Chickpea hydrolysate as a supplement to growth factors in cell-culture media

Introduction:

The global human population is projected to increase by 25% to ~9.5B by 2050, creating a proportional increase in nutritional requirements worldwide.[1] The livestock industry contributes to 18% of total global greenhouse gas emissions, as well as 8% of global human water use and 55% of soil erosion and sediment.[2]

While policies can and have been enacted to reduce the environmental impact of animal agriculture, the transition and substitution of livestock with alternative protein sources is necessary to solving the climate crisis.[3] The most likely candidate for a widely accepted alternative protein is one which results in the creation of the same products through different, more environmental and ethical, means. Cellular agriculture is the process of farming animal products from cells instead of animals.[4] This new form of agriculture allows for a more controlled and precise way to create meat while excluding the environmentally harmful byproducts of livestock.

Over the past 7 years, the cell cultured meat industry has boomed, growing from 5 companies in 2015 to over 100 companies in 2021.[5] While there has been an increase in research and industry in this field, cell cultured meat products have not found their way into grocery stores yet, primarily due to the high cost of manufacturing. Therefore, the crucial next step in expanding cellular agriculture is to reduce the cost of production.

In both bench-side and industry-scaled cell culture, the most costly component of the cell growth process is the cell media. This media is a nutrient-rich liquid that the cells live in, and is used to provide all of the necessary nutrients to allow cells to proliferate. Traditionally, components in media have been derived from fetal bovine serum (FBS). As the name suggests, FBS is animal-derived. FBS also varies from its nutrient composition between batches, and is becoming increasingly scarce. From an economic and ethical standpoint, it has become critical for industry stakeholders to move towards an animal-free and chemically defined media.

Creating such media, however, has proven to be challenging. In chemically defined and serum-free formulations, biomanufactured growth factors contribute to over 99% of the associated costs - namely FGF-2 and TGFβ.[6] Because of this, one of the primary challenges within the cellular agriculture industry is to reduce the cost of growth factors in media. Many researchers and cell-cultured meat startups have looked towards substituting growth factors with chemical and plant-based alternatives. Ideally, substitutes should completely replace growth factors in media at a lower price-point with little to no reduction in media performance. However, there has been little success with this area, as research on potential growth factor alternatives have failed to provide robust and long term results in cell growth.[7] An alternative approach to reducing media costs involves supplementing growth factors in media with growth-stimulating compounds, rather than replacing them. The objective of this approach is to increase cell yield in the production process, meaning that the same amount of media will yield a greater number of cells. Though the price points of growth factors and other expensive

components may remain the same, an overall cost reduction is achieved. In this proposal, we take on this method of approach.

Chickpea (*Cicer arietinum*) is an annual plant of the pea family, often consumed for its nutritional properties. It typically contains between 20-25% protein, primarily in the form of albumins, and is often consumed as an alternative protein source in countries where animal protein is scarce or expensive.[7,8] Through hydrolysis, the processes of chemical breakdown by reaction with water, these proteins are converted into bioactive peptides. These peptides have the potential to replace proteins in media if they contain the right analogs for the active domain of a desired protein.[9] Currently, there are certain chickpea derivatives being used in the cultivated meat industry; Future Meat Technologies is replacing serum albumin with chickpea protein homologs to reduce media costs.[10] Previous studies have also found that the addition or substitution of chickpea hydrolysate to growth factors or serum in cell culture media has a growth-stimulation effect on cells.[10,11,12]

Here, we propose the supplementation of chickpea hydrolysate in serum-free cell media to accelerate the growth of cells, thereby increasing cell yield and reducing overall cost of production. In the sections below, we will describe our own research proposal that will study the proliferative effect of chickpea hydrolysate in the context of cell-culturing meat and detail our findings of related studies in a literature review. Then, we will provide an industry-scaled cost-analysis to our proposed resource, as well as practical considerations for the supply of chickpea and an environmental impact statement of our resource.

