticipate that the development of faster translation speed in the o-ribosome [127] would be valuable for recombinant protein production in *E. coli*.

Another cell engineering approach to increase protein production would be decoupling cell growth from recombinant protein production. When *E. coli* is used to produce recombinant protein, the initial stage involves cultivation to attain high biomass, known as the growth phase. Subsequently, an inducer is introduced, marking the onset of the induction phase where recombinant protein production takes place. Despite this being the production phase, growth continues to occur, and the metabolic burden issue may arise during this phase. Hence, decoupling growth and production can be a favorable strategy. Various approaches exist to achieve this goal. Recently, a novel growth switch was implemented in *E. coli* by permanently removing the origin of replication (oriC) from the chromosome, halting cell division while maintaining metabolic activity [128]. Likewise, in another work, a bacteriophage-derived *E. coli* RNA polymerase (RNAP) inhibitor peptide was expressed. By doing this, cell division and host mRNA transcription was inhibited while allowing for transcription of genes by the orthogonal T7 RNAP. The RNAP inhibitor peptide prevents σ-factor 70 mediated formation of transcription complex, inhibiting transcription of host genes driven by σ-factor 70 and directing metabolic resources exclusively towards synthesis of the protein of interest (POI). Co-expression of a phage-derived xenogeneic regulator enhanced recombinant protein production under industrial fed-batch conditions, leading to up to 3.4-fold improvement in total and soluble protein yields compared to the reference system [129].

Innovations in bioprocess for cost-reduction.

Bioprocess optimization is crucial for enhancing the yield and titer of recombinant GFs and SPs from optimized strain. It is also essential in screening experiments to identify best performing strain. As bioprocess optimization is a step-by-step, time-consuming, and resource-intensive process, it must be employed judiciously to minimize costs. Innovations in bioprocessing are critical as they shorten the time from concept to commercialization, ultimately saving time and expense. Increased yield and titer can significantly reduce overall production costs.

Small-scale bioreactor applications and protein detection methods

Optimizing bioprocesses often involves refining process conditions and media composition, which requires numerous experiments. Screening experiments are essential to identify the best strains for further optimization. The BioLector, a baffled microtiter plate capable of monitoring cell growth, dissolved oxygen (DO), and pH has become a staple in high-throughput experiments due to its ability to replicate bench-scale bioreactors [130]. Similarly, there are open-source projects available that make bioreactors more affordable for laboratories by using commonly available hardware. Companies like Pioreactor offer more comparatively cost-effective, small-scale (20 ml) reactors for labs that lack the resources to purchase expensive bioreactors. Likewise, many groups are working on reducing the size of bioreactors suitable for batch [131], fed batch [132], and chemostat processes [133]. Smaller bioreactors come with the benefit of lower operational costs. However, its small volume poses a challenge, particularly for replicating fed-batch scenarios, a critical factor in many optimizations. Despite this, advancements have been made, such as a microtiter plate cultivation protocol for *E. coli* in a micro-bioreactor system, which uses an enzymatic glucose release medium to simulate carbon-limited growth in a fed-batch process [134].

While small-scale bioreactors streamline experimentation, protein quantification remains a bottleneck. Traditional protein visualization techniques, such as Western blot, dot blot, or SDS-PAGE, are not easily scalable and

hinder the efficiency of optimization experiments. To overcome these obstacles, scalable technologies are needed to quantify protein without purification to facilitate efficient bioprocess optimization. Commercially available microfluidic chip-based electrophoresis systems provide another powerful tool for protein quantification in optimization experiments. These systems require small sample volumes, offer rapid detection, and are highly sensitive, complementing high-throughput bioprocess optimization [135].

Mass Photometry, a recently developed technique, measures the light scattered by individual proteins and other biomolecules. This scattered light signal is utilized to count the molecules and accurately determine their mass [136]. This method has proven effective in quantifying the mass of individual molecules and has been tested to analyze protein–protein interactions [137]. This promising technique, which is sensitive, rapid, and requires minimal sample consumption, is well-suited for protein quantification for high-throughput optimization and screening experiments. Similarly, Aptamer-Based biosensor provides a promising method for detecting target proteins and can be integrated with high-throughput experiments due to their fluorescent readout [138, 139]. However, its application in bioprocessing has yet to be explored (Fig. 4).

