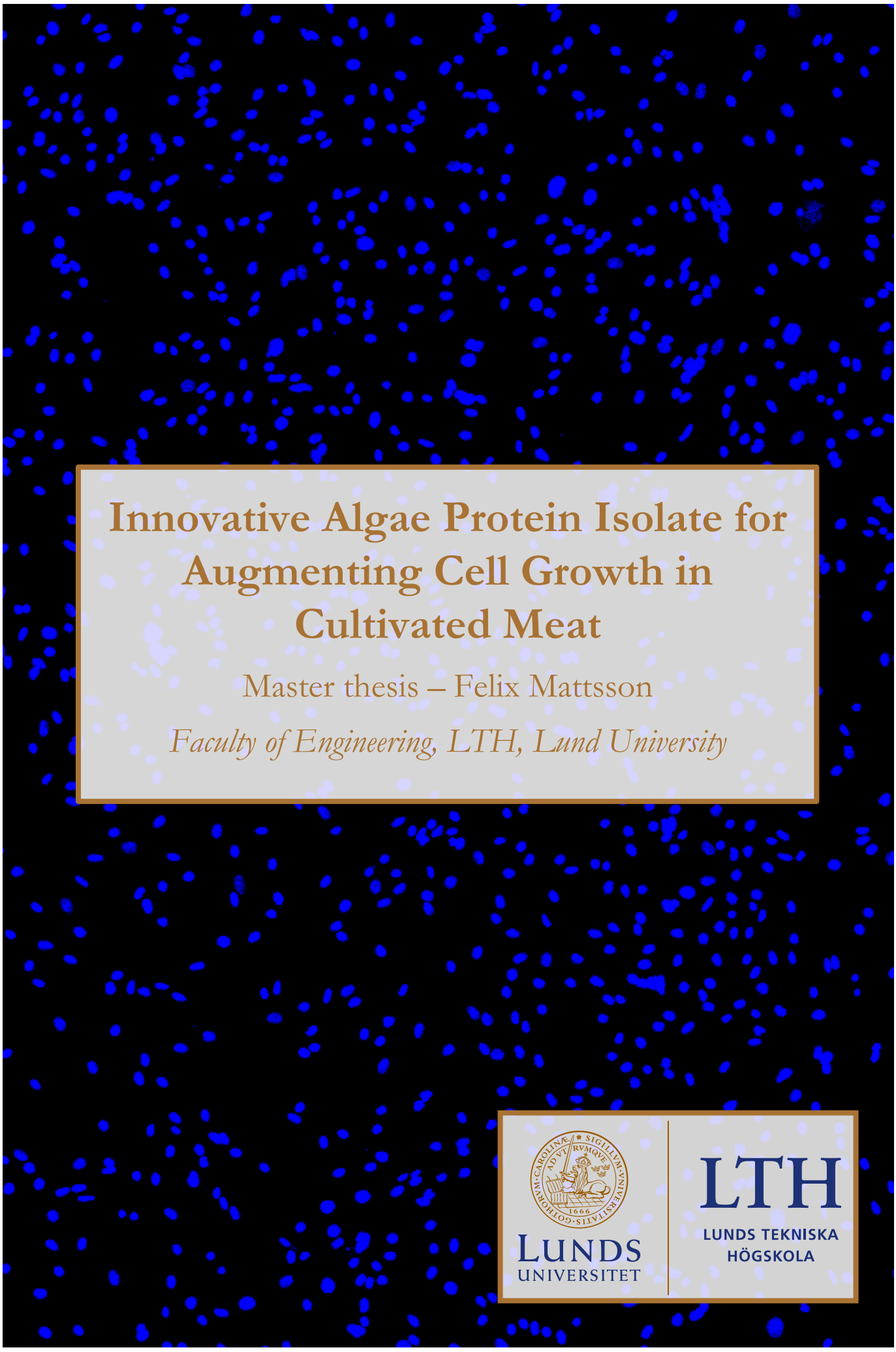

# Innovative Algae Protein Isolate for Augmenting Cell Growth in Cultivated Meat

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# Innovative Algae Protein Isolate for Augmenting Cell Growth in Cultivated Meat

**Master's degree project in Applied Microbiology – KMBM05**

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Project executed at Nordic Virtual Pastures, Bio Innovation Institute, Copenhagen

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*Front page: DAPI Stained Nucleus of Bovine Satellite Cells*

## ABSTRACT

Cultured meat production presents a sustainable solution to the environmental, ethical, and antibiotic-related challenges of conventional meat production. This master's thesis explores the pivotal role of cell culture media in cultivating meat, emphasizing the importance of developing cost-effective and sustainable alternatives to fetal bovine serum (FBS). The study aims to extract functional proteins from various microalgae, assess the proliferation of bovine satellite cells (BSCs) under different FBS concentrations, and explore algae protein isolates (APIs) as potential FBS replacements.

Through a series of experiments, the thesis revealed that ultrasonication time influences protein yield from microalgae, with optimal durations varying by sample type. Combining ultrasonication with alkaline extraction emerged as the most effective method for API development, offering a balance between protein yield and quality. Additionally, reducing the osmolality of APIs through raw material washing significantly improved BSC growth, suggesting that high osmolality contributes to observed toxic effects.

The study demonstrated that while high concentrations of APIs initially exhibited toxicity, lowering the concentration allowed for cell growth, particularly when paired with reduced FBS concentrations. The consistent performance of API 2.0, developed through optimized extraction methods, highlighted its potential in reducing FBS dependency. Furthermore, the development of algae peptide mixtures (APMs) showed promising results in enhancing cell proliferation at optimal concentrations. Despite some experimental variations, the findings suggest that algae-derived protein and peptide extracts could become viable, sustainable alternatives to FBS, supporting the ethical and environmental goals of cultured meat production and advancing its scalability and economic competitiveness.

# PREFACE

All laboratory work was conducted at Bio Innovation Institute at the company Nordic Virtual Pastures (NVP). I want to deeply thank the whole team for taking me in and made me feel part of the team from day 1. To this I would like to show a little extra appreciation for all who have helped me throughout this project:

Thor - for being an amazing supervisor. You have given me a lot of freedom in what I want to do, but not without challenging my ideas to make sure I know what I am doing (which is not always the case). You have been an incredible source of knowledge and I have always ended up smarter after a conversion with you.

Jonas – From the first weeks when you painfully had to teach a clueless swede how to handle cells without everything dying to amazingly nerdy conversations about basically anything. You have been a blessing to work with.

Pilar – For always answering my questions and providing me with strong expertise that you have. You have been mentoring me throughout this project and I am truly grateful.

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Jan – For taking a chance on a Swedish biotech engineer and provided me with this opportunity. I have great respect for you and the team that you have built.

Jenny – A big thanks for always looking out for my best and all your valuable feedback on my work throughout the entirety of this project.

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# INTRODUCTION

Cultured meat production has emerged as a promising solution to address the growing concerns related to the environmental impact, ethical considerations, and the overuse of antibiotics in conventional meat production (Stout, 2023). With a rapidly increasing global population and the associated rise in demand for animal products, there is an urgent need to explore alternative, sustainable approaches to meat production (Godfray et al., 2018). This master thesis aims to delve into the pivotal role of cell culture media in the cultivation of meat, focusing on its environmental significance, ethical implications, and the imperative to reduce antibiotic usage.

From an environmental standpoint, conventional meat production is a resource-intensive process, contributing significantly to deforestation, greenhouse gas emissions, and water pollution (Godfray et al., 2018). Cultured meat offers a sustainable alternative that could significantly reduce the ecological footprint associated with conventional livestock farming (Post et al., 2020). Ethically, concerns about animal welfare and the moral implications of mass-scale slaughter are driving the exploration of cultured meat as a more humane and compassionate option. Additionally, the potential for reduced antibiotic usage in cultured meat production addresses the global challenge of antibiotic resistance, enhancing food safety and security.

As the demand for cultured meat increases to meet the rising global population's protein needs, it becomes crucial to address the scalability of production processes. One of the major components influencing scalability is the volume of cell culture media required (Stout, 2023). This thesis recognizes the challenges associated with large-scale production and emphasizes the necessity for cost-effective media formulations. Achieving cost-effectiveness is essential for the cultured meat industry to compete economically with traditional meat production, ensuring its widespread adoption and impact on the food market.

The foundation of cell culture media is pivotal in ensuring the sustainability of cultured meat production. This thesis underscores the importance of utilizing sustainable sources for the components of cell media to mitigate environmental impact. By sourcing ingredients responsibly, the cultured meat industry can contribute to the broader goal of establishing a more sustainable and environmentally friendly food production system.

Fetal bovine serum (FBS) is a commonly used component in cell culture media, providing essential nutrients and growth factors for cell proliferation and differentiation (Gstraunthaler, 2003). However, the use of FBS raises ethical concerns and poses challenges related to its high cost and potential variability (Gstraunthaler, 2003). This thesis explores the composition of FBS, highlighting expensive components such as insulin, FGF-2, transferrin, and albumin. Understanding the intricacies of FBS and its associated costs is crucial for developing alternatives that align with the ethical and economic goals of cultured meat production.

To fully realize the benefits of cultivated meat, it is imperative to transition away from serum-based media. This thesis emphasizes the need to explore and develop serum-free alternatives that not only enhance the ethical and environmental aspects of cultured meat production but also contribute to making it a viable, economically competitive option in the future food industry. Replacing serum-based media is a critical step towards achieving a more sustainable and ethical food production system, ensuring the long-term success and acceptance of cultured meat as a mainstream food source.

## AIM

There are three main objectives for this master's thesis. First, this thesis aims to develop an effective method for extracting functional proteins from various microalgae raw materials. This will be accomplished by experimenting with different extraction techniques and evaluating their performance based on the protein yield. Second, the thesis aims to assess the proliferation of bovine satellite cells, considering the amount of fetal bovine serum used in the cell culture media. Lastly, this thesis intends to explore the use of the algae protein extracts as a potential replacement for fetal bovine serum in cultivating bovine satellite cells.

# BACKGROUND

## Bovine Satellite Cells

In the context of cultivated meat, bovine satellite cells (BSCs) play a pivotal role. Cultivated meat entails in vitro growth of muscle tissue as an alternative to conventional animal agriculture. Bovine satellite cells serve as a key component in this process, as their unique capacity for differentiation into muscle cells allows for the generation of meat without the need for extensive land, water, and feed resources (Stout et al., 2022).

BSCs are quiescent muscle progenitor cells located at the periphery of muscle fibers, integral to the growth and maintenance of skeletal muscle in cattle (Gonzalez et al., 2020). Upon activation, these cells undergo proliferation and subsequent differentiation into myoblasts, forming multinucleated myotubes and contributing to muscle growth, repair, and regeneration (Gonzalez et al., 2020). Harvesting bovine satellite cells typically involves muscle biopsies, wherein a small tissue sample is extracted for subsequent in vitro cultivation (Gonzalez et al., 2020).

The use of bovine satellite cells in cultivated meat production holds significant promise for creating a more sustainable and efficient method of meat production. By harnessing the regenerative capabilities of these cells, large quantities of muscle tissue can be generated, addressing the environmental concerns associated with conventional livestock farming. Moreover, the incorporation of bovine satellite cells into cultivated meat processes aims to replicate the taste, texture, and nutritional profile of traditional meat products (Stout et al., 2022).

In the pursuit of in vitro meat production, understanding the optimal growth conditions for bovine satellite cells is crucial. A recent study investigated the effect of growth medium components on BSC proliferation. Notably, the concentration of fetal bovine serum (FBS) emerged as a critical factor. The addition of bovine serum at a concentration of 20% resulted in the highest proliferation rate (Zygmunt et al., 2023).

## Fetal Bovine Serum - The Component That Must Be Replaced

Fetal bovine serum (FBS) is a nutrient-rich medium that has long played a pivotal role in cell culture, especially in the growth and maintenance of bovine satellite cells, contributing significantly to the field of cellular agriculture. FBS is derived from the blood of fetal calves, typically collected during the slaughter of pregnant cows (Godfray et al., 2018). In the context of culturing bovine satellite cells, FBS serves as a crucial supplement in the culture media (Godfray et al., 2018).

The components within FBS, including insulin, transferrin, albumin, and fibroblast growth factor-2 (FGF-2), play key roles in supporting cell growth and differentiation. Insulin regulates glucose metabolism, transferrin facilitates iron transport, albumin serves as a carrier for various molecules, and FGF-2 promotes cell proliferation (Godfray et al., 2018). This intricate blend of components mirrors the natural environment necessary for the optimal growth of cells.

The production of FBS involves a multi-step process. After collection from slaughtered pregnant cows, the blood undergoes centrifugation to separate the serum from blood cells. The serum is then filtered and subjected to additional processing steps to ensure the removal of contaminants. The final product is typically heat-inactivated to eliminate the risk of introducing infectious agents (Godfray et al., 2018).

The reliance on FBS has raised ethical and sustainability concerns within the field of cellular agriculture. As the demand for FBS continues to grow, researchers are exploring alternative serum-free media and supplements (Godfray et al., 2018).