Proposed methods:

Hydrolysate isolation:

Chickpea flour, alcalase, and flavourzyme will be purchased as detailed in subsequent sections. Alcalase and flavourzyme will be cleaned using PD-10 Sephadex G25 columns to remove preservatives and any contaminants. Hydrolysis will be performed at 50°C under constant stirring with pH of 8.0 for hydrolysis with alcalase and pH of 7.0 for hydrolysis with flavourzyme. Adjustments of pH will be made as necessary with addition of NaOH or HCl. Chickpea flour will be suspended in distilled water at 2% (w/v) and 0.03 Anson Units of alcalase per gram of flour. After 75 minutes, 5 Anson Units of flavourzyme per gram of flour will be added. To terminate hydrolysis, the enzymes will be inactivated through heating of the solution at 95°C for 20 minutes. The pH will then be adjusted to 7, and the remaining suspensions will be clarified by centrifugation for 10 minutes at 3000g. The supernatants will then be filtered through 0.45 and 0.22μm nitrocellulose filters before being added to the cell culture media.

Cell culture and treatment:

Induced pluripotent stem cells (iPSCs) will be kept at 5% (v/v) CO_2 and 37°C in Essential 8 Medium (Thermo Fisher) in six-well plates. For routine maintenance, media will be replaced every other day, and cells will be passaged at 80% confluence through singularization with Accutase (Invitrogen) and seeded at 10,000 cells cm^{-2}. Experiments will be initiated by replacing the medium with fresh medium supplemented with 10% (v/v) chickpea hydrolysate one day after passaging the cells.

Cell growth characterization:

Cell counting:

The cells will be singularized with Accutase (Invitrogen), and viable cells will be counted on a manual hemocytometer based on Trypan blue (Corning) exclusion. Cells will be counted on days 2, 15, and 30 of growth. On days 15 and 30, as the cells will likely span more than 1 well, the result of cell count will be based on all wells that originated from the same well at the start of the experiment.

Immunocytochemistry:

Immunocytochemistry will be performed on days 2, 15, and 30 to ensure that the stem cells do not differentiate. The following are bovine induced pluripotent stem cell markers and will be evaluated: SSEA-1, SSEA-3, SSEA-4, OCT-4.[13]

Preliminary Results:

As referencing Girón-Calle et al:

Girón-Calle et al. studied chickpea protein hydrolysate as a substitute or supplementation for serum in cell culture of Caco-2 cells.[8] They tested the supplementation of chickpea hydrolysate at different degrees of hydrolysis (DH) ranging from 0-32%, and with varying amounts of serum added (0-10%) (Figure 1). The standard culture protocols called for 10% serum, and this condition caused normal growth in the cells. The addition to the standard media of chickpea hydrolysate at 22% DH was found to have an enhanced effect on the proliferation of both Caco-2 and THP-1 cells. Therefore, in our proposal, we will use a supplementation of chickpea hydrolysate in the cell culture media, as opposed to a substitution. We will also use serum-free media. With these adjustments, we still anticipate similar results.

The researchers next evaluated the ability of the cells to be cultured at longer timepoints. They cultured the cells with 10% FBS and different concentrations of chickpea hydrolysate with a 22% DH for 9 days. They found that on day 9 of culture, the cells grown in media supplemented with 10% chickpea hydrolysate had grown approximately 50% more than the cells with no hydrolysate added. While these results were not significant due to large variance, with the continued exponential trend and with more replicates we expect there to be a significant difference at later time points. We therefore anticipate that cells grown in growth factor-based media supplemented with 10% chickpea hydrolysate will also have 50% more cells on day 9, with a continued exponential trend.