Optimizing fed-batch processes and automation in bioprocess development

In fed-batch processes, managing the feeding process is complex because metabolic activity slows down over time, reducing nutrient uptake demand. Overfeeding must be avoided due to the risk of acetate production. Therefore, implementing a proper feeding profile is crucial. A model-based feeding approach, where the kinetics of the strain is characterized beforehand, can help optimize the feeding profile to maximize product titer while avoiding overflow metabolism [140]. This approach also facilitates the implementation of Process Analytical Technology (PAT), which is an added advantage. Multiple at-line sensors, such as Raman spectroscopy and various glucose sensors, can help establish an effective feeding profile [141]. Incorporating these sensors makes the fermentation process more robust and less prone to failure. Utilizing model-based decisions, enhanced with data from at-line sensors and techniques like Kalman filters, promises higher fermentation titers and reduced failure rates [142].

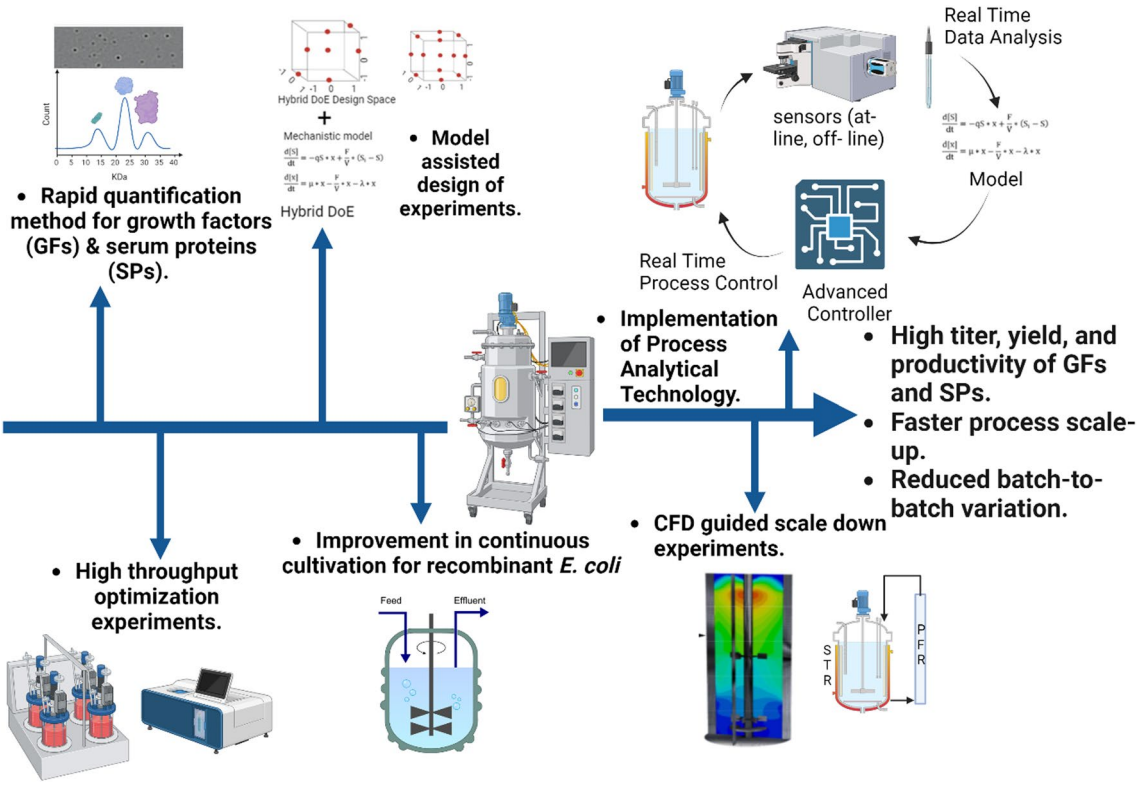

Fig. 4 Schematic highlighting key areas in bioprocess that can reduce recombinant Growth Factors (GFs) and Serum Proteins (SPs) production cost for cultivated meat. (Created with www.biorender.com, CFD image is taken from lecture slide of BioTechDelft course "Modelling and Computation for Micro-organisms in Bioprocesses")