## Recombinant produced growth factors

The rise of recombinant protein technology has significantly influenced the development of serum-free growth media as an alternative to traditional fetal bovine serum (FBS)-based formulations. Recombinant

proteins are synthesized by genetically engineered organisms, typically bacteria, yeast, or mammalian cells, to produce proteins with specific functions. This methodology has been widely employed to produce growth factors for use in serum-free cell culture media (Stout et al., 2022).

However, it is important to note that the production of recombinant growth factors can be cost-intensive. The technology involved in genetically engineering microorganisms, scaling up production, and ensuring the purity and functionality of the final product contributes to the overall expenses. In particular, the recombinant production of insulin, FGF-2, transferrin, and albumin involves intricate processes that may necessitate significant financial investment (Stout et al., 2022).

The cost intensity of producing above mentioned recombinant growth factors remains a significant challenge. As the demand for serum-free growth media continues to rise, there is an increasing need for exploring and developing more cost-effective alternatives to ensure the widespread adoption of sustainable and ethical practices in cellular agriculture and cultivated meat production.

## Alternatives to Serum and Recombinant-based Nutrients and Growth

Rapeseed, also known as canola, boasts a rich nutritional profile that holds promise for usage in cell culture media. Abundant in proteins, lipids, and carbohydrates, rapeseed provides a diverse array of nutrients essential for cellular growth and proliferation. Additionally, it contains vitamins and minerals, making it a compelling candidate for formulating serum-free growth media (Stout et al., 2022).

Beyond its direct nutritional contributions, rapeseed presents an opportunity for upcycling within the agricultural landscape. By-products from rapeseed processing, such as meal and oil, can be repurposed for use in cell culture media. This approach aligns with principles of sustainability and circular economy, turning waste streams into valuable components for cellular agriculture (Stout et al., 2022).

Studies have shown that rapeseed contains albumin-like proteins, positioning it as a potential substitute for recombinant albumin in cell culture media. Preliminary research indicates that the use of rapeseed albumin yields comparable results to recombinant albumin in the cultivation of bovine satellite cells. This finding suggests a promising avenue for developing cost-effective and sustainable alternatives in serum-free media formulations (Stout et al., 2022).

The success of rapeseed albumin sparks interest in exploring additional alternatives. Microalgae, with their diverse nutritional content, emerge as a promising new avenue. From high lipid content in certain strains or high protein in others. Some microalgae varieties have demonstrated a content of transferrin like proteins, a pivotal growth factor in cell culture (Schwarz et al., 2003). By harnessing transferrin like proteins from microalgae, a sustainable and more cost-effective alternative to traditional sources could be explored. The unique nutritional composition of microalgae adds a layer of complexity to the formulation of growth media, showing potential as a source for serum-free media, presenting exciting possibilities for innovation in the cellular agriculture landscape.

## Algae Samples Used in This Thesis

In this thesis three different samples of algae were used. Due to IP rights the exact strain and origin of these algae samples cannot be disclosed, however a short summary of the most important characteristics is presented below. The different algae used will for the purpose of this thesis be called X1, Y and X2.

### Algae X1

Algae X1 is distinguished by its lighter coloration and rich nutrient profile, which includes high levels of proteins, vitamins, minerals, and antioxidants. It thrives under specific, controlled growth conditions and has a robust cell structure that enhances nutrient retention. Despite its strong cell walls, X1 is processed to improve digestibility, making its nutrients more bioavailable. Its versatility extends to use in dietary supplements, functional foods, and skincare products, all while maintaining an environmentally friendly cultivation process that supports sustainability and reduces carbon dioxide levels.

A 40x picture of Algae X1 dissolved in Milli-Q water is shown in **Figure 1**.

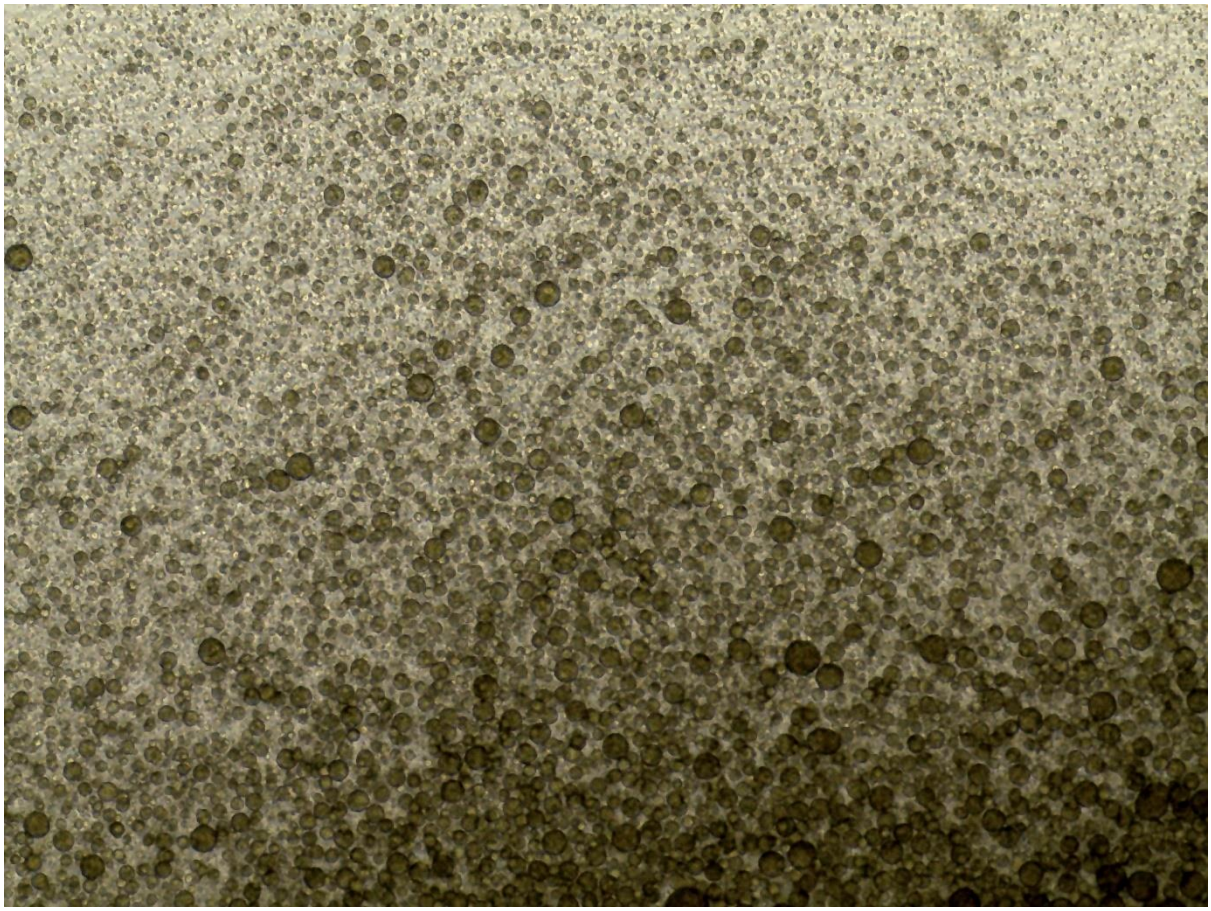

**Figure 1. 40x picture of Algae X1 dissolved in Milli-Q water.**

## Algae Y

Algae Y is a small, unicellular microorganism known for its high lipid content, which can make up to 60% of its dry weight, primarily in the form of polyunsaturated fatty acids. This characteristic makes it a candidate for biofuel production, highlighting its significant potential in the field of renewable energy.

The sample used in this thesis is spray dried, with a clearly ruptured cell wall. A 40x picture of Algae Y dissolved in Milli-Q water is shown in **Figure 2**.

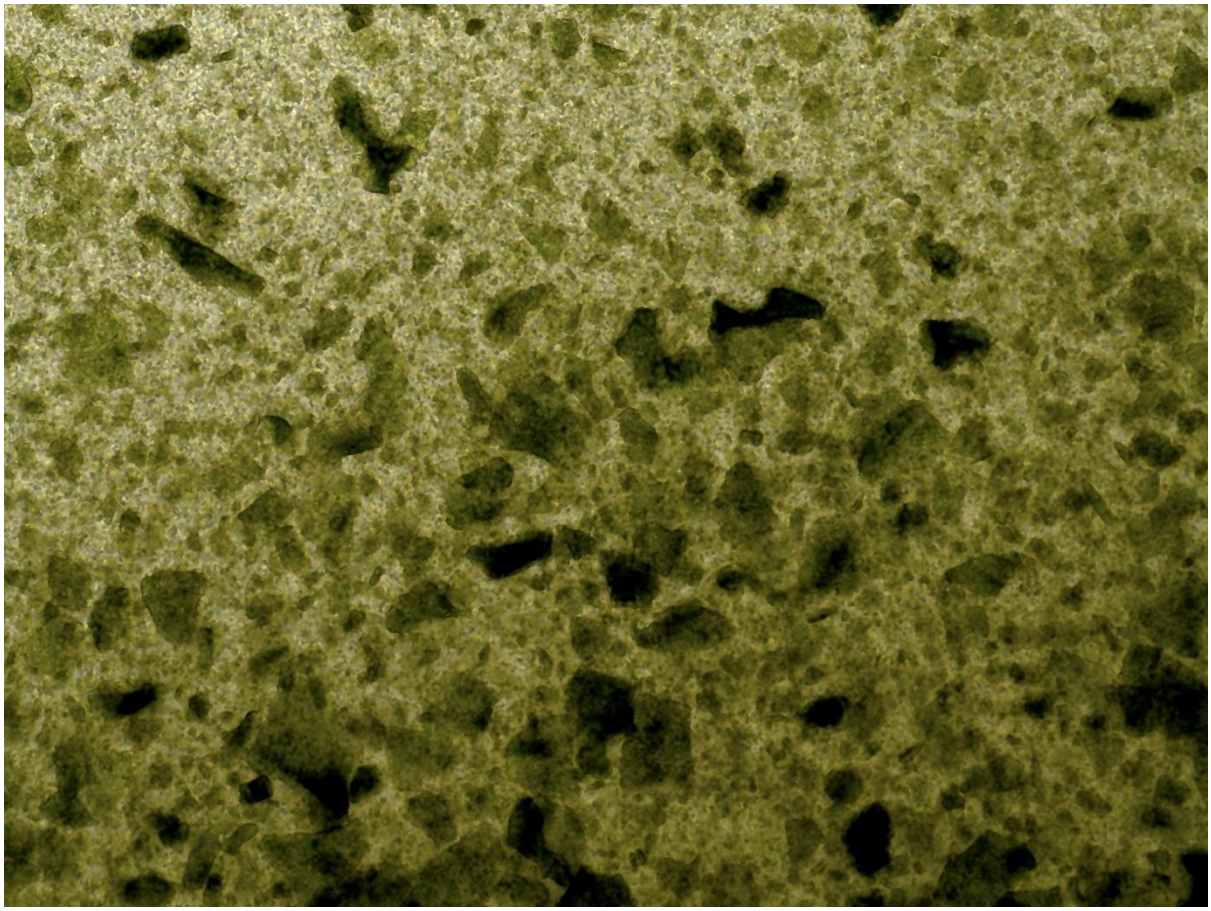

**Figure 2. 40x picture of Algae Y dissolved in Milli-Q water.**

## Algae X2

The sole difference between Algae X1 and X2 is that the holding time (in this case time without exposure to UV light) for X2 is increased compared to X1. When the holding time for microalgae in each system is increased, several critical effects can occur, particularly the degradation of chlorophyll. However, the degradation process can also lead to the recycling of nutrients, potentially making them more available for the remaining cells, which can influence the population dynamics and metabolic profiles of the culture. However, accumulation of waste products and pH fluctuations may also occur and could add stress on the algae (Figueroa-Torres, Pittman and Theodoropoulos, 2021).

A 40x picture of Algae X2 dissolved in Milli-Q water is shown in **Figure 3**.

