Recommendations for Fungal Production of Bovine Albumin, a Component for Fetal Bovine Serum Substitutes

Executive Summary

A significant proportion of the cost of producing cultivated meats is due to the need for FBS (Fetal Bovine Serum). We propose using fungi to produce the largest component of FBS, bovine albumin, to reduce cost and reduce the need for animal products.

The aim of this study is to explore the potential of the filamentous fungi *Neurospora crassa* to produce bovine albumin, a critical component of FBS. The fungal production of bovine albumin is critical in producing FBS alternatives. *Neurospora crassa* is so far being used in biotechnology and healthcare applications as a cell factory and shows immense potential to be used in cell ag applications, especially in the case of cultivated meat. For the proof-of-concept study, we compared the cell densities of cultures grown with and without FBS at the end of three days. Our research represents an opportunity to create a serum-free growth media alternative from modified fungi that is cost-effective and reduces a reliance on animal products.

Objective Overview

To create cultivated food products that are available at price parity with traditional animal products, several challenges need to be overcome to produce at scale and bring down costs. As the production of growth factors and recombinant proteins for preparation of culture media contribute to 99% of overall production costs[16] (i.e. due to the need for FBS), we propose using modified fungi as a natural bioreactor to produce an alternative to FBS, bovine albumin, to reduce cost and reduce the need for animal products. This study aims to explore the potential of the filamentous fungi *Neurospora crassa* to produce bovine albumin, a critical component of FBS.

Existing protein production methods for FBS

Economics of bovine albumin production methods today

FBS is collected in meat processing plants at the evisceration stage. The FBS is later collected from the fetus through a catheterised umbilical vessel or a 12-16 gauge needle into the fetal heart[34]. Alternatively, the production of bovine serum albumin (BSA) is based on the method developed by Cohn et al., 1946[7]. The chromatography-based process has been selected as the principal method for the production of high-purity BSA[8,9]. Concentration of serum albumin is 1.3% in whole blood with an average recovery of 2% from plasma. The cost of plasma production is on average $0.92/kg of plasma[15]. Considering a 2% yield, the cost of bovine serum albumin would be on average $46/kg.

Existing Fungal Protein Production Methods

Use of fungi for protein production

Filamentous fungi are a large group of multicellular fungi that form branching tubular structures called hyphae. They are commonly referred to as molds. We propose using *Neurospora crassa,* a species that is currently used for heterologous (non-native) fungal protein production.

Filamentous fungi secrete large amounts of proteins due to their native ability to secrete enzymes and other metabolites, which has attracted interest in utilizing them as methods to produce a wide range of products. Filamentous fungi are able to grow on simple and inexpensive substrates, making them valuable for decreasing costs of protein production. Therefore, they could be a valuable tool in decreasing the costs of producing meats in cellular agriculture by producing components needed to produce a serum-free media.

There have been a number of efforts to increase the expression levels and production of recombinant proteins in fungi. However, there are still a number of challenges in expressing heterologous proteins.

Technical aspects of fungal protein production

Yields of fungal proteins are up to 25g/L in mixed stirred tank reactors[39]. With an estimate of 1 million liters of FBS consumed per year and an average of 45g albumin/L this would require approximately 1.8 million liters of bioreactor space a year to produce enough albumin to replace the global supply of albumin in FBS. Increase in the yield of fungal proteins would decrease the space and cost needed for albumin production.

Challenges in fungal production

Yields of heterologous proteins in fungi can be orders of magnitude lower than that of homologous proteins, though this decrease varies depending on the protein[36]. Limitations happen at all levels of protein production. Therefore, in order to increase albumin yields from fungal hosts, modifications can be made at all levels of protein production.

Intracellular Process Modifications

ERAD (Endoplasmic reticulum associated protein degradation) is a process in which abnormally folded proteins, including the heterologous protein, triggers repression of protein secretion, which decreases the production of desired products and is a major obstacle to heterologous protein production. Deletion of the genes involved in ERAD such as *doaA* and *sttC* increased the secretion of heterologous proteins[37]. Therefore, a potential fungal host should have modifications to offset the aforementioned complications arising from ERAD.

Fusion Proteins increase stability of heterologous proteins and encourage exudation

A number of authors have used fungal hosts to produce camelid single-domain antibodies, specifically adalimumab (VHH). The addition of 0.25% BSA increases the production of VGG 5-fold.[32] The highest yield in *Aspergillus oryzae* reached 73 mg/L at most; this was accomplished by fusion of the target protein with α-amylase AmyB, which is an exuded protein; with this technique, a productivity of 39.7 mg/L adiamab was acquired[23]. Fusion of the heterologous proteins to naturally secreted proteins enhances protein stability, promoting translocation and protection from degradation; Xylanase A (XYNA) was found to be an effective carrier protein for fusion with the desired heterologous protein[37].