The bioprocess is evolving rapidly toward greater integration, automation, and data-driven optimization. The adoption of advanced parallel mini-bioreactor systems [143–145] and their integration with digital infrastructures [146] is necessary to streamline the development process. The platforms that can do high-throughput experimentation and implement control strategies, such as Model Predictive Control [143, 144] to address challenges arising from nonlinear dynamics, parameter uncertainties, and process constraints. The use of modular and automated workflows, incorporating robotics and feedback-driven operations, enables integration of cultivation, data generation and analysis [145]. Additionally, model-based experimental design and optimization approaches [144, 147] should be implemented to explore complex design spaces efficiently, making the characterization of strains and processes robust, rapid and scalable. Collectively, the fully integrated, autonomous bioprocessing platforms can accelerate development timelines of strain and processes for GFs and SPs production from *E. coli*, reducing costs, and giving confidence in the production processes.

Advancing bioprocess optimization: intensified doe and hybrid modeling for reduced experimentation

Statistical Design of Experiments (DoE) is a widely utilized in bioprocess optimization, with recent improvements enhancing its effectiveness [148]. In a study by Von Stosch and colleagues, they proposed a novel approach called intensified Design of Experiments (iDoE). This method involves shifting the conditions during each experiment, reducing the total number of experiments required compared to traditional DoE. iDoE compresses the evaluation of several DoE combinations into fewer experiments, enhancing efficiency [149]. Similarly, hybrid modeling integrates process knowledge with data-driven methods, can accelerate optimization processes. In hybrid modeling, complex parameters in a mechanistic model are replaced with data-driven models [150]. Furthermore, the combination of iDoE and hybrid modeling enhances optimization efficiency. Specifically, a dynamic hybrid model incorporating differential equations, and a feedforward artificial neural network was employed to describe data from iDoE experiments, where conditions were varied during the experiment. This model was trained using data from an *E. coli* fermentation. The case study results indicate that the combined use of iDoE and hybrid modeling can reduce the total number of required experiments by approximately 40% compared to traditional DoE [151]. This approach improves optimization efficiency and helps lower the cost of experimental procedures.

Continuous bioprocessing for *E. coli*

Continuous bioprocess operation is beneficial for scaling up, as it ensures uninterrupted production and minimizes downtime in downstream operations, thereby enhancing productivity. However, implementing continuous operation for engineered *E. coli* presents challenges. Over time, their nutrient uptake capacity decreases because of metabolic burden, necessitating adjustments to the flow rate, which can be challenging. Additionally, cell stability is a concern, as cheater cells that do not produce protein can outcompete productive cells over the long term [152, 153]. Recently, a cascade approach for continuous bioprocessing has shown promise. In this method, cells for growth and production are segregated into two bioreactors. Fresh, uninduced cells are continuously supplied from the first reactor to the second, where they are induced and both reactors operate in continuous mode. This approach has improved protein yield and titer compared to conventional fed-batch processes [154].

Scale-down experiments and CFD models for efficient bioprocess scaling

Cells must eventually be grown in industrial bioreactors to achieve cost-effective production of GFs and SPs at scale. Scaling up is a challenging process, often leading to a decrease in productivity. It requires significant time and resources, leaving little room for failure. Therefore, a rational approach to scaling up is crucial. Industrial bioreactors present issues with mass transfer, leading to areas of high and low substrate concentrations, as well as varying oxygen demands. These substrate and oxygen gradients can reduce biomass and product yield. The lower oxygen transfer rate in large bioreactors compared to lab-scale ones creates aerobic and anaerobic zones, subjecting cells to aerobic-anaerobic [155] and feast-famine cycles [156]. Understanding these heterogeneities is essential for expediting the scaling-up process. Carefully designed scale-down experiments can mimic the environmental heterogeneity of large-scale bioreactors, making them practical for investigating the effects of these gradients on cell physiology and process efficiency.