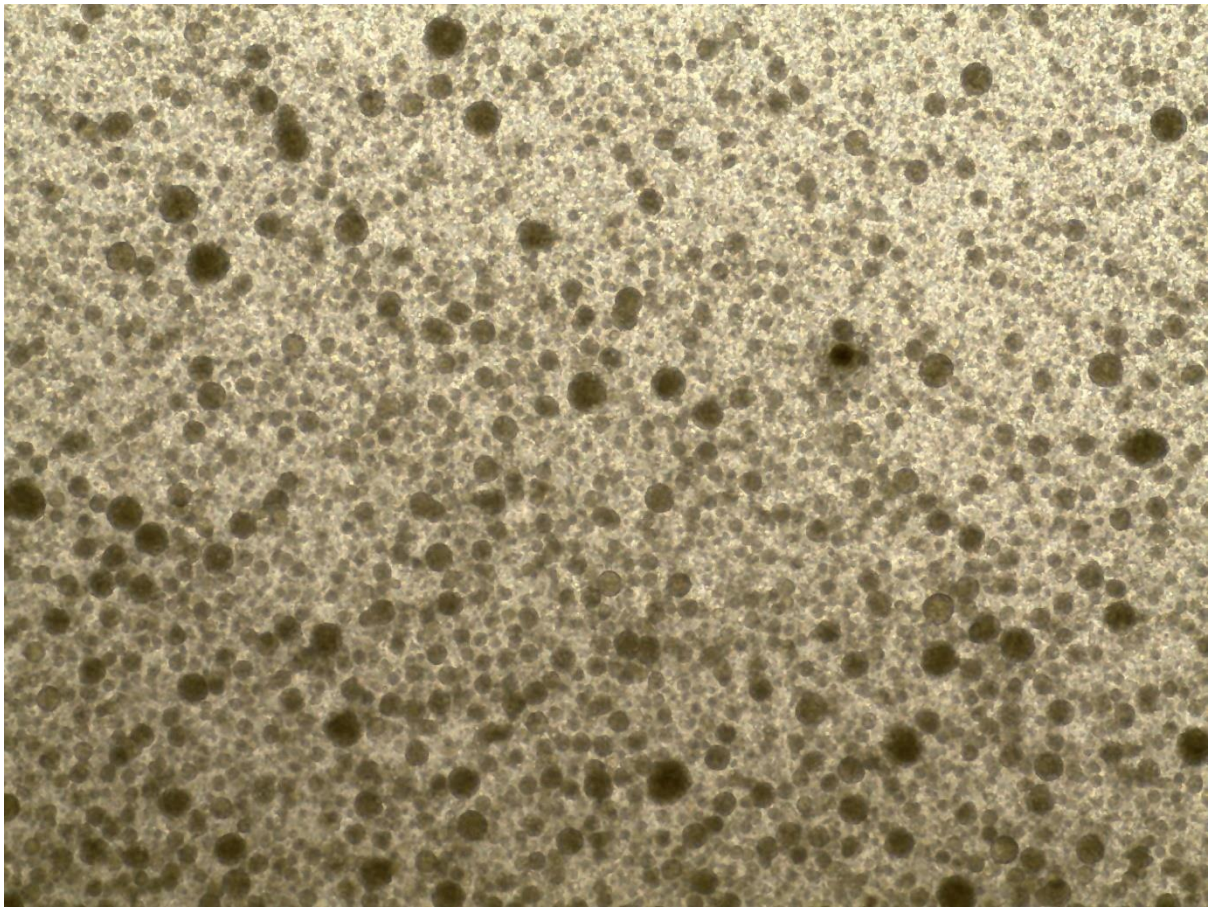

**Figure 3. 40x picture of Algae Y dissolved in Milli-Q water.**

## Extraction of Biomolecules from Micro Algae

### Enzymatic Hydrolysis

*Protease*

Enzymatic pretreatment using proteinases is a potential approach to break down the cell walls of microalgae. These cell walls, composed of polysaccharides and glycoproteins, act as protective barriers. By applying proteinases, it is possible to selectively weaken the cell walls, leading to the release of intracellular organic matter. This process can enhance the biodegradability of microalgal biomass, making bio molecules such as proteins more accessible. Enzymatic pretreatment offers advantages over mechanical or thermal methods due to its lower energy demand (Córdova, Passos and Chamy, 2019).

In a recent study, different proteases were used to prepare hydrolysates to break down isolated proteins from rapeseed (Gérard Chabanon et al., 2008). These hydrolysates were then used to successfully cultivate Chinese Hamster Ovarian (CHO) cells. It is suggested that the cell proliferation effect observed in the study is due to these hydrolysates contain a mix of peptides obtained from the hydrolysis of the isolated rapeseed proteins (Gérard Chabanon et al., 2008).

*Cellulase*

Cellulase enzymes have the potential to facilitate protein extraction from algae, particularly in breaking down the cell wall to release intracellular biomolecules. The cell wall of most micro algal species mainly consists of cellulose. Under the action of cellulase, cellulose is hydrolysed into short-chain cellulose, promoting the release of intracellular proteins from the algal cells (Le Nguyen Doan et al., 2022).

### Ultrasonication

Ultrasonication, a powerful method based on intense ultrasonic energy, offers a solution by breaking down the protective cell walls of algae (Liu et al., 2022).

Algae cell walls provide structural integrity but also pose a challenge for extracting intracellular compounds. Ultrasonication exploits the phenomenon of cavitation—the rapid formation and collapse of microscopic bubbles in a liquid. When exposed to intense ultrasonic energy, these bubbles generate shockwaves that disrupt cell walls. The mechanical forces of cavitation tear the cells into smaller fragments, facilitating the release of bioactive molecules (Liu et al., 2022).

Within the liquid, longitudinal vibration waves create pressurized air formations that radiate and form cavitation shocks. These shocks impact the cell walls, leading to their rupture. The process is highly efficient and can be scaled up from lab-scale experiments to large commercial production. By harnessing ultrasonication, researchers can efficiently extract lipids, proteins, antioxidants, and other valuable compounds from algae biomass (Liu et al., 2022).

### Alkaline Hydrolysis for Protein Extraction

Alkaline hydrolysis is a common technique for extracting proteins from different biomass materials, including algae. It involves treating the algal biomass with alkaline solutions, such as sodium hydroxide (NaOH). The process exploits the solubilization principle of proteins in alkaline media. Proteins have an overall negative net charge in alkaline conditions, which increases their solubility. As a result, they gradually interact with water molecules and become more soluble. Alkaline hydrolysis is particularly effective for extracting proteins from various plant sources, including algae (Álvarez-Viñas et al., 2020).

### Acidic Hydrolysis for Protein Extraction

Much like alkaline hydrolysis, acidic hydrolysis is a commonly employed method for extracting proteins from micro algae. The algal biomass is incubated in an acidic solution, often hydrochloric acid (HCl). The acid disrupts the cell walls and protein structures, making them more accessible for extraction (Álvarez-Viñas et al., 2020).

## The Bicinchoninic Acid (BCA) Assay

The bicinchoninic acid (BCA) assay is a widely used method for determining the total protein concentration in an unknown sample. In this assay, copper (II) ions ($Cu^{2+}$) are reduced to copper (I) ions ($Cu^{1+}$) in the presence of peptides or proteins. BCA molecules can chelate (bind to) $Cu^{1+}$, resulting in the formation of a purple-colored complex. The intensity of this purple color is directly proportional to the protein concentration in the sample, and the maximum absorbance occurs at 562 nm. The BCA assay offers high sensitivity (detecting proteins at low concentrations), is less affected by protein composition, and is compatible with various sample types encountered in protein research (qb3.berkeley.edu, n.d.).

## DAPI Staining & Fluorescence Microscopy

DAPI (4',6-diamidino-2-phenylindole) is a fluorescent stain commonly used in biological research, particularly in fluorescence microscopy. It binds specifically to adenine–thymine (AT)-rich regions in DNA. These regions are characterized by a high AT base pair content. When DAPI binds to double-stranded DNA, it emits blue light upon excitation by UV light. This property allows visualizing cellular components, such as nuclei, which can be used to quantify cell count and assessment of overall cell growth (Andrade and Arismendi, 2013).

From a technical standpoint, DAPI has an absorption maximum at 358 nm (ultraviolet) and emits blue light with a maximum at 461 nm. Microscopists excite DAPI with UV light and detect its emission through a

blue/cyan filter. Although DAPI can also bind to RNA, its fluorescence is not as strong when bound to RNA (Andrade and Arismendi, 2013). An image of BSCs stained with DAPI using fluorescence microscopy is shown in **Figure 4**.

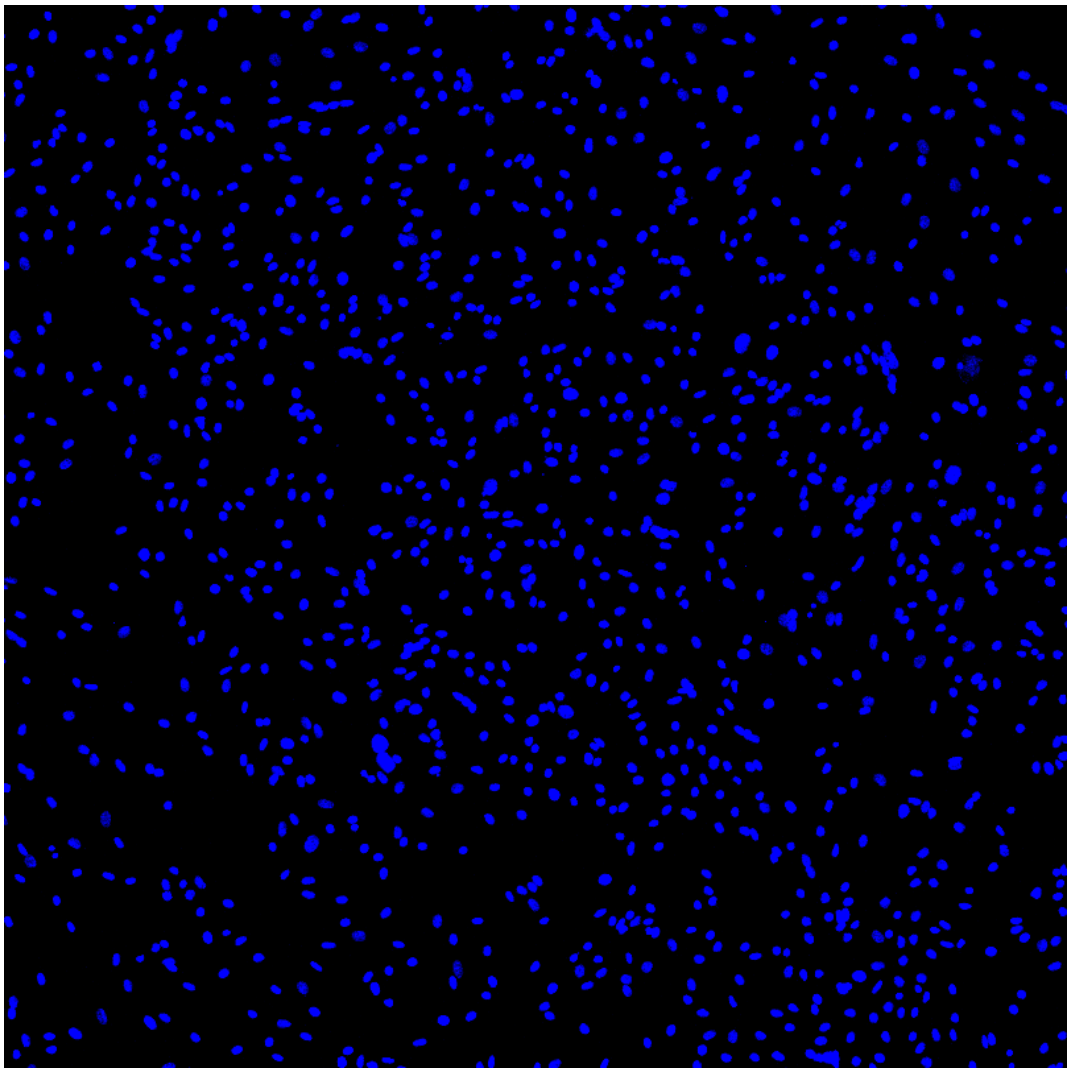

**Figure 4. Bovine Satellite Cells fixated and stained with DAPI (4',6-diamidino-2-phenylindole).** Image taken using ImageXpress Confocal HT.ai and processed using MetaXpress software.

# MATERIALS & METHODS

## Algae Protein Isolate (API) Development

A descriptive figure of how the API was made is presented below.

### Protein Extraction

*Washing the Raw Material*

2.5g of each algae species (X1, Y And X2) dissolved in Milli-Q water to a w/v ratio of 5% and centrifuged (Megafuge™ ST4) at 4300 g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in Milli-Q water to a w/v ratio of 10% in a 50 ml falcon tube.

*Ultrasonication*

Algae power dissolved in Milli-Q water (10% w/v) was sonicated at 70% AMP, 2 seconds on, 2 seconds on using a probe sonicator (VCX 500, Sonics©), for a total running time of 5 minutes. A comparison of sonication on yielded protein is presented in the results section.

*Alkaline Extraction*

The pH of each sample was adjusted up to roughly pH 12 using 1M NaOH and incubated at 37°C for 60 minutes, 250 RPM followed by centrifugation (Multifuge X4FR Pro, Thermo Scientific) at 10 000 g for 10 minutes. The supernatant was collected and stored in at 4°C overnight.

*Ultrasonication and Alkaline Extraction*

All the steps described in *Ultrasonication* followed by all the steps described in *Alkaline Extraction.*

*Washed Raw material and Alkaline Extraction*

All steps described in *Washing the Raw Material* followed by the steps described in *Alkaline Extraction*.

*Washed Raw material, Ultrasonication and Alkaline Extraction*

All steps described in *Washing the Raw Material* followed by the steps described in *Ultrasonication* followed by the steps *Alkaline Extraction*.