Increasing Solubility of Heterologous Proteins

For ease of purification, the target protein should be soluble, meaning that it is not localized to a membrane. Solubility improvements were demonstrated by Allgaier[2], who used the filamentous fungi *Neurospora crassa* to produce subunit vaccines for influenza. Specifically, the authors demonstrated secretion and partial purification of hemagglutinin (HA) and neuraminidase (NA), two influenza membra. Soluble proteins were produced by modifying and truncating full length hemagglutinin and neuraminidase proteins. The native signal peptide was replaced with a fungal leader sequence from the *Cel12A* gene, which is a region of an mRNA molecule that precedes the coding sequence of a gene. The proteins were made soluble (and therefore easier to purify) by deleting the C-terminus. Finally, a 9-His tag was added on the C-terminus for Ni-NTA protein purification techniques. These modifications resulted in an easily purified protein for vaccine production. Similar techniques could be applied to our bovine albumin producing fungi.

Extracellular Process Modifications

Extracellular degradation by fungal proteases decreases the yield of heterologous proteins.[32] Deletion of secreted proteases has been proposed[37]. The construction of a protease-deficient strain increased the secreted cellulase in Myceliophthora thermophila to five times higher than the original strain[23].

Fungal Bioprocessing Modifications

Fungal product yields can also be improved by optimizing the growth conditions and morphology of the fungal culture. Higher numbers of branches in filamentous fungi are associated with higher yields of secreted proteins; deletion of *racA*, a Rho GTPase involved in actin control resulted in an increase in mycelial tips and a corresponding increase in the secreted protein glucoamylase in *A. niger.*[35]

Other bioprocessing techniques include modification of the bioreactor. Fungi cultivated in layered packed-bed bioreactors have been demonstrated as a more efficient reactor design. This design consisted of three layers of fungi in screen baskets separated by air which allowed for more efficient heat removal and therefore higher yields of cellulase[6]. Though this experiment was not for heterologous protein production, the increased yield of exuded proteins implies that

exuded heterologous proteins may also be observed. Additionally, the solid-state status of the packed-bed reactors produce a more homogenous culture with similar size and gene expression of colonies, which produces less batch-to-batch variation[40]. These cultivation techniques for our proposed system should be considered to increase yield.

Transformation of Filamentous Fungi

Fungal genetic transformation has been challenging due to the presence of the fungal cell wall. However, there exists a number of techniques for fungal transformation. Protoplast-mediated transformation is the most common fungal transformation method in which enzymes are used to remove the cell wall for transformation; the resulting protoplasts then uptake the provided exogenous DNA for transformation[37]. This technique has been used to transform newly born hyphae in *Neurospora*, the target organism of this work.

Output streams

Waste or contaminants present in the production of biopharmaceuticals from fungi and could also be present in the production of bovine serum albumin includes host cell, process, and product related impurities. Host cell impurities encompass host cell proteins and DNA, process related impurities could be buffers, leached ligands and antifoaming agents, and finally product related impurities include aggregates or fragments of the produced protein. Steps to remove and purify the compound include an initial recovery step which is the separation of the microorganism and the supernatant. The separation is usually achieved through centrifugation, filtration, sedimentation, and flotation. Later the supernatant is ultrafiltrated and the protein or compound of interest purified or concentrated following precipitation or chromatography[3]. An alternative approach to chromatography, such as precipitation through the bovine serum albumin isoelectric point, could be employed to avoid increasing the production cost by using chromatography which is generally used for high purity compounds. Industry experts are still determining the minimum BSA purity needed for the myoblast growth and differentiation.

Potential application areas of the resource

To replace FBS in growing cultured meat, there are a number of advantages to using animal-free serum.

The largest advantage is that fungal proteins would be significantly less expensive to use as an FBS alternative as FBS makes up a huge part of the production cost. Compositionally, producing FBS with fungi could result in a more consistent cell-based performance since serum-free media allow a more controlled culture environment that results in greater productivity. The removal of animals from the system is also more ethical and decreases the risk of contamination with endotoxin, hemoglobin effects, and infectious agents.

However, fungal-produced albumin also has some drawbacks. Currently, it is only reproducible lab-scale and will require more time to scale up and lower costs of production. Scaling up will also result in eventual impact on environmental costs and human health. These effects are currently uncertain. Thus, it may be difficult to create a business case for this.

FBS is used in cell culture for biopharmaceuticals, vaccines, as well as cellular agriculture (e..g cultivated meats). Having a FBS-free media option would be useful in any application where we use cell culture.

Roadmap for resource utilization for application area(s) or current resource that it could replace

Previous studies looking to replace FBS have found alternative FBS-free and serum-free media that enable the growth and proliferation of bovine embryos and chick myoblast cells [24,31]. In both studies bovine serum albumin, insulin, transferrin, and a cell specific growth factor was utilized. Interestingly, the FBS-free media enable the production of bovine embryos and the proliferation and differentiation of the chick myogenic cells [24,31]. Both studies support our proposal of producing bovine serum albumin as a supplement for FBS-free media which would be cheaper to produce than the conventional chromatographic methods through the filamentous fungi *Neurospora crassa.* A recent study found the differentiation of bovine satellite cells in absence of FBS possible by activating the receptors expressed during early stages of differentiation.[1] The receptors Insulin Like Growth Factor 1 Receptor (IGF1R), Transferrin Receptor (*TFRC*), and Lysophosphatidic Acid Receptor 1 (LPAR1) were activated with ligands to induce differentiation into myogenic cells[11] It is still to be elucidated the receptor that interacts with bovine serum albumin which could be used to identify other cell cultures from different sources that could use

bovine serum albumin as a supplement in the absence of FBS. Such sources are chicken or seafood satellite cells which are included in current startup companies such as GOOD Meats and BlueNalu, representing a potential market for the BSA cell media supplementation.