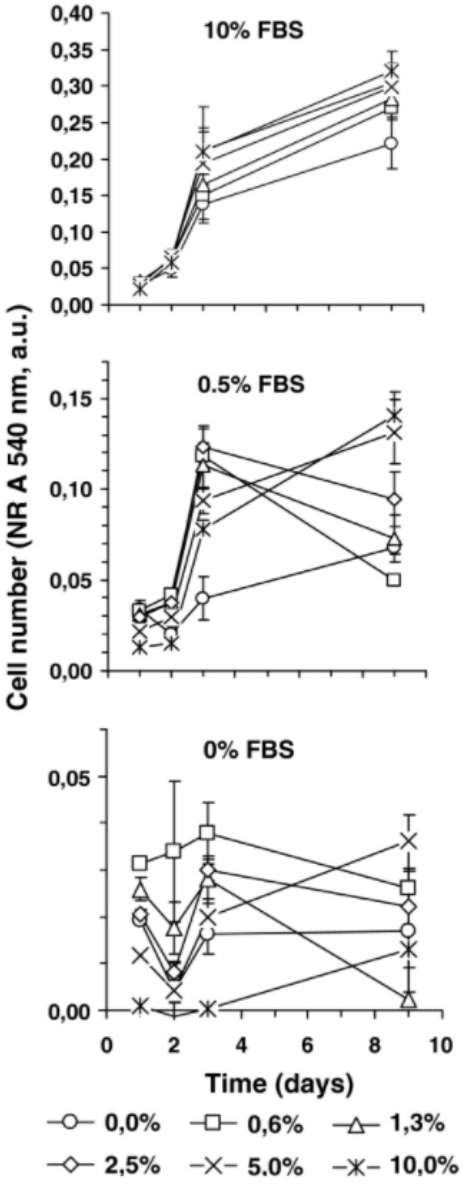

Figure 1: Proliferation of Caco-2 cells cultured in the presence of different serum (FBS) levels and chickpea hydrolysate of increasing degree of hydrolysis. Cell number is estimated by measuring cellular uptake of vital stain neutral red.

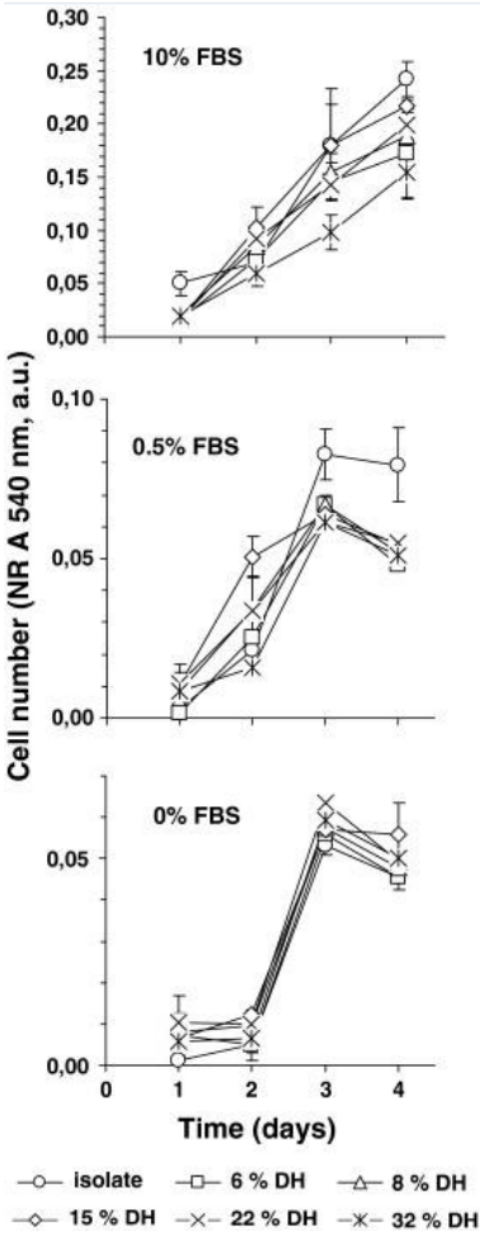

Figure 2: Proliferation of Caco-2 cells cultured in the presence of different serum (FBS) levels and 22% DH chickpea hydrolysate of increasing concentration in the media. Cell number is estimated by measuring cellular uptake of vital stain neutral red.