*Acidic Extraction*

The pH of each sample was adjusted up to roughly pH 3 using 1M HCL and incubated at 37°C for 60 minutes, 250 RPM followed by centrifugation (Multifuge X4FR Pro, Thermo Scientific) at 10 000 g for 10 minutes. The supernatant was collected and stored in at 4°C overnight.

*Hydrolysis Using Cellulase Blend*

The pH of each sample was adjusted up to roughly pH 9.5 using 1M NaOH and incubated with 250 µl Cellulase Blend (ref. #SAE0020, Sigma-Aldrich) at 50°C for 5 hours, 175 RPM followed by centrifugation (Multifuge X4FR Pro, Thermo Scientific) at 10 000 g for 10 minutes. The supernatant was collected and stored in at 4°C overnight.

### Protein Concentration Estimation Using BCA

Samples for a standard curve prepared using the protocol provided by the BCA kit (ref. #23225, Thermo Fisher) with known concentrations (Thermo Fisher Scientific (2024)). The reference protein in this context was human albumin. Duplicates (25 µl of each) of standard were pipetted onto a 96-well plate. A dilution series of 1x, 5x, 25x & 125x was made for each sample and duplicates (25 µl of each sample) were pipetted on the same 96-well plate.

The working reagent and the copper sulfate solution (both provided by the BCA kit) were mixed to a ratio of 50:1 and 200 µl were pipetted into each sample/standard. The whole plate was shaken lightly for 30 seconds, incubated at 37°C for 30 minutes. The plate was placed in a room for 5 minutes and the absorbance was measured at 562 nm. The data was collected and processed using GraphPad Prism 10.2.3. Corresponding figures are med using GraphPad Prism 10.2.3.

### Filtration

After protein extraction, the samples from the highest yielding protein isolates were filtered through a 5.0, 1.2, 0.8, 0.45 and 0.22 µm filter (ref. #SMWP02500, #RAWP04700, #AAWG04700, #HAWP02500 & #GVWP09050, Sigma-Aldrich) using a vacuum pump. The samples were sterilized using a 0.2 Millipore filter (ref. #10362452, Fisher Scientific) inside a LAF bench.

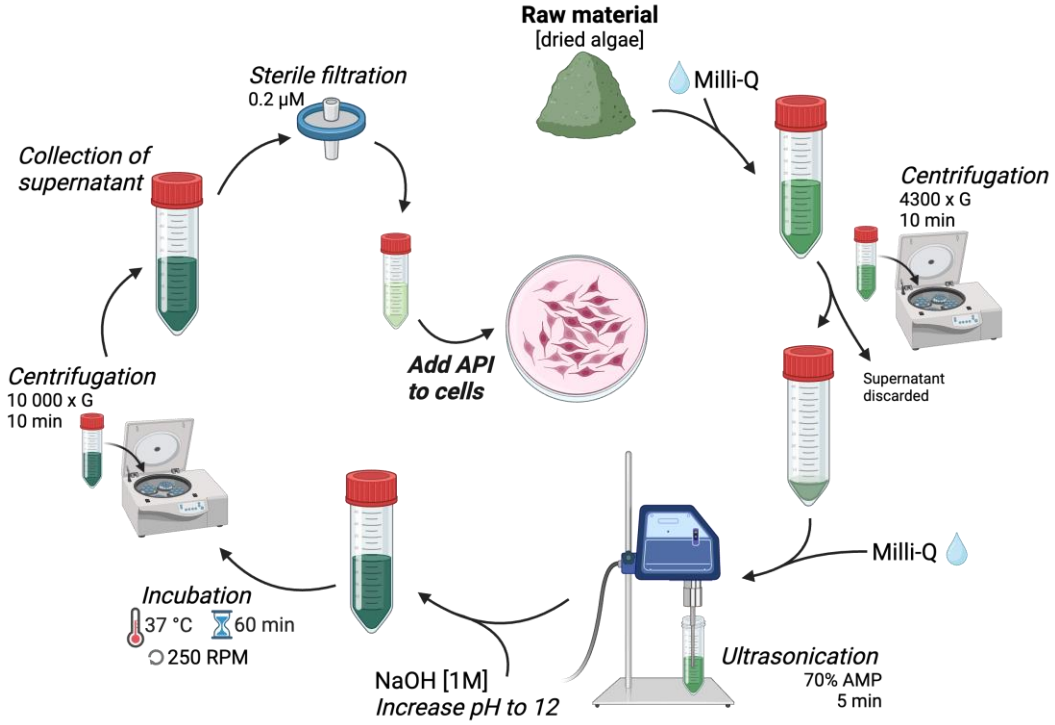


**Figure 5. Overview of how the algae protein isolate is made.** (Figure designed in BioRender).

## Algae Peptide Mixture (APM) Development

### Washing the Raw Material & Ultrasonication

The same procedure is used in the development of API presented above.

### Hydrolysis Using Protease

The pH of the washed and ultrasonication-treated samples was adjusted to roughly 9.2 using 1M NaOH and 250 µl Alcalase® (ref. # 126741, Sigma-Aldrich) was added to each sample and incubated at 50°C, 175

RPM for 5 hours. All samples were centrifuged (Multifuge X4FR Pro, Thermo Scientific) at 10 000 g for 10 minutes and the supernatant was collected and stored at 4°C overnight.

### Filtration

Each sample was filtered through a 5.0, 1.2, 0.8, 0.45 and 0.22 µm filter (ref. #SMWP02500, #RAWP04700, #AAWG04700, #HAWP02500 & #GVWP09050, Sigma-Aldrich) using a vacuum pump. Each sample was then centrifuged (Megafuge™ ST4) at 4300 RPM, 4°C overnight through a 100 kDa spin column filter (Vivaspin, Cytiva) and the permeate was centrifuged (Megafuge™ ST4) at 4300 RPM, 4°C overnight through a 30 kDa spin column filter (Vivaspin, Cytiva) and the permeate was then sterilized through a 0.2 Millipore filter (ref. #10362452, Fisher Scientific) inside a LAF bench.

## Harvesting Bovine Satellite Cells

A sterile biopsy from a 15-month-old calf was used to harvest the bovine satellite cells. Biopsy provided by *TEKNOLOGISK INSTITUT, DK.*

## Cell Cultivation

### List of Reagents for Cell Cultivation

A list of reagents used in cell cultivation and staining experiments is presented in **Table 3** in the Appendix.

### Seeding Bovine Satellite Cells

PBS, Trypsin, and stock mixture of DMEM) + 20 (v/v) % FBS + 1 (v/v) % PS were put a water bath set at 37°C for 15 minutes, all heated mixtures were then sterilized with 70% ethanol and put inside a LAF bench. A 75 cm2 cell culture flask with roughly 80% confluent BSCs was taken out of the incubator and put on the LAF bench. The media in the flask was removed by suction and 10 ml of PBS was added to wash the cells from any remaining media. The PBS was then removed by suction and 2 ml of trypsin was added to dislodge the cells from the flask. The flask was then incubated at 37°C for 3 minutes and put back into the LAF bench. 8 ml of DMEM + 20 (v/v) % FBS + 1 (v/v) % PS was added to the flask and pipetted into a 15 ml Eppendorf tube. A 250 µl sample of the cell suspension was taken and the live cell count was measured using NucleoCounter NC-202. Based on the cell concentration given, the cell suspension was diluted to a cell concentration of 3344 live cells/ml. 500 µl of diluted cell suspension was then pipetted into each well of a 24-well plate (ref. #82426, ibidi) and incubated at 37°C, 5% CO2 overnight.

## Fetal Bovine Serum titration

A 24-well plate (ref. #82426, ibidi) seeded with 3344 cells/ml the day before was used to carry out the FBS titration experiment. Stocks of DMEN + 1 (v/v) % PS with 30%, 20%, 12.5%, 10%, 7.5%, 5%, 2.5% and 0% FBS were prepared, and the plate was taken out of the incubator and put into a LAF bench. The media in each well was removed by suction and 250 µl PBS was added to each well to wash away any remaining media. The PBS was then removed by suction and 500 µl of FBS stock mixture was added to each well according to **Table 1**.

**Table 1.** Experimental setup in a 24-well plate for FBS titration experiment. Each well contains DMEM + 1 (v/v) % + the respective FBS concentration.

| | | | | | |
|---|---|---|---|---|---|
| 30% FBS | 30% FBS | 30% FBS | 20% FBS | 20% FBS | 20% FBS |
| 12.5% FBS | 12.5% FBS | 12.5% FBS | 10% FBS | 10% FBS | 10% FBS |
| 7.5% FBS | 7.5% FBS | 7.5% FBS | 5% FBS | 5% FBS | 5% FBS |
| 2.5% FBS | 2.5% FBS | 2.5% FBS | 0% FBS | 0% FBS | 0% FBS |

API/APM in cultivation of Bovine Satellite Cells

PBS, DMEM + 1 (v/v) % and FBS were put in a water bath set at 37°C for 15 minutes, all heated mixtures were then sterilized with 70% ethanol and put inside a LAF bench. Stock of DMEN + 1 (v/v) % PS with 20%, 4% and 0% FBS were prepared samples containing DMEM + 1 (v/v) % + 4 % FBS and different protein concentrations of API/APM from the three algal species. A 24-well plate (ref. #82426, ibidi) seeded with 3344 cells/ml the day before was taken out of the incubator and put inside the LAF bench. The media in each well was removed by suction and 250 µl PBS was added to each well to wash the cells from any remaining media. The PBS was removed by suction and 500 µl each sample was added to each well. The cells were then incubated for three days at 37°C, 5% CO2. Several concentrations were tested over several experiments and are all presented in the results section. An example plate setup is presented in **Table 2.**

**Table 2. Example experimental setup for testing different API/APM concentration on bovine satellite cells.** The first row contains control samples containing DMEM + 1 (v/v) % PS + 20%, 4% and 0% FBS respectively. The second row contains DMEM + 1 (v/v) % PS + 4% FBS + samples of API/APM from X1 at different concentrations. The third row contains DMEM + 1 (v/v) % PS + 4% FBS + samples of API/APM from Y at different concentrations. The fourth row contains DMEM + 1 (v/v) % PS + 4% FBS + samples of API/APM from X2 at different concentrations.

| Controls | 20% FBS | 20% FBS | 4% FBS | 4% FBS | 0% FBS | 0% FBS |
|---|---|---|---|---|---|---|
| **API/APM from X1** | 0.015 [mg/ml] + 4% FBS | 0.015 [mg/ml] + 4% FBS | 0.025 [mg/ml] + 4% FBS | 0.025 [mg/ml] + 4% FBS | 0.050 [mg/ml] + 4% FBS | 0.050 [mg/ml] + 4% FBS |
| **API/APM from Y** | 0.015 [mg/ml] + 4% FBS | 0.015 [mg/ml] + 4% FBS | 0.025 [mg/ml] + 4% FBS | 0.025 [mg/ml] + 4% FBS | 0.050 [mg/ml] + 4% FBS | 0.050 [mg/ml] + 4% FBS |
| **API/APM from X2** | 0.015 [mg/ml] + 4% FBS | 0.015 [mg/ml] + 4% FBS | 0.025 [mg/ml] + 4% FBS | 0.025 [mg/ml] + 4% FBS | 0.050 [mg/ml] + 4% FBS | 0.050 [mg/ml] + 4% FBS |

## DAPI Staining

After three days of incubation, the media is removed from each well and washed with 250 µl of PBS. To fixate the cells, 250 µl of paraformaldehyde, 4% in PBS is added to each well and is incubated at room temperature for 10 minutes. The paraformaldehyde was removed, and each well was washed one time with 250 µl PBS. To permeabilize the cells to facilitate later staining, 250 µl of 0.1 v/v % of Triton X-100 in PBS was added to each well and incubated at room temperature for 15 minutes. The Triton X-100 was then removed and 250 µl of DAPI stain was added and incubated at room temperature away from light for 20 minutes. After incubation, the DAPI stain was removed, and each well was washed with 250 µl PBS three times. Lastly, 500 µl of PBS was added to each well and the plate was stored at 4°C away from light until imaging.

## Imaging, Data & Figures

Using ImageXpress Confocal HT.ai, nine images per well of the 24-well plate were taken and analyzed using MetaXpress software. The number of nuclei per image was then counted and the sum of all nine images was calculated. This sum corresponds to the live cell count of the given well. The data was collected and processed using GraphPad Prism 10.2.3. Corresponding figures are med using GraphPad Prism 10.2.3.

# RESULTS

## About the Results

The development of API was initiated by experimenting with various protein extraction methods. The primary objective was to identify the method that yields the highest protein concentration from the different micro algas. However, simplicity was also a factor that was considered. The ease of implementation, recognizing that cost effectiveness and practicality plays a significant role in real world applications.

Having established an efficient protein extraction method, the focus was shifted to API concentration. Determining the optimal concentration is critical for achieving robust cell growth. Through systematic testing and analysis, the aim was to find the sweet spot where the API promotes cell proliferation without causing adverse effects.