Evaluation of the sustainability impact

Sustainability Comparison: FBS versus recombinant albumin from fungi

Consider whether producing a protein media supplement from filamentous fungi would be more sustainable than the FBS production process. For the purposes of this analysis, a production process is sustainable if its raw materials are in ample supply, its waste streams are minimal and/or can be valorized, and production is economical in time and cost. It is also important to consider how each process contributes to greenhouse gas emissions through energy demands and the carbon cycle.

There is plenty of data on these components for FBS and its source, the cattle industry. However, there is a lack of data on industrial-scale protein production in filamentous fungi because the largest applications so far are pilot-scale. Our sustainability data for fungal protein comes from bench and pilot-scale studies of using filamentous fungi to produce biomass such as protein. A full Life Cycle Assessment of protein production in filamentous fungi is beyond the scope of this report. Further scientific enquiry and data generation about fungal protein production at scale would enable a quantitative comparison of sustainability with FBS. The comparisons to follow are quantitative whenever data is available.

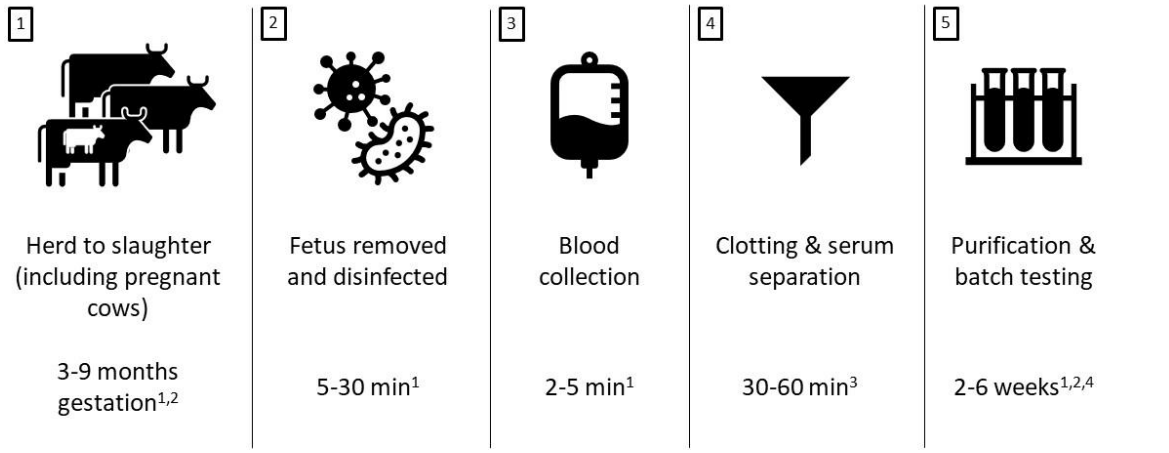

Figure 1: Overview of the FBS production process with the typical timeline for each step.

FBS comes from the fetuses of beef cows who are pregnant at the time of slaughter [18,20,33]. This is not uncommon in the harvest of mixed-sex free-grazing cattle herds[20]. Fetuses are harvested for serum if they are between 3-9 months gestation. After the slaughter of the mother, the reproductive tract and fetus are removed and disinfected [18,20]. The harvester extracts fetal blood via the heart or umbilical cord [20]. After clotting, technicians separate serum and plasma components of the blood. The serum undergoes purification and irradiation to remove bacteria and viruses[18]. FBS suppliers evaluate the performance of each batch on cell lines, as there is high batch-to-batch variability.[18,33,34] The purification and quality control process is labor-intensive and can take 2-8 weeks.[18,20,33] Fetal serum is preferable to calf or cow serum because it is low in immunoglobulins and high in growth factors and key serum proteins that promote growth and proliferation in cell culture.[18,20,34] Albumin is the most abundant protein in FBS at 50 mg/mL (about 50% of total serum protein mass).[12]