Cost benefits of recommendation:

Cost comparison							
	Cell no. (NR A 540 nm, a.u.)		Total cost		Cost / cell		*Cost reduction*
Day	Status quo	With hydrolysate	Status quo	With hydrolysate	Status quo	With hydrolysate	
2	0.05	0.05	0.02	0.02	0.42	0.39	*-8.6%*
15	0.31	0.48	0.13	0.14	0.42	0.28	*-33.1%*
30	0.62	0.97	0.26	0.27	0.42	0.28	*-34.7%*

Source of cell numbers: Girón-Calle et al. research paper[9]

Based on secondary research, we identified the cost benefit of our recommendation with regard to growth factors to be over 30%. Such benefit was computed by comparing the per-cell cost of growth factors under the status quo with that under the scenario of using chickpea hydrolysate as a catalyst.

The driver behind such cost decrease was an increase in cell growth by over 50% upon using the hydrolysate. It is to be noted that the focus of our research was solely on the change in growth factor cost. If other costs associated with the media or cell-lines change as a result of introducing the hydrolysate, the final cost reduction may be less than or greater than 30%.

The above calculations are based on the following assumptions:

- That the supplementation of chickpea hydrolysate in growth factor-based media will have the same effect of that in serum-based media
- That there is not an increase in media required due to the increased cell growth
- That there is unlimited exponential cell growth
- That the average chickpea is 23% protein[15]

Procurement of resource:

Chickpea flour is widely available and it is priced as a commodity. India produces about 70% of chickpeas globally, and is also the largest importer. Australia is the largest exporter of chickpea[16]. For ease of procurement, however, we are considering chickpea prices in the USA. As we scale the process, we can consider importing larger quantities from countries such as Australia, Turkey or India. It is important to note that chickpea prices are volatile with strong dependencies on cropping choices, global demand, and consumer choices. However, within the context of our proposal, it is still a major cost reduction agent.

Major suppliers of chickpea flour include Ardent Mills, Chickplease, AGT Foods, etc. As we scale the process, we can consider direct procurement of chickpeas to cut processing costs.

Sustainability impact:

Chickpea agriculture is a highly sustainable process in terms of emissions, land degradation and water usage.

Chickpea, like other legumes, can be used as a cover crop. Cover cropping is a well established regenerative agriculture method to reduce soil erosion, and enhance nitrogen and carbon fixing[17,18]. Apart from enhancing soil health, it can also generate new streams of income for farmers as regenerative agriculture becomes more prevalent in the carbon credit markets.

Chickpeas also have a very low water footprint. They require about 4,000 liters of water per 1 kg, as compared to 15,000 liters per 1 kg of beef.[19]

Conclusion and next steps:

Though plant hydrolysates hold promise in the cultivated meat industry, it is important to recognize the lack of literature on plant hydrolysates and their applications. Consequently, hydrolysate products are not fully characterized and no single standard method of production for chickpea hydrolysate in the literature exists. For future directions, we suggest that preliminary research should be conducted to fully understand the chemical composition and protein profile of chickpea hydrolysate. Additionally, the engineering of a standard cost-effective and streamlined production process for chickpea hydrolysate may be valuable if chickpea is proven to be a viable resource. Further studies on these topics are warranted.

As for conducting a deeper techno-economic analysis of chickpea as an industry resource and to further validate the efficacy of chickpea hydrolysate as a growth catalyst, we recommend conducting primary studies of its impact on cell growth and other costs associated with production. Such tests would need to be conducted at different production volumes to validate its performance consistency and stability. Once efficacy is established, long-term contracts can be negotiated with chickpea flour producers to ensure consistent supply while hedging against price volatility.

We believe that chickpea hydrolysate represents a viable solution to the challenge of high growth-factor cost in cell-cultured food production. Given the numerous concerns around the ethical and environmental impacts of animal agriculture, cellular agriculture will likely be a key focus area of several food manufacturers in the coming decades. Solutions that reduce the cost of producing cell-cultured food will be vital to driving growth of the industry. In addition to reducing the cost of production, chickpea is a highly sustainable resource that offers several environmental benefits as noted above. Hence, we believe it holds substantial promise as an environmentally-friendly resource to the cell-cultured meat industry.

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