## Algae Protein Isolate (API) Development

### Protein Extraction

*Ultrasonication Time Comparison*

An efficient way of disrupting the cell wall of micro algae has been the use of ultrasound (Liu et al., 2022), however, the use of ultrasonication can be time consuming and energy intensive. For this reason, a small experiment comparing sonication times on protein yield was conducted and the amount of protein yielded after sonication is presented in **Figure 6**. The protein concentration was measured on the supernatant using BCA.

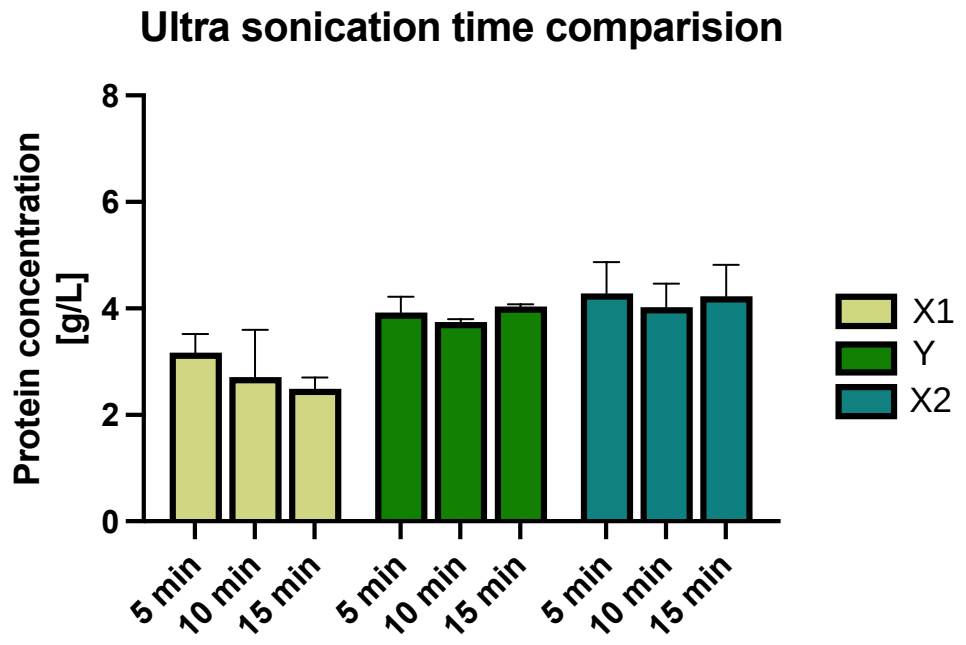


**Figure 6. Comparison of sonication times on yielded protein.** X1, Y and X2 were sonicated 5, 10 and 15 minutes and protein concentration [g/L] was measured using BCA. Data presented are the mean value of duplicate measurements ± SD.

Images of each algae type before and after ultrasonication treatment are presented in **Figure 7.**

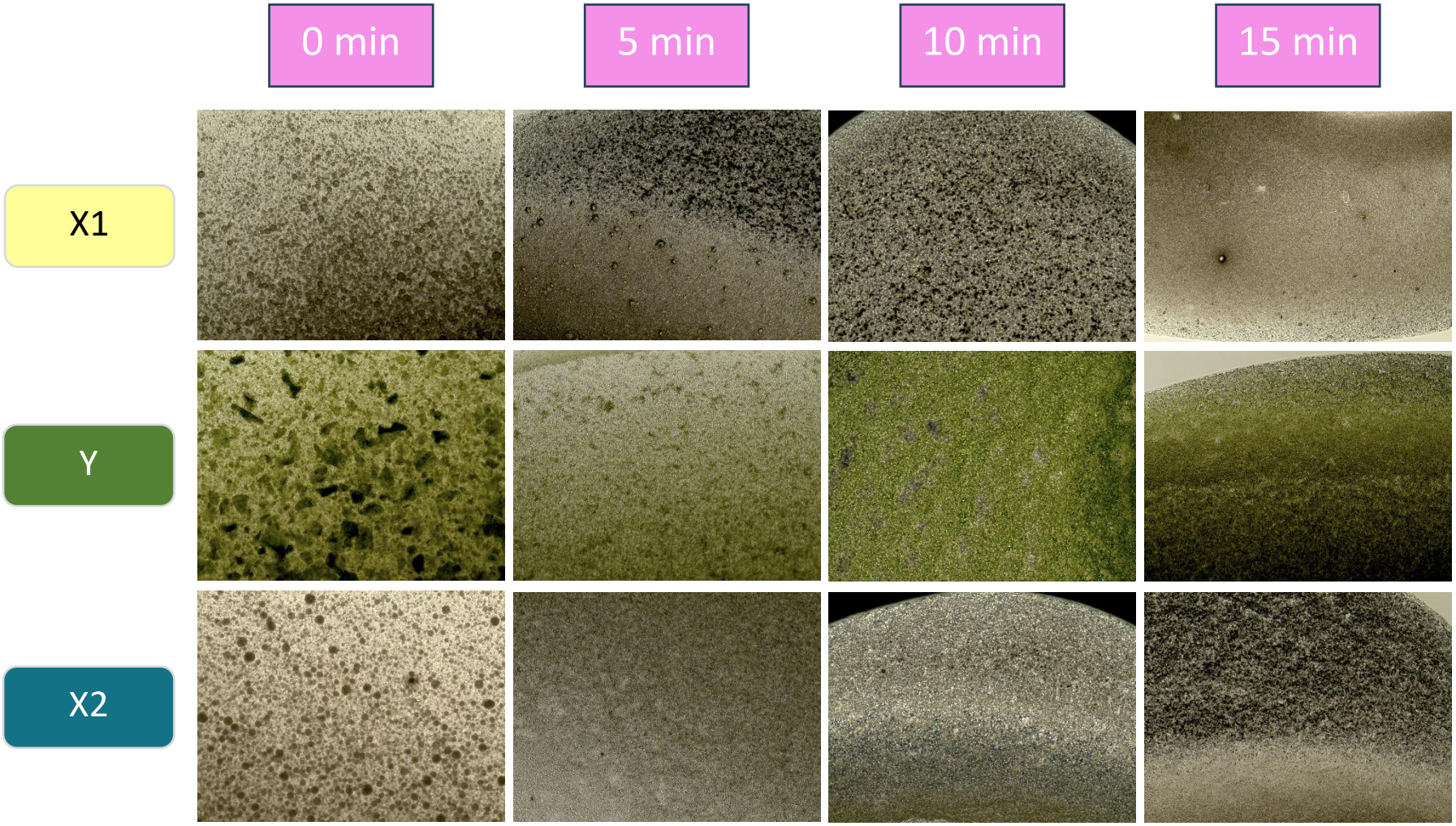


**Figure 7. Visualization of cell disruption before and after ultrasonication.** Microscope images of X1, Y and X2 that were sonicated 0, 5, 10 and 15 minutes and the cell disruption is visualized in this figure.

*Hydrolysis Using Cellulase Blend*

Another promising method of disrupting the cell wall is enzymatic hydrolysis using a cellulase enzyme (Le Nguyen Doan et al., 2022). Each algae sample was incubated with the enzyme and the protein concentration was measured in the supernatant using BCA. The result is presented in **Figure 8A**.

*Ultrasonication, 5 minutes*

To test the accuracy of the ultra-sonication comparison on protein yield, a new experiment was conducted aiming to get a more precise grasp of the protein present in the supernatant after sonication. The result is presented in **Figure 8B**.

*Alkaline Extraction*

Attempting to increase extraction yield further, the pH of each sample was to potentially increase the solubility of the proteins. The protein concentration was measured on the supernatant using BCA. The result is presented in **Figure 8C**.

*Acidic Extraction*

Attempting to capture proteins that are soluble in acidic conditions, the pH was decreased samples were incubated. The protein concentration was measured on the supernatant using BCA. The result is presented in **Figure 8D**.

*Ultrasonication + Alkaline Extraction*

To first disrupt the cell wall of the algae and then to increase the solubility of the proteins more available proteins, each sample was first by the Alkaline Extraction described above. The protein concentration was measured on the supernatant using BCA. The result is presented in **Figure 8E**.

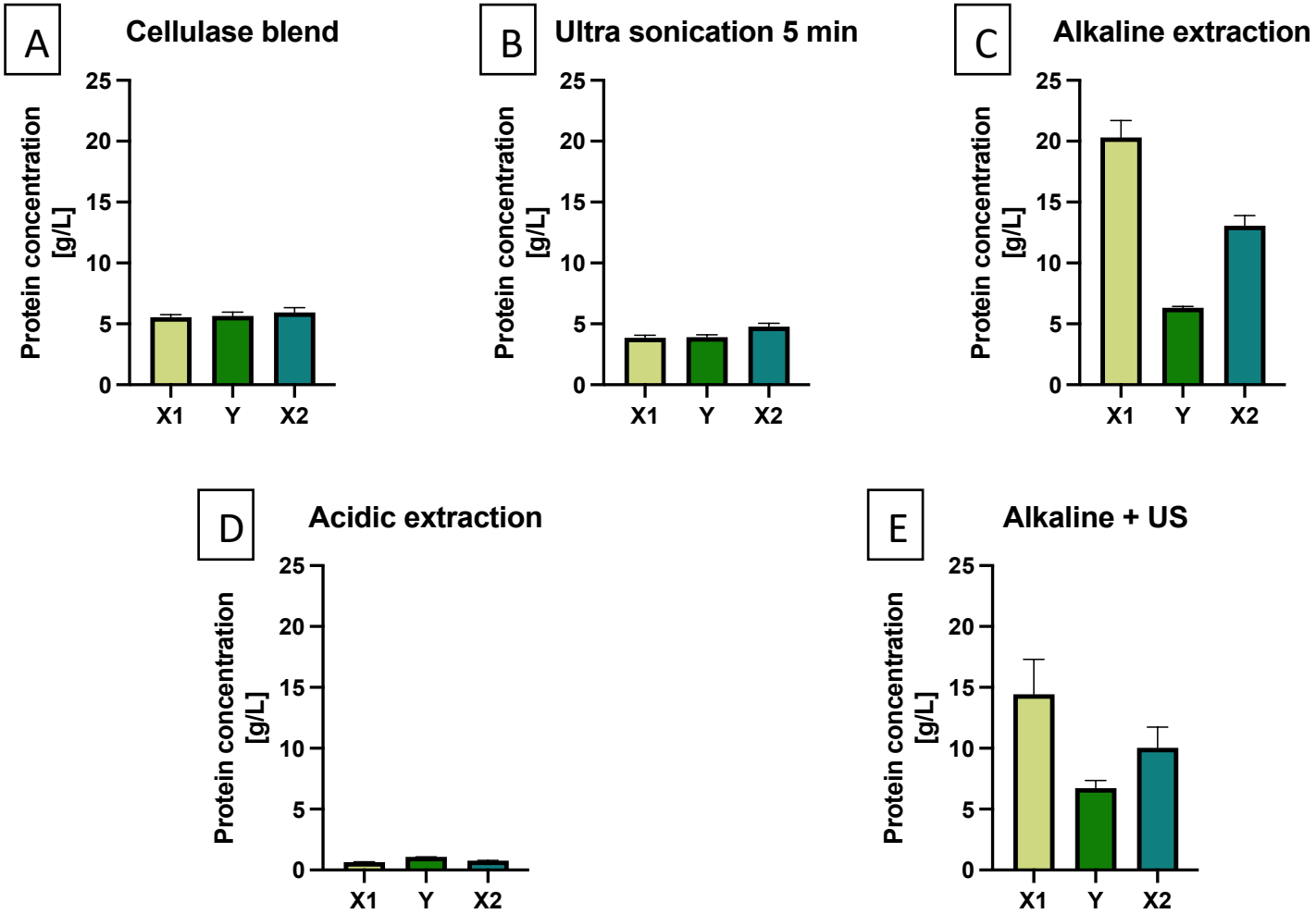


**Figure 8. Protein yield of supernatant after different extraction methods.** A: Samples of X1, Y and X2 were incubated with cellulase blend for 5 hours and protein concentration [g/L] of the supernatant was measured using BCA**.** B: Samples of X1, Y and X2 were ultrasonicated for 5 minutes and protein concentration [g/L] of the supernatant was measured using BCA. C: Samples of X1, Y and X2 were incubated in alkaline solution for 60 minutes and protein concentration [g/L] of the supernatant was measured using BCA. D: Samples of X1, Y and X2 were incubated in acidic solution for 60 minutes and protein concentration [g/L] of the supernatant was measured using BCA. E: Samples of X1, Y and X2 were sonicated for 5 minutes and incubated in alkaline solution for 60 minutes and protein concentration [g/L] of the supernatant was measured using BCA. Data presented are the mean value of duplicate measurements ± SD.

## FBS Concentration Titration

To enable the investigation of serum replacement properties of the API, an FBS titration experiment was conducted to find a range where the BSCs seem to be more fragile and sensitive to an increase or decrease in FBS concentration. The result is presented in **Figure 9**.