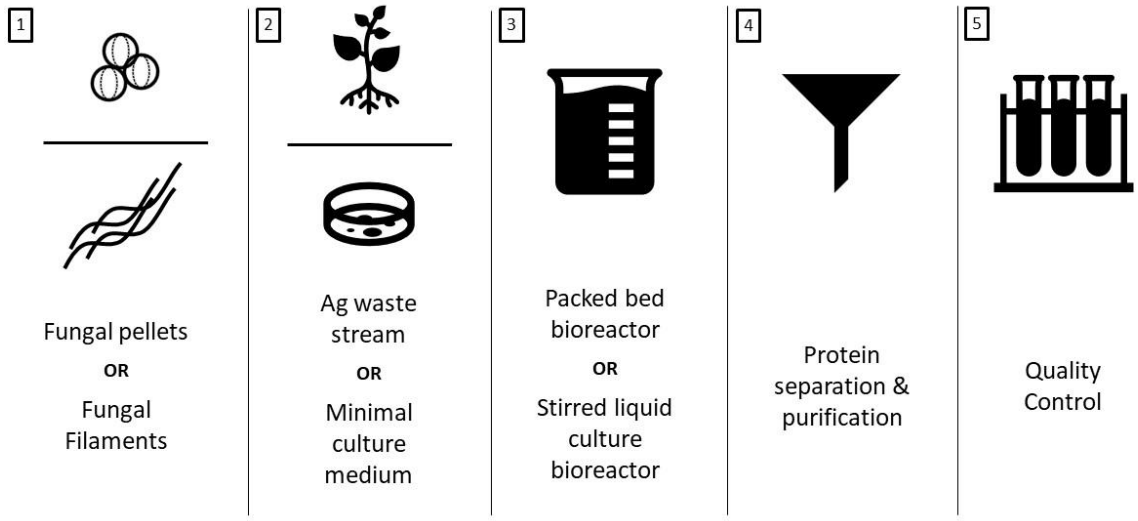

Figure 2: Overview of protein production with filamentous fungi. Fungal cultures are started from spores in the form of filaments or pellets. The proposed production process could be fed by minimal medium[28], or a waste biomass slurry from agriculture or industry such as rice polishings, wheat bran, lumber, or paper pulp.[1,14,21] Culture time for optimal protein yield can range from 3-7 days.[1,3,17,21] To produce a media supplement via this method, the target protein would need to be separated from the culture medium and byproducts and undergo quality control checks to verify adequate purity.

Filamentous fungi are decomposers with the ability to excrete proteins and other useful biomolecules.[32] Researchers have utilized this ability to express antibodies for vaccines and valorize agricultural waste to produce protein-rich biomass and useful chemicals.[1,2,6,10,19,21] Fungal spores often start in a pellet or filament-like morphology.[35] They grow easily on minimal media and a variety of carbon and nitrogen sources.[14,28,38] Incubation time for maximum product yield ranges from 3-7 days depending on the culture medium, fungal strain, and bioreactor design.[1,3,17,21] At harvest, one must separate fungal biomass from the culture medium through various techniques (centrifugation, sedimentation, filtration, etc).[1,21] A laboratory-grade media supplement such as recombinant albumin would require additional purification steps through chromatography or precipitation. Protein yield from filamentous fungi culture can be up to 25 g/L in stirred tank bioreactors[38]. Protein biomass yield from packed bed fungal culture can reach up to 10 g biomass/L agricultural waste.[3]

Comparison: Raw Materials Availability

FBS is a byproduct of the beef industry. Therefore, factors affecting beef and cattle affect the availability of FBS. Major cattle regions include the United States, New Zealand, Australia, and South and Central America[12]. Globally, over 300 million beef cattle are slaughtered annually; this includes between one to two million bovine fetuses from pregnant cows at the time of slaughter.[20] Fetuses at three, six, and nine months gestation yield approximately 150, 350, and 550 mL of FBS respectively. FBS supply can be highly variable due to conditions affecting cattle herds, such as drought, storms, herd health, demand for meat and dairy, and food prices.[20] These factors also influence batch-to-batch FBS variability and the viability of cells cultured with a given FBS batch.

In comparison, raw materials for fungal culture are more diverse and reliable in supply. Fungal cultures optimized to express target proteins have been grown on Minimal Media and Yeast Extract Peptone Dextrose Media (YEPD).[28,39] Filamentous fungi also grow well on agricultural and industrial residues. Examples of culture substrates include (not exhaustive) rice polishings, lumber waste, sugar beet pulp, wheat bran, oat hulls, and paper pulp waste.[1,3,4,14,21] Several of these studies optimized fungal strains and bioreactor design to produce protein (via biomass or excretions) using 40-60% dilution of agricultural waste streams.[1,3,4,14] Agricultural and industrial wastes are not typically considered a resource and their valorization into a valuable substance is highly desirable for environmental and economical reasons. Literature suggests fungal strains and bioreactor design can be adapted for optimal substrate utilization, so this production method is flexible for a number of raw material types.[14,21,35] A variety of plant-based substrate sources also ensures production can continue if one source is scarce. Filamentous fungi themselves are abundant in nature and easily isolated from the environment or recycled from previous incubations.[1,19,21] Common filamentous fungi strains used for protein production are *Neospora crassa, Aspergillus niger, Aspergillus oryzae,* and *Trichoderma harzianum,* which are commercially available and easily cultivated.[1,2,4,14,17,28,38]

Comparison: Timeline

The typical timeline to harvest and process FBS for sale is between 2-6 weeks. Slaughter and blood collection are complete within 5-35 minutes of entry to the slaughterhouse, depending on

the type of facility.[20] It takes 30-60 minutes for the blood to clot and to separate the serum from the plasma.[27] From there, labor-intensive purification and testing last for weeks. Raw FBS may contain bacteria, viruses, and prions that must be removed via filtration and irradiation.[18] FBS has high batch-to-batch variation, so suppliers must test each batch to confirm its growth factor and protein content are acceptable.[20] Cell banks also test FBS batches on a range of cell lines to evaluate their efficacy on different tissue types and applications. Cell line testing lasts for at least 2 weeks, as one may not be able to see the differentiating or apoptotic effects of an FBS batch immediately.[33] FBS is kept frozen unless carefully thawed for a purification step; purification protocols include at least 2 thaw/refreeze periods for prefiltration and sterile packaging per ISIA recommendations[18].