A hydrodynamic model, such as Computational Fluid Dynamics (CFD), can guide these scale-down experiments. For instance, research conducted at Delft University, though not specifically for *E. coli*, provides valuable insights into connecting CFD, kinetic models, and scale-down approaches for efficient scaling up. Initially, a kinetic metabolic model is developed to capture both the short-term feast-famine cycle for substrates and the long-term effects of this cycle [157]. This kinetic model was then integrated with the CFD model. Using the Euler–Lagrange approach, CFD simulations calculate the

organism's trajectory in an industrial bioreactor (e.g., a 140 m^3 reactor). The "lifeline" of glucose uptake along this trajectory is determined by coupling the kinetic model, allowing for metabolic regime analysis that identifies the type and duration of substrate fluctuations [158]. These calculations represent actual fermentation processes and inform the design of scale-down experiments, as demonstrated by Haringa et al. [159] and Kuschel et al. [160] These experiments, guided by the CFD model, provide data on timescales from seconds to hours under industrially relevant conditions. From this data, precise cell kinetics can be inferred, leading to the development of models that elucidate the impact of environmental oscillations on *E. coli* over short and long fermentation periods.

While Computational Fluid Dynamics (CFD) models are accurate for understanding scale-up challenges, they can be complex and resource-intensive to implement. A more accessible alternative is the use of simpler mechanistic models. For example, mechanistic models for *E. coli* have been parameterized to describe physiological behaviour under environmental gradients in two-compartment scale-down experiments combining Stirred Tank Reactor (STR) and Plug Flow Reactor (PFR) setups. Once parameterized, these models can be used to extrapolate the strain performance at different circulation times and pulse feeding profiles, offering a less computationally demanding but lower-accuracy alternative to CFD modelling [161]. For scenarios where a complex STR-PFR setup is unavailable, simplified mechanistic models parameterized through glucose pulse experiments using enzymatic glucose feeds can replicate glucose concentration gradients observed in larger bioreactors in a small-scale parallel bioreactor. This approach reduces computational burden and simplifies experimental setups while retaining the ability to mimic the physiological responses seen in industrial-scale systems. This approach can accelerate bioprocess characterization incorporating scale-up considerations and can also screen strain suitable for scale up conditions [162].

Circular bioprocess

Another potential avenue for cost reduction in bioprocessing is the utilization of spent cultivated meat media. Studies have shown that spent media retains residual nutrients, including amino acids, glucose, and likely vitamins, which can be fortified and repurposed as growth media for microbial cell cultivation. These microbial cultures can, in turn, be used for the production of recombinant GFs and SPs [163]. As cultivated meat production scales up, large volumes of spent media will be generated. Repurposing this spent media for the production of recombinant GFs and SPs promotes sustainability, and also reduces the costs associated with microbial cell culture media.

To sum up, employing either or all of, the high-throughput experiments, the iDoE approach, hybrid modeling, kinetic modeling, and CFD-guided scale-down experiments will expedite the screening, optimization, and scaling-up processes. This integrated strategy will significantly shorten the time from concept to reality and enhance confidence in scaling processes from lab scale to industrial reactors. Ultimately, this will reduce time and costs, significantly boosting the large-scale production of GFs and SPs.

While *E. coli* remains the most technologically mature platform for producing recombinant GFs and SPs, it does have limitations that highlight the need for continued innovation. For example, challenges persist with high molecular weight proteins like transferrin. In such cases, alternative host like *Pichia pastoris* is preferrable. Additionally, endotoxin contamination remains a concern, necessitating extra purification steps. However, since these products are intended for food applications, the regulatory requirements are generally less stringent than those for pharmaceuticals. The formation of inclusion bodies continues to be an issue with *E. coli*, and its secretion efficiency is not on par with other industrial workhorses like *Pichia pastoris*. Alternative production platforms, such as other microbial hosts, plant-based systems, and cell-free synthesis, show promise but are less developed compared to *E. coli*. Despite its drawbacks, the advantages of *E. coli*—including its well-established technology, scalability, and cost-effectiveness—make it a suitable platform for producing recombinant GFs and SPs for cultivated meat applications.