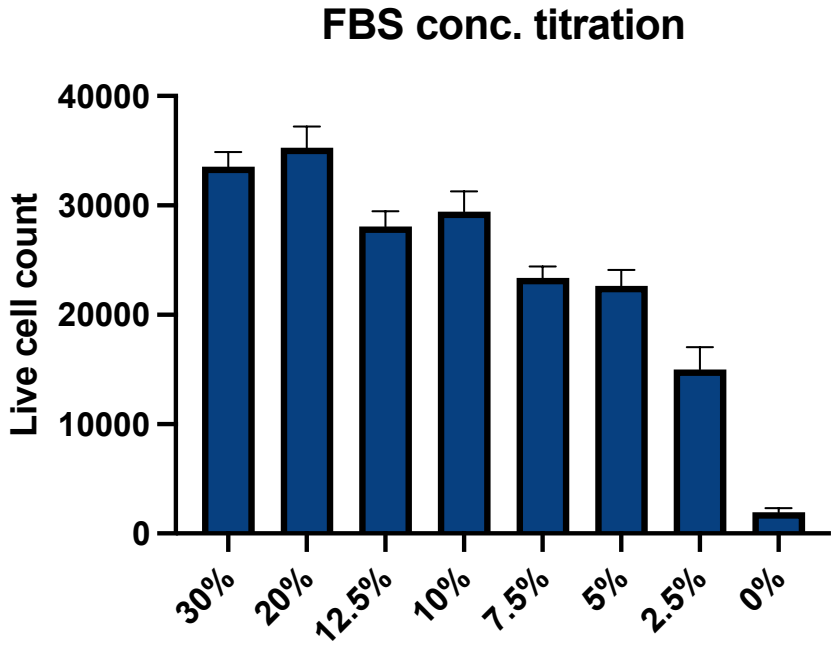


**Figure 9. FBS concentration titration on Bovine Satellite Cells (BSCs), passage 0.** BSCs cultured in 30, 20, 12.5, 10, 7.5, 5, 2.5 or 0% FBS. Data presented are the mean value of duplicate measurements ± SD.

## API Concentration Optimization

### *Initial Testing - BSCs Cultivated in API*

The initial test of API was an extract using only alkaline extraction described in the *Materials & Methods* section. The aim of this test was to establish concentration range where the API has a toxic effect on BSCs to enable further optimization and to find the optimal concentration range to potentially reveal serum replacement properties of the API. The result from this test is presented in **Figure 10**.

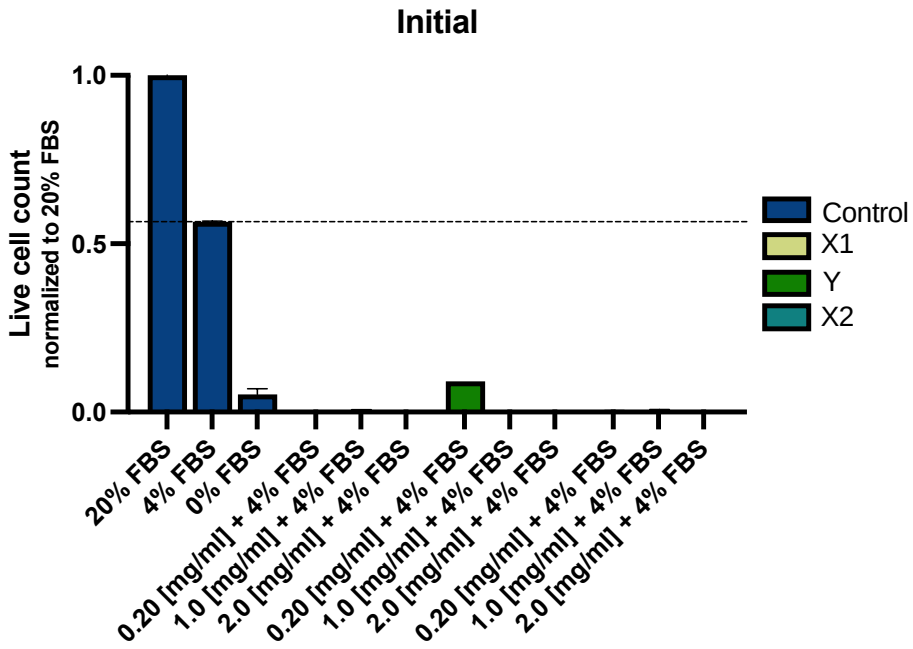


**Figure 10. Initial test cultivating bovine satellite cells (BSCs), passage 3, in algae protein isolate (API).** BSCs cultured in API from X1, Y and X2 at 2.0, 1.0, 0.2 [mg/ml] protein + 4% FBS. Controls with 20, 4 and 0% FBS all without any API added. Black dotted line corresponds to the mean value of the 4% FBS control. Data presented are the mean value of duplicate measurements ± SD.

*BSCs Cultivated in Reduced Concentration*

From the results obtained from the initial API experiment, a new experiment was conducted with heavily reduced API (from the same extraction as the previous experiment) concentrations with the goal of reducing the toxic effect of the API. The result is presented in **Figure 11**.

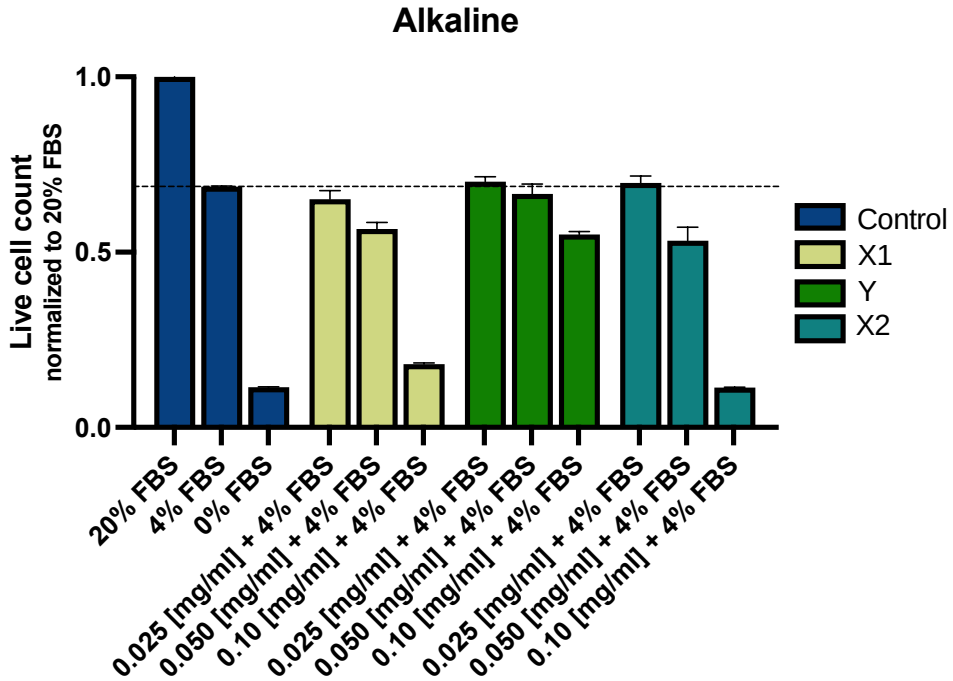


**Figure 11. Bovine satellite cells (BSCs), passage 1, cultivated reduced concentration of algae protein isolate (API).** BSCs cultured in API from X1, Y and X2 at 0.10, 0,050, 0.025 [mg/ml] protein + 4% FBS. Controls with 20, 4 and 0% FBS all without any API added. Black dotted line corresponds to the mean value of the 4% FBS control. Data presented are the mean value of duplicate measurements ± SD.

*Reduced Concentration – API From Ultrasonication + Alkaline Extraction*

An API was developed where the algae samples were first sonicated followed by alkaline extraction. This API was tested in the same concentration range as the previous experiment (**Figure 11**). The goal with this experiment was to compare the two extracts performance on the BSCs cell growth. The result is presented in **Figure 12**.

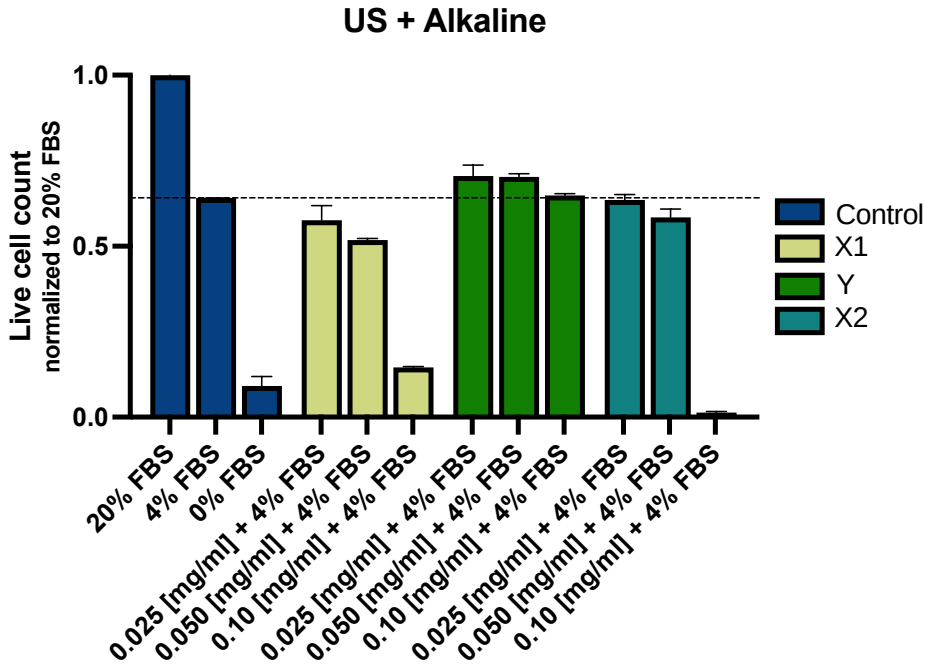


**Figure 12. Bovine satellite cells (BSCs), passage 1, cultivated reduced concentration of algae protein isolate (API) from ultrasonication + alkaline extraction.** BSCs cultured in API from X1, Y and X2 at 0.10, 0,050, 0.025 [mg/ml] protein + 4% FBS. Controls with 20, 4 and 0% FBS all without any API added. Black dotted line corresponds to the mean value of the 4% FBS control. Data presented are the mean value of duplicate measurements ± SD.

*Reducing Osmolality in the API*

A strong buffer capacity was observed when attempting to increase the pH to enable alkaline extraction of the algae samples. The large amount of NaOH added could potentially inhibit cell growth (Romanova et al., 2022). To reduce the amount sodium hydroxide (NaOH), the dried algae was first washed with Milli-Q water, centrifuged, and resuspended before proceeding with above mentioned extraction methods. The aim of this experiment was to reduce the buffer capacity in the algae raw material. The APIs osmolality was measured, and the results are presented in **Figure 13**.

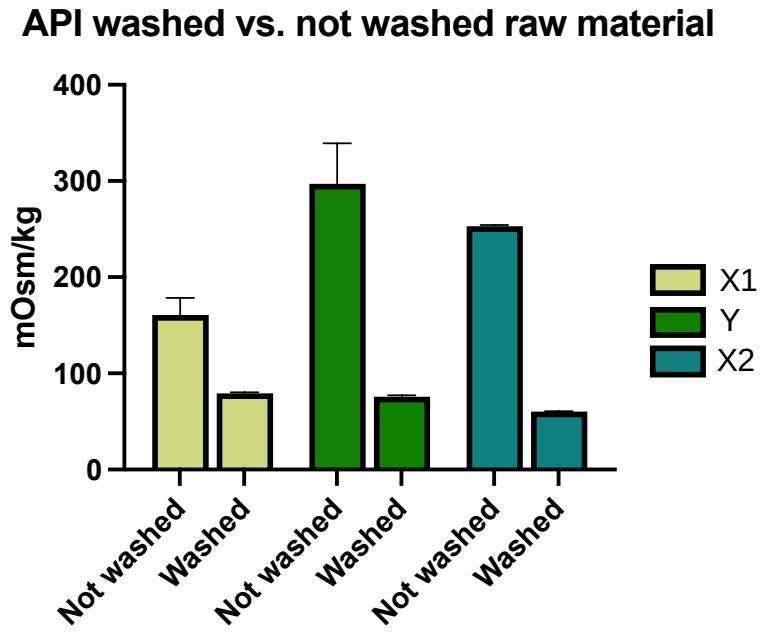


**Figure 13. Osmolality of algae protein isolate with and without washed raw material.**

*Protein Concentration in the Supernatant After Washing*

To see how much protein is lost due to washing the raw material, the protein concentration of the supernatant of the washed algae was measured using BCA. The result is presented in **Figure 14**.