Filamentous fungal strains can produce biomass or protein from raw materials within 3-10 days. Culture time and other conditions such as temperature and pH can be tuned with machine learning models to optimize yield.[19,35] Biomass production usually requires shorter incubation periods than specific target products like antibodies. Below is a survey of filamentous fungal strains used to produce protein biomass and their incubation times for maximum yield. Target products range in specificity from crude protein to specific antibody subunits. Substrates include defined media used in routine laboratory fungal culture, agricultural waste products, and blends of the two.

Paper	**Strain**	**Culture media**	**Max yield (protein/culture medium vol.)**	**Incubation time**
Ward et al. 2004	*Aspergillus niger*	Corn Steep Liquor (CSL)-Fructose Medium	0.9 g/L antibodies	60 hours
Huynh et al. 2020	*Aspergillus oryzae*	Potato Dextrose Agar (PDA)	0.397 g/L antibody subunits	6 days
Khamrai et al. 2019	*Phanerochaete*	Yeast Extract Peptone Dextrose (YEPD)	3.9 g/L biomass	7 days

	chrysosporium			
Richter et al. 2014	*Aspergillus niger*	Minimal Medium (MM)	4.5 g/L peptides 24.9 g/L biomass	66 hours
Ahmed et al. 2017	*Trichoderma harzianum*	Rice polishings, Vogel's medium	22 g/L biomass	72 hours
Asadollahzadeh et al. 2018	*Aspergillus oryzae*	Spent sulfite liquor (SSL), PDA	10 g/L biomass	4 days
Futamura et al. 2001	*Aspergillus oryzae*	Cotton seed meal, gluten meal, soybean meal, wheat germ sungrain, PDA	110 g/L (PDA) 31 g/L (wheat germ) biomass	6 days

Figure 3: Survey of timelines for maximum protein yield in filamentous fungal culture.

Comparison: Cost

FBS is the most costly component of cell culture media. FBS cost has increased over 300% since 2015 due to increased demand and low supply.[11] In 2015, the price for 500 mL of FBS increased from €80 to €1200.[33] Demand is expected to increase as researchers embrace cell lines as a tool to study disease and develop pharmaceuticals[18]. One would expect the cost of FBS to continue to increase into the future.

There is little data on the cost of the fungal protein production method proposed. However, there are technical aspects of this approach that could reduce hypothetical costs. Filamentous fungi excrete proteins, which is cheaper and easier to purify than other microbial sources.[1] One can also recycle fungal filaments and pellets when a batch finishes incubating to inoculate the next, as fungi exhibit indeterminate growth.[1,19] There is also evidence that filamentous fungi can produce useful proteins from agricultural and industrial waste streams. Valorizing waste streams

turns them into a revenue source instead of a remediation cost sink. For example, *Aspergillus niger* is a filamentous fungal strain used in food, pharmaceutical, and other industries to produce citric acid from a variety of culture media. The estimated global market value of this application is over $2 billion.[4] An optimized fungal strain for producing recombinant proteins from waste streams could follow a similar trajectory.

Comparison: Regulatory Considerations

FBS is regulated by the USDA in the US and the European Commission in the EU. If a supplier's FBS is approved for sale in the EU, it can generally be sold legally in Asian markets.[25] The main risk of FBS use is spreading viruses to humans and cell culture through contaminated batches. Regulations require that FBS be irradiated during purification to minimize risk of viral spread. Bacteria can also be removed via sterile filtering. The International Serum Industry Association (ISIA) accredits FBS batches with its Traceability Certification, which certifies that an FBS batch came from healthy, virus-free cattle and its serum has been properly purified, irradiated, and passed quality control checks.

The proposed heterologous protein expression in fungi falls in a regulatory gray area. This product is not food for human consumption, nor feedstock for oral consumption by animals, nor a medicine for disease prevention or treatment. Thus, existing US and EU regulatory structures do not capture the products of this process. Best practice would be to purify the protein product to remove unwanted chemicals, viruses, and bacteria. Filamentous fungal strains would be easiest to use if Generally Regarded as Safe (GRAS) by the US FDA. *Aspergillus oryzae* is a filamentous fungi that has been shown to produce heterologous proteins at high yield and is GRAS by the FDA due to its use in Japanese foods for millenia. A wrinkle could develop if the culture media included an industrial or agricultural waste stream. If culture media for cell-cultivated foods were considered a feed additive, current regulation would suggest the use of waste streams is permissible. Substances sourced from agricultural and industrial waste streams (i.e. hemicelluloses, lignin sulfonate) may be safely used as food additives in animal feed without purification per US CFR. A fungal protein produced from a waste stream could be allowed to contain additional substances found in the waste stream as long as these substances are not detectable in food for human consumption. In summary, the fungal protein production

methods proposed are several degrees removed from producing food or oral-fed animal feed, which makes regulatory hurdles unlikely. It is important to note that regulations on cell-cultured animal products and their "feed" (media) are still being developed at the time of writing.