Conclusion and outlook

Cultivated meat presents significant challenges in achieving cost-effectiveness, but these hurdles also open exciting avenues for innovation. This review has focused on addressing the primary cost drivers of cultivated meat media, particularly recombinant GFs and SPs, which constitute a substantial portion of production expenses. Utilizing *E. coli* as a platform for recombinant GFs and SPs production emerges as a promising, cost-effective solution.

The review serves as a comprehensive resource for researchers across diverse disciplines dedicated to reducing the cost of recombinant GFs and SPs, ultimately advancing the scalability and affordability of cultivated meat production. Organized around four key objectives—yield enhancement, product improvement, purification innovation, and process innovation— this review highlights a blend of practical, cutting-edge, and forward-looking approaches to address these challenges:

Yield Enhancement using fusion tags Strategies such as fusion tags to improve protein solubility are widely implemented, straightforward, and effective.
Purification Innovation using aggregating tags Methods to reduce purification costs, including the use of aggregating tags, hold great promise despite limited widespread adoption.
Product Improvement Approaches to enhance GFs and RPs stability, including conventional methods and in silico techniques for thermostability, were thoroughly explored.
Yield enhancement using cell engineering strategies Innovations such as eliminating the need for antibiotics or IPTG, decoupling growth and production, enhancing *E. coli's* protein production capabilities, and glycosylation strategies were reviewed. While some strategies, like antibiotic elimination, are achievable in the near term, others, such as glycosylation in *E. coli* and preparing *E. coli* for industrial-scale bioreactors, require further research and development.
Process Innovation Bioprocess advancements, including fermentation process optimization and leveraging high-throughput experimentation integrated with predictive modeling, were emphasized as crucial to streamlining decision-making and improving outcomes.

In a hypothetical future, the innovative approaches discussed in this review could converge to revolutionize the cost-effective production of recombinant GFs and SPs from *E. coli*. For instance, thermostable GFs could be designed using computational tools and integrated with fusion tags to enhance expression and solubility. These GFs could then be produced in *E. coli* strains that do not require antibiotic selection systems, capable of achieving high levels of induction through temperature control. Additionally, these strains would be well-suited for operation in heterogeneous industrial bioreactors, efficiently secreting GFs into the culture medium. Bioprocess advancements, such as real-time monitoring and advanced process control, could optimize feeding strategies to maximize GFs production. Reversible aggregating tags could streamline protein concentration from large volumes, simplifying downstream purification and polishing steps. By leveraging these synergistic innovations, the production of recombinant GFs and SPs could achieve a significant reduction in costs, making them appropriate for cultivated meat production.

In conclusion, the strategies outlined in this review, when implemented synergistically, offer the potential to significantly reduce the cost of producing GFs and RPs, paving the way for more affordable cultivated meat media. While it should also note that not all the strategies should be implemented. While most of the existing technologies have been tested individually or for different modalities or processes, their integrated use remains challenging. Bridging multiple disciplines to enable a concerted approach is still a complex task. Realizing this vision will require research, innovation, and collaboration across multiple scientific and engineering disciplines. The path forward is challenging, yet the opportunities for breakthroughs make the journey worthwhile.

Abbreviations

BRT17	β-Roll Tag
CBD	Cellulose binding domain
CspB	Cell surface protein B
CFD	Computational fluid dynamics
CNBr	Cyanogen bromide
DOE	Design of experiments
DO	Dissolved oxygen
DsbA	Disulfide bond oxidoreductase A
ELP	Elastin like polypeptide
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GFs	Growth factors
gor	Glutathione reductase
GST	Glutathione S-transferase
HlyA60	Hemolysin A
IGF	Insulin-like growth factor
IFNα	Human interferon α-2b
iDOE	Intensified design of experiments
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MBP	Maltose binding protein
NRG	Neuregulin
oriC	Origin of replication
PDGFB	Platelet-derived growth factor subunit B
PET	Polyethylene terephthalate
PROSS	Protein repair one stop shop
POI	Protein of interest (POI)
RNAP	RNA polymerase
RPs	Recombinant proteins
sGFP	Superfolder green fluorescent protein
SUMO	Small ubiquitin-like modifier
trxB	Thioredoxin reductase
TGFβ	Transforming growth factor beta

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Ethics approval and consent to participate
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