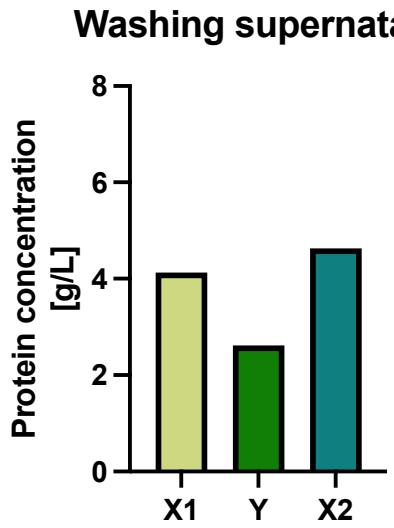


**Figure 14. Protein concentration in the supernatant after washing the algae raw material.** Samples of X1, Y and X2 were washed with milli-Q water, centrifuged and the protein concentration [g/L] was measured using BCA. Data presented from a single replicate.

*Comparing APIs – High vs Low Osmolality*

APIs where only alkaline extraction had been used and APIs with ultrasonication + alkaline extraction, where the raw material had been washed with milli-Q water, thus reduced osmolality, were tested on BSCs. The result is presented in **Figure 15**.

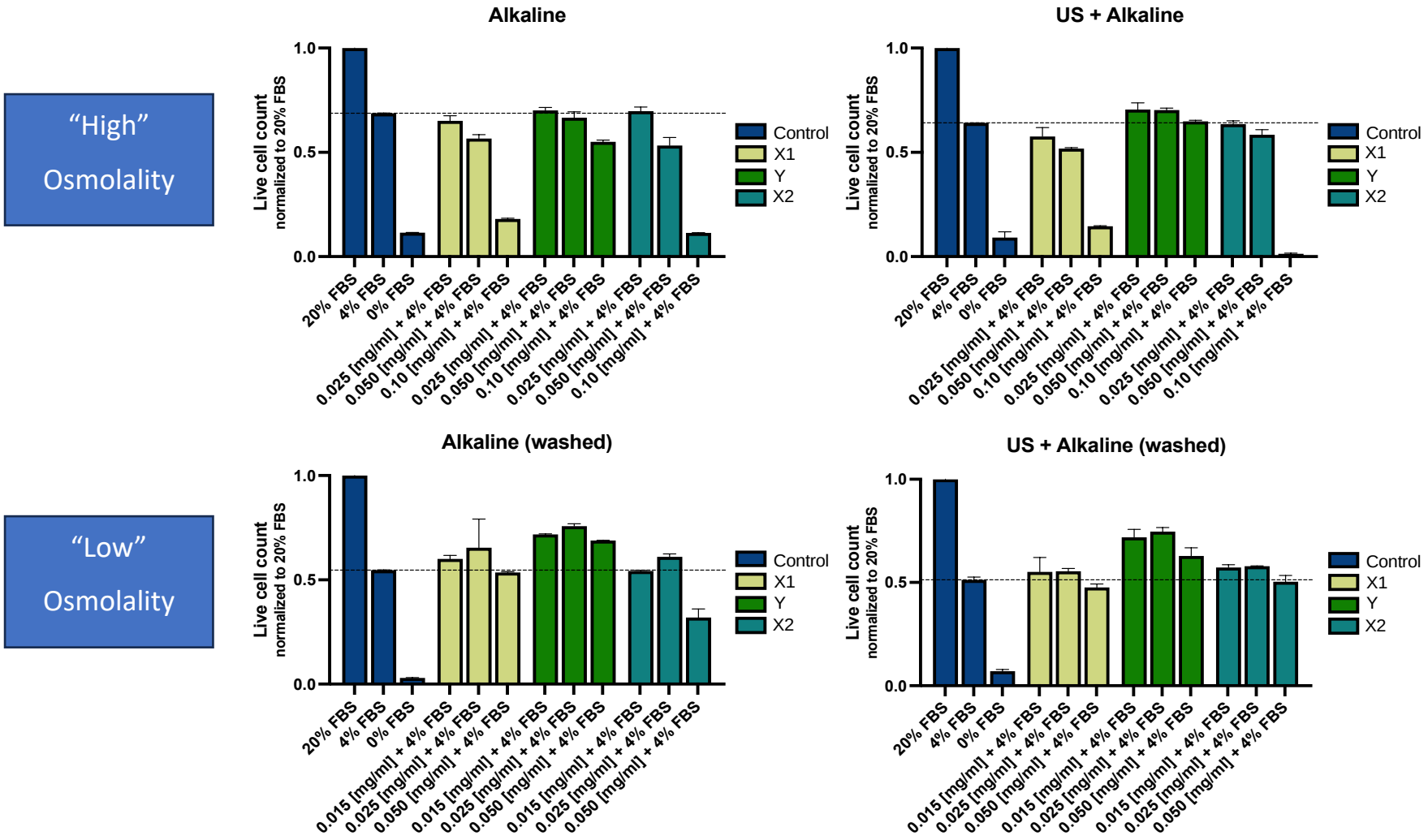


**Figure 15. Comparison of extraction methods & osmolality on BSCs growth (all passage 4).** BSCs cultured in API from X1, Y and X2 at 0.10, 0,050, 0.025 [mg/ml] protein + 4% FBS. Controls with 20, 4 and 0% FBS all without any API added. Black dotted line corresponds to the mean value of the 4% FBS control. Data presented are the mean value of duplicate measurements ± SD.

*Replicating The Results*

A new API, named API 2.0, was made using the same extraction protocol as previously (washing raw material + ultrasonication + alkaline extraction) and tested on BSCs. The result is presented in **Figure 16**.

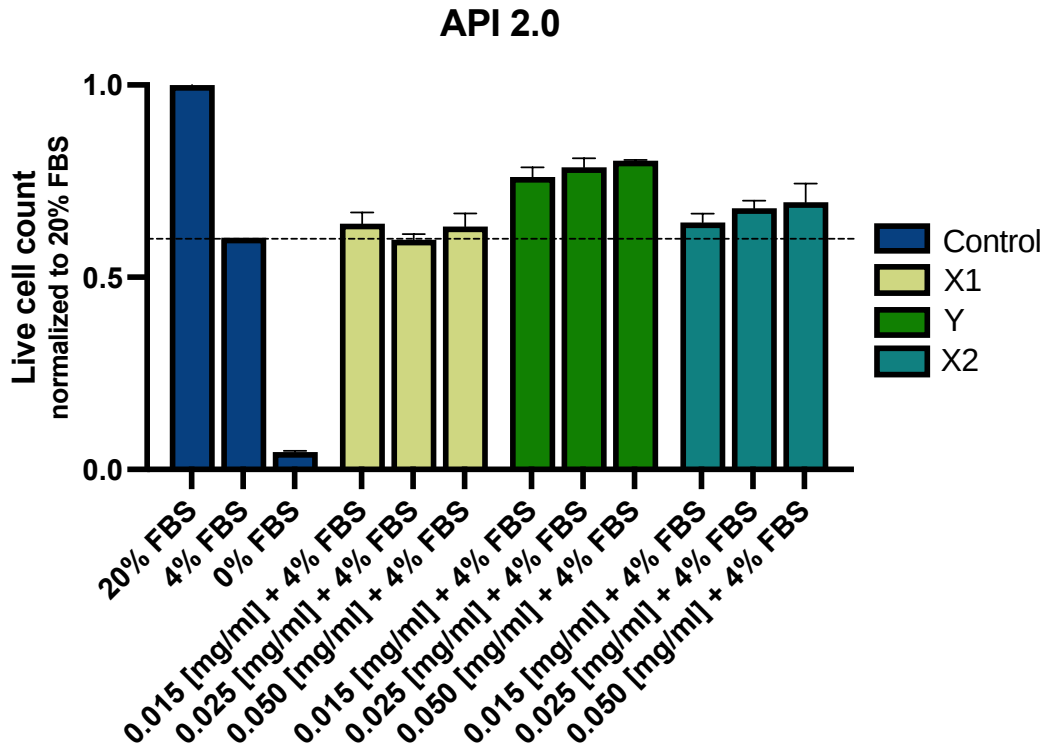


**Figure 16. Bovine satellite cells (BSCs), passage 3, cultivated in API 2.0 from ultrasonication + alkaline extraction.** BSCs cultured in API from X1, Y and X2 at 0.10, 0,050, 0.025 [mg/ml] protein + 4% FBS. Controls with 20, 4 and 0% FBS all without any API added. Black dotted line corresponds to the mean value of the 4% FBS control. Data presented are the mean value of duplicate measurements ± SD.

*FBS Concentration Titration with API*

The performance of the different APIs had thus far only been tested in combination with 4% FBS. To test the performance with a wider concentration range of FBS, a titration experiment was conducted using the first iteration of the API with reduced osmolality, sonicated followed by alkaline extraction. The so far, best performing concentration of API (0.025 mg/ml protein) was used in this setup. The result is presented in **Figure 17**.

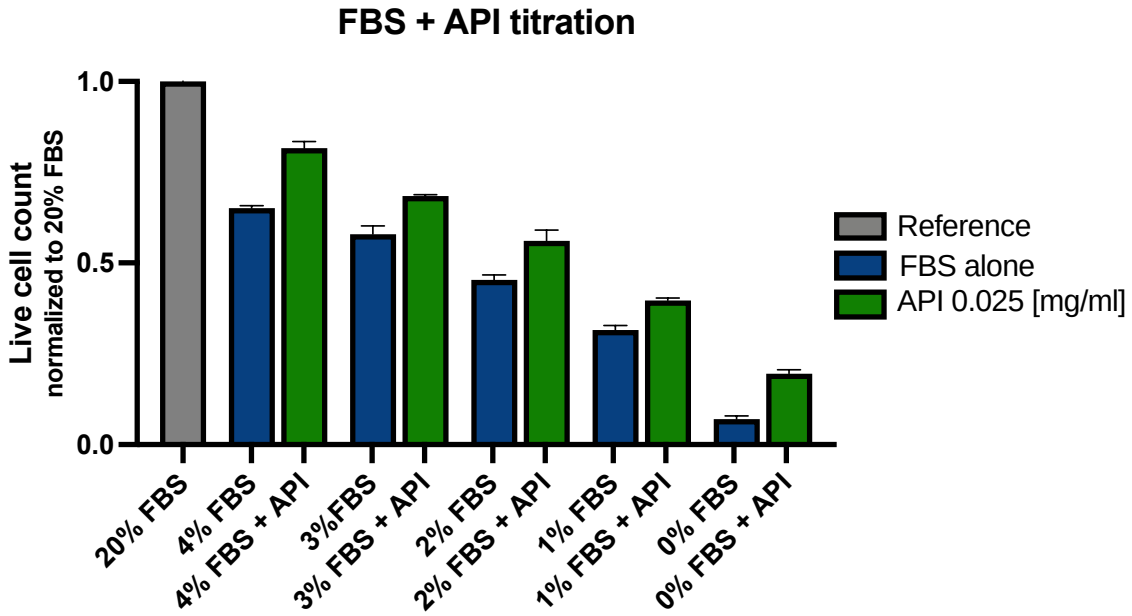


**Figure 17. FBS concentration titration with added algae protein isolate (API) from Y on bovine satellite cells), passage 2.** API developed from algae Y with concentration of 0.025 [mg/ml] protein in media with 4, ,3 ,2 ,1 and 0% FBS. Data presented are the mean value of duplicate measurements ± SD.

## Algae Peptide Mixture (APM) Development

### About the Development of APM

In a recent study, dried rapeseed was hydrolyzed using different proteinases and the hydrolysates were used to cultivate CHO cells successfully. Among the proteases used in the study, Alcalase® enzyme proved to have the best effect.

These findings sparked the idea of attempting to develop an algae peptide mixture (APM) by using a similar approach. Algae samples were washed, ultrasonication and incubated with Alcalase® at a pH optimal for the enzyme. Next, the APM was filtered down, keeping the permeate of a 30 kDa spin column. Lastly, the protein/peptide concentration was measured, and the optimal concentration was estimated to achieve maximum cell proliferation boosting effect.

### Protein/Peptide Concentration

The protein/peptide concentration of the APM was estimated using BCA and the result is presented in **Figure 18**.

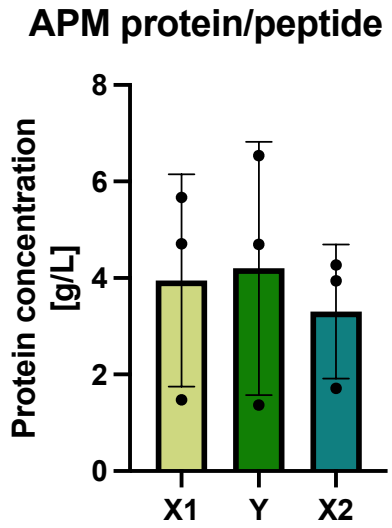


**Figure 18. Protein/peptide concentration of algae peptide mixture.** Samples of X1, Y and X2 were washed with Milli-Q water, ultra sonicated for 5 minutes, and incubated with Alcalase® enzyme and the protein concentration was measured on the supernatant using BCA. Data presented from a single replicate, with the mean of triplicate measurements from 1x, 5x and 25x dilutions.