Comparison: Environmental Considerations

Since FBS comes from beef cattle, its carbon footprint includes emissions from beef farming in addition to energy inputs and greenhouse gas emissions from harvest and purification. Livestock emit a quarter of agricultural greenhouse gas emissions, mostly due to methane emissions from cows.[5] Globally, beef production emits 10-20 kg of CO2 per kg of live weight marketed beef cattle.[26] Beef CO2 emissions are highest in the US at 18.3 kg CO2/kg carcass weight[29] and slightly lower in representative EU operations at 11-13 kg CO2.kg live weight beef[5]. A Thailand beef model estimated to be representative of operations in developing countries emitted 10-14 kg CO2/kg live weight; this lesser amount is due to local feed availability instead of importing feedstocks.[26] Thus, the true carbon footprint of a batch of FBS depends on the region it was harvested from. FBS also requires non-trivial amounts of energy for refrigeration and freezing during processing, cold-chain transport, and storage between -5 to -20 degrees Celsius.[12] There is also biohazard risk if FBS enters the environment or is not handled properly.

Fungal protein production presents opportunities of many varieties to reduce environmental impact. Filamentous fungi have an ecological role as a carbon sink in nature, fixing carbon in the soil.[13,22,35] Several studies have cultured fungal strains on agricultural waste streams, which valorizes waste and decreases nitrogen runoff to the environment. Cultivation can be optimally conducted at room temperature, which negates the need for energetically expensive heating or cooling of the production space[1]. Agricultural streams also include sequestered carbon through plant biomass and emit smaller amounts of CO2 during their life cycle than animals such as cows.[30] The burning of crop residues is currently 5% of agricultural greenhouse gas emissions; utilizing these waste streams to create a valuable product prevents them from being burned.[5] Once waste streams are used in culture, most leftover media can be processed as the waste stream normally is. As most applications are currently bench scale, there is a lack of data on quantitative environmental effects of fungal fermentation. The metabolic characteristics of fungi

and potential to utilize waste streams suggests this process could be carbon-negative, or optimized to be carbon-neutral or low carbon footprint.

Conclusion: Sustainability Analysis

Fungal protein production is highly likely to be more sustainable than current FBS production. Fungal culture can be done quickly with a variety of substrates, including agricultural and industrial waste streams. This diversity and flexibility ensures stable raw material supply. It also has the environmental perks of valorizing waste streams and reducing greenhouse gas emissions. Since FBS is a byproduct of beef production, supply is dependent on herd health and regional conditions, herds need time to mature before harvest, and greenhouse gas emissions are high. FBS is also highly regulated and must be traced and purified carefully. The majority of cell culture media costs are due to the expense of FBS. There is little data on cost and regulatory structures for fungal proteins because applications thus far are pilot-scale at the largest. Filamentous fungi have been used to produce other chemicals for great economic gain. Overall, protein production in filamentous fungi appears to be an economically, socially, and environmentally-sustainable opportunity.

Feasibility and real-life application of resource (Regulation)

As regulation plays a huge role in the costs and resources, including time, required in the development process, it affects the feasibility and real-life application of our proposal.

GMO and its products regulation in US

Genetically Modified Organism (GMO) products are currently regulated by three agencies: Food and Drug Administration (FDA), Environmental Protection Agency (EPA), and United States Department of Agriculture (USDA). The FDA regulates the food that are GMO or contain GMO ingredients under the Coordinated Framework for the Regulation of Biotechnology along with the USDA and EPA. Moreover, the FDA has a Plant Biotechnology Consultation Program in which the producer submits his product information to evaluate its safety before it enters the market. Bioengineered foods derived from GMOs are also required to comply with the National Bioengineered Food Disclosure Standard which requires a label such as “bioengineered food” (FDA, USDA, & EPA, 2017). Additional regulations include the Code of Federal Regulations

under the Federal Food, Drug, and Cosmetic Act Title 21 Chapter 1 Subchapter B (Food for Human Consumption), Subchapter C (Drugs: General), Subchapter D (Drugs for Human Use), Subchapter F (Biologics), and current good manufacturing practice (cGMP) (FDA, 2022).

GMO and its products regulation in EU

Products obtained from mycelium of the same species may be subject to Regulation (EU) 2015/2283 on novel foods. This entails the proof of safety via solid and robust scientific evidence, for example numerous toxicity studies or animal models or human data. However, if fungi are used to produce novel food but are not part of the food or not directly ingested, it does not fall under the Novel Food Regulation.[25] Two routes could possibly be used to regulate the production of BSA from fungi. First, the BSA could be subjected to the Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. The regulation states in subsection 11 that "where a GMO used in the production of food and/or feed has been authorized under this Regulation, foods and/or feed containing, consisting of or produced from that GMO will not need an authorisation under this Regulation, but will be subject to the requirements referred to in the authorisation granted in respect of the GMO". Considering BSA is a feed in the cell culture media for the growth of cell cultures. Subsection 24 details that if the product derived or produced from a GMO is present in trace amounts in conventional food or feed as a result of unavoidable presence it is not subjected to the GMO labeling requirements from the regulation (Parliament & Union, 2003). Another pathway is to create a new regulation based on existing Good Manufacturing Practices of cell media reagents for Advanced Therapy Medicinal Products (ATMP), similar to the United States CFR Title 21, Subchapter H - Medical Devices part 864 subpart C - Cell and Tissue Culture Products (European Commission, 2017; FDA, 1980). One of the requirements of the Good Manufacturing Practices (C(2017) 7694) is the adoption of standards to ensure quality and aseptic production of the product. Future regulations could consider a merger between both the food and feed and the ATMP regulations to ensure the quality of BSA not from a pharmaceutical standpoint but from a food production perspective.

GMO regulation elsewhere

The first chicken lab grown meat regulation approval was obtained in Singapore (BBC, 2020). The regulation is based on Singapore's novel food regulation in which the product needs to undergo a rigorous screening for food safety. If GMOs are used a detailed procedure of the genetic modification process needs to be performed along with an assessment to evaluate if the modifications give rise to any food safety hazards. Other requirements include a risk assessment and management, safety information of the host/recipient strain, genome characterization to evaluate absence of virulence-related genes, and documentation of absence of adverse effects for humans (SFA, 2021). It is to be determined by Singapore regulatory entities if the BSA produced from Fungi would fall in the novel food category if it is used as a nutrient source for the production of cultured meat.

Experimentation

Methods

Optogenetic C2C12 cells, a mouse skeletal muscle myoblast cell line, were grown in 6 mL of growth media with and without 10% v/v FBS (Dulbecco's Modified Eagle's Medium; 1% v/v penicillin/streptomycin; 1% sodium pyruvate; 1% L-glutamine). 0.5 million cells were thawed from stocks stored in vapor-phase liquid nitrogen and added into each flask with media either containing 10% FBS or containing no FBS. Cells were maintained at 37°C and 5% CO_2 into T75 flasks.

0.5 million cells from the same cryogenic vial were transferred to a t25 flask after thawing into 6mL media either with 10% v/v FBS or with no FBS. Cells were grown to 80% confluency in each flask, then 0.5 million cells were transferred into a fresh flask to begin the experiment. At the beginning of the

Results and Conclusion

The cells without FBS did not proliferate to 80% confluency; therefore, the cell monitoring experiment was unable to start. In contrast, the cells with FBS reached 100% confluency at the 3 day time point. Due to cell death and lack of growth in the FBS free flask, the experiment was

ended. We conclude that FBS or a substitute is necessary for the proliferation of C2C12 myoblast cells.

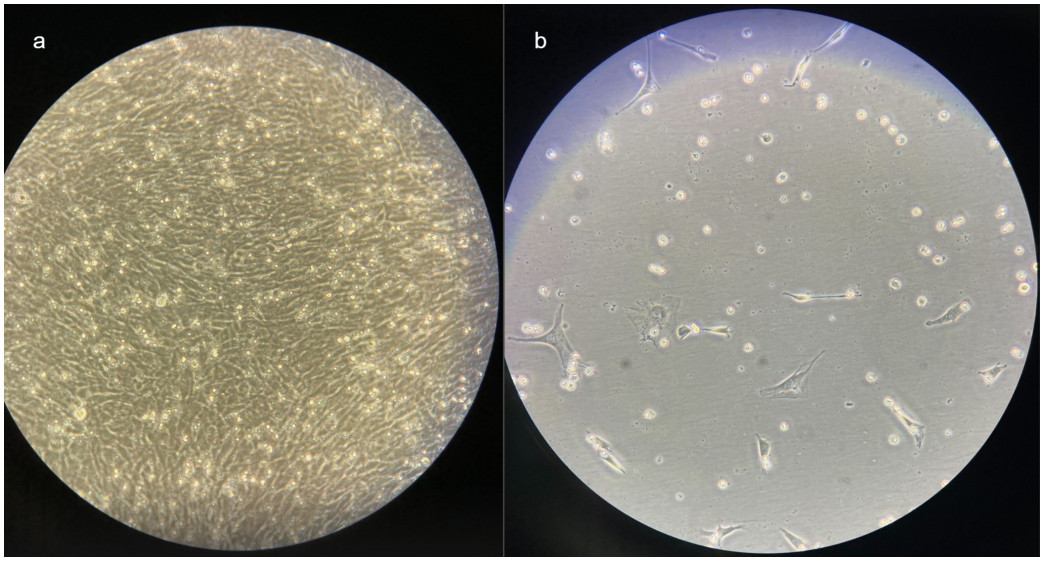

Figure 4: Comparison of cell population density with (a) and without (b) 10% v/v FBS after 72h. Round cells are non-adherent and considered dead.

Next Steps

Risks and challenges

There have been a number of efforts to increase the expression levels and production of recombinant proteins in fungi but there are challenges associated with it.

- Challenges in expressing heterologous (non-native fungal proteins) fungal production
- Yields of heterologous proteins in fungi can be orders of magnitude lower than that of homologous proteins[36]. Limitations happen at all levels of protein production. Therefore, in order to increase albumin yields from fungal hosts, modifications should be made at all levels of protein production.

In terms of risks:

- Currently, it is only reproducible lab-scale and will require more time to scale up and lower costs of production. There might be other costs associated with the scale up process not applicable to or seen in lab-scale experiments.

- Absolute quantitative impact on environmental costs and human health (e.g. What are the short term and long term costs? How will this alternative affect the final quality and safety of cultured meat?) are uncertain. The presence of such unknown factors presents challenges in terms of crafting a comprehensive business model.

These, however, help inform the future steps we can take to dive deeper and find solutions to overcome these challenges.

Our competitive advantage

Rigorous market research supports that our idea is novel and fills an important gap in the cultivated meat process. Among the cultivated meat producers and ingredient manufacturers, we found that producing an FBS growth media alternative that can support the development of a cultivated protein product was unique to the industry, compared to most companies which focused on producing a product. Companies who were using mycelium as part of the development process were harvesting non-GMO fungi to produce fungi-derived products (e.g. supplements), not growth factors. A large majority of companies are focusing on end product formulation[16] while a handful are focusing on ingredient optimization, and even fewer using fermentation-derived processes. Among these, there are no companies that are using fermentation of fungi to specifically produce growth factors, thus our competitive advantage.

Path to data generation and product development

Additional Experimentation

In order to generate more data, we will conduct more experiments in the following order (Note: these are roughly described and subject to modification and more detail, according to the results):

I. Compare growth of cells with FBS vs. without FBS

II. Obtain 2-3 fungal strains and optimize their production of our proposed recombinant bovine serum albumin. Pick the fungal strain that has the best result and collect the purified recombinant bovine serum albumin (PRBSA)

III. Grow cells with PRBSA and other nutrients to complete media formulation.

IV. Grow cells with FBS and compare this growth rate with the cells grown on the PRBSA to determine if there is an improvement. Modify and optimize if necessary.

V. Compare growth of cells using other novel sources of culture media (e.g. plant-based hydrolysates) vs. with FBS vs. our PRBSA. Optimize such that our PRBSA can result in the greatest yield or growth.

Time scale for production and utilization

A benchmark was followed from EVERY or FUMI ingredients which are companies producing recombinant proteins as ingredients for consumer end products. FUMI ingredients started an accelerator program in summer 2019 while holding their first pilot production in 2022.

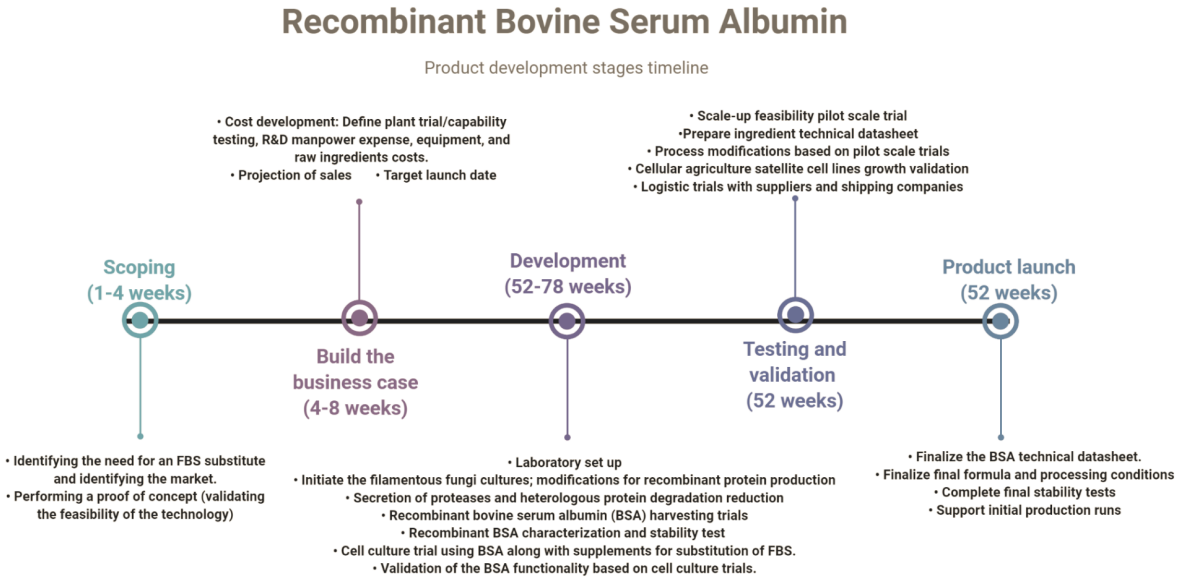

Figure 5: Product development estimated timeline for the production of recombinant bovine serum albumin from *Neurospora crassa.*

Conclusion

Creating cultivated food products that are available at a price parity with traditional animal products presents several challenges that need to be addressed - these include the use of growth factors, which can take up a huge part of the overall production costs. Using modified *Neurospora crassa*, an underutilized resource, as a natural bioreactor to produce an alternative to FBS, bovine albumin, shows promise to reduce cost and the need for animal products.

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