### APM Concentration Optimization

BSCs were cultivated in APM with the same concentration range used as in the most successful experiments using API. Later, a similar experiment was conducted, further reducing the concentration. A comparison of the results of these two tests of the APM is presented in **Figure 19**.

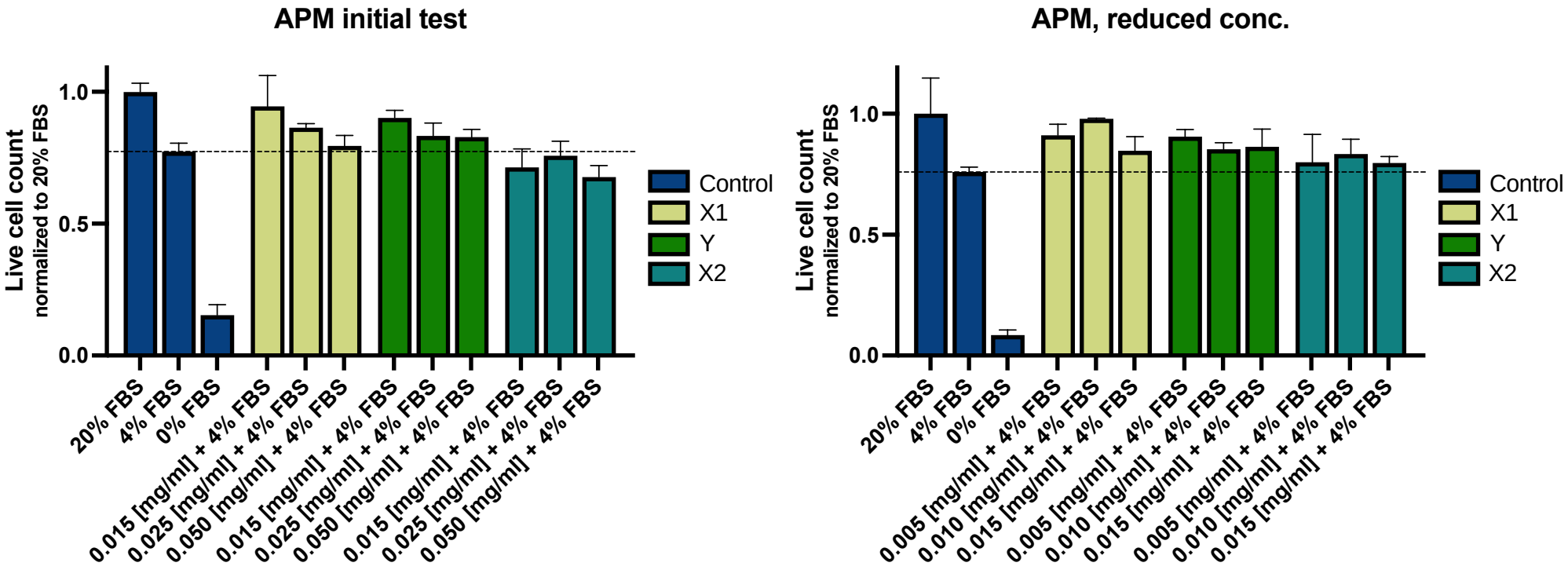


**Figure 19. Comparison of initial test of algae peptide mixture (high concentration) and algae peptide mixture with reduced concentration on bovine satellite cells), passage 4.** BSCs cultured in APM from X1, Y and X2 at 0.015, 0,025, 0.050 [mg/ml] protein/peptides + 4% FBS (APM initial test) and 0.005, 0,010, 0.015 [mg/ml] protein/peptides + 4% FBS (APM, reduced con.). Controls with 20, 4 and 0% FBS all without any APM added. Black dotted line corresponds to the mean value of the 4% FBS control. Data presented are the mean value of duplicate measurements ± SD.

# DISCUSSION

## Algae Protein Isolate (API) Development

### Protein Extraction

*Ultrasonication Time Comparison*

Results presented in **Figure 6** indicate that the amount of protein extracted varies with the duration of ultrasonication and the type of sample (X1, Y, and X2).

For samples X1 and X2, an increase in ultrasonication might correlate with higher protein concentrations. Longer sonication durations enhance protein extraction efficiency for these types. However, for Y samples, the highest protein extraction occurs at 10 minutes of ultrasonication. This suggests that Y responds optimally to this specific duration.

It is essential to consider the balance between yield and protein quality when choosing ultrasonication times. The observed trends align with existing literature. A study proposes an optimal ultrasonication time beyond which protein degradation may occur (Liu et al., 2022). At longer durations, proteins might denature or fragment, affecting their quality. Therefore, while maximizing yield, protein integrity must be considered as the main factor.

The results from this experiment indicate a low difference in amount of protein extracted between sonication times, thus the milder treatment of 5 minutes of ultrasonication was used in consequent experiments.

It should also be noted that the Y raw material, prior to being used in this context, had been freeze dried and thus given the fragmented appearance due to cell rupture during the drying process.

*Comparing Extraction Methods*

One of the main things to point out is the fact that mainly protein quantity and not protein quality is used to determine the best extraction method. The goal of this comparison was to obtain one or two good protein-yielding extraction methods that in theory would not put to much stress on potential functional proteins.

When examining the results in **Figure 8**, it is evident that proteins seem go well into solution in alkaline conditions. This result is similar to what Stout observed when attempting to extract proteins from rapeseed (Stout, 2023) and stood out as a promising candidate. While Yamanaka had recent success in nutrient extraction from micro algae using acidic hydrolysis (Yamanaka et al., 2023), this result indicate that proteins were poorly dissolved in low pH and resulted in very low protein concentration when measured using BCA.

The use of a cellulase to break down the cell wall seem to fall in the middle of the two previous discussed extraction methods. The pH was increased to roughly 9 and incubated for 5 hours at elevated temperature. Though the use of cellulase blend yielded a higher protein concentration compared to ultrasonication on its own, the relatively long procedure, compared with ultrasonication, made this method deemed as inferior.

In addition, it was suspected that further incubation in alkaline conditions would start to degrade proteins instead of extracting them. It must be noted that this suspicion was not thoroughly assessed and in hindsight would have been interesting to investigate further. Another consideration of using an enzyme to break down the cell wall is the fact that the amount of protein extracted will appear higher than what is true. This

is because BCA will measure the enzyme as part of the total protein concentration and thus give a somewhat elevated result. The exact amount of enzyme added could be effectively subtracted but adding functional ingredients (such as enzymes) introduce additional variables to consider.

Using the information obtained from this experiment, the API was further developed using alkaline extraction on its own as well as ultrasonication followed by alkaline extraction as two methods of obtaining proteins to formulate the API.

## FBS Concentration Titration

The findings reveal distinct sensitivity patterns across different FBS concentrations. Between 5% and 0% FBS, BSCs demonstrate heightened responsiveness. Higher FBS concentration seems to enhance cell growth which, where 20% seems to be the most optimal concentration. This is what is expected and is in line with the finding of Zygmunt et al., (2023). Conversely, lower concentrations inadequately support cellular activities. The argument of deciding upon both slightly higher and slightly lower than 2.5% FBS concentration is valid. The BSCs used in this experiment were passage 0 cells and because of a limitation of the quantity of cells available to conduct experiments, consequent BSC cultivations were likely to be of higher passage number, thus arguably more difficult to cultivate and more sensitive to limited growth factors. For this reason, the 4% FBS concentration was chosen for future experiments instead of a lower even FBS concentration.

## API Concentration Optimization

### *Initial Testing - BSCs Cultivated in API*

It is evident that all APIs in all concentrations tested held a potent toxic effect and killed most of not all cells. Though some cells cultivated in the lowest concentration of the Y API survived; it was clear that the concentration had to be significantly lowered to reveal any serum replacement effects perhaps hidden by the apparent toxicity.

### *BSCs Cultivated in Reduced Concentration*

In this experiment, a reduced concentration of all APIs was tested and a clear improvement in cell growth was achieved, compared to the initial test of the APIs. A clear pattern of increased cell growth of APIs from X1, Y and X2 respectively with lower concentration was observed. However, when compared to the 4% control without any API added, only the lowest concentrations of all three APIs revealed similar live cell count. This indicates that a low enough concentration to remove some or all toxicity was found, though without serum replacement effect. Though, deemed unlikely, the growth boosting property might still be suppressed by a still present toxicity.

### *Reduced Concentration – API From Ultrasonication + Alkaline Extraction*

With APIs developed by samples first treated with ultrasonication followed by alkaline extraction, BSCs were cultivated in the same concentrations as the previous experiment. A similar trend where the toxic effect seems to diminish with lower API concentration was revealed. Also, the two lowest Y API concentrations yielded a greater cell growth than the 4% control. It should be noted that this increase is small and with limited testing, no conclusion about this can be drawn. However, one could speculate that some cell growth-boosting bio molecule could be present due to the increased processing of the raw material (ultrasonication) and that it could contribute to the observed effect.

### *Reducing Osmolality in the API*

In **Figure 13** it can be clearly observed that washing the raw material had a large impact on the osmolality of all APIs. Though not thoroughly investigated, the buffer strength can vary between micro algae strains (Dolganyuk et al., 2020). When discussed with the manufacturer of the algae samples, it is stated that no additional buffer is added in the production process, which indicates that the buffer strength originates from the algae itself.

### *Protein Concentration in the Supernatant After Washing*

**Figure14** indicates a somewhat significant amount of protein is lost due to the washing step even though no additional processing has been done. Interestingly, the supernatant of the thus far lowest protein yielding

algae, Y, seems to contain the most protein. This is likely due to the cell wall of Y algae already been ruptured by freeze drying, making proteins more available.

Comparing this result to previous protein extraction methods, the amount of protein in the supernatant after washing is on a comparable level to all tested extraction methods, except for the attempted alkaline extraction.

*Comparing APIs – High vs Low Osmolality*
From the comparison on cell growth boosting effect of APIs with and without washed raw materials a clear improvement can be observed. Both the APIs (alkaline extraction only and ultrasonication + alkaline extraction) with reduced osmolality improved cell growth compared to APIs with higher osmolality. It has been shown that osmolality upwards of 545 [mOsm/kg] force CHO cells to abort proliferation (Romanova et al., 2022). Though not the same cell type, the highest measured osmolality in any of the APIs were not close to that range, the highest being API from Y just below 300 [mOsm/kg], which interestingly is the API with the greatest performance of the different API candidates.

Comparing the best performing API to the 4% control, a clear improvement can be observed in live cell count. This indicates that high osmolality seems to have been part of or the whole previously observed toxic effect. This result also indicates that with the lower toxicity, a serum replacement effect is observed.

One thing that should be kept in mind when comparing the figures, is that fact that the level of 4% control compared to the 20% control varies between experiments. A fluctuating between experiments is expected, though the same passage (4) is used in both experiments which indicates some uncertainty of the results.

*Replicating The Results*
The result of testing API 2.0 in the same concentration range as the previous experiments revealed a similar result, meaning that no obvious toxic effect was observed and that the API 2.0 from Y had a greater live cell count compared to the 4% control.

Interestingly, the higher concentration of API 2.0 from Y seems to yield the greatest live cell count. This indicates a shift in optimal performance concentration and that for API 2.0, a higher cell growth enhancing effect could be unlocked.

*FBS Concentration Titration with API*
The API has thus far only been tested in combination with 4% FBS, and a new experiment was designed to test the best performing API (API from Y, washed, ultra sonicated and alkaline extraction) with a falling FBS concentration. This experiment revealed that the API enhances the live cell count for all tested FBS concentrations. Most notably, the 0% FBS + API increased the live cell count by approximately 2.5X compared to control with 0% FBS. Though the goal would be to reach live cell count levels compared to the 20% FBS control, this result indicates potential in use of API to reduce the amount of FBS used when cultivating BSCs over a wider range of FBS concentrations.

## Algae Peptide Mixture (APM) Development

### Protein/Peptide Concentration

Though BCA is a method that has high accuracy in a wide range of protein sizes and concentrations (Thermofisher.com, 2024), the result from measuring protein/peptide concentration in the APM presented a large variation between the different replicates. Ideally, other protein determination methods should have been used, to potentially obtain results with lower variation. Such methods include the Lowery protein assay or Bradford assay (Thermofisher.com, 2024). This would enable a good comparison in protein concentration and an answer closer to the truth.

Though this result adds a large amount of uncertainty, the mean of the measurements was used to compute concentration for later cultivation experiments of BSCs. The results of these experiments could then be compared to get a rough estimation of the ideal concentration range for the use of the APM.

## APM Concentration Optimization

*Initial Cultivation Experiment Using APM*

When examining **Figure 19** a few key observations must be pointed out. Comparing the 20% and the 4% FBS control it is clear that the live cell count is quite similar, indicating that a large increase in FBS does not boost cell proliferation a large amount in this experiment. Considering previous cultivation experiments discussed in this report, the relationship between the 4% control and the 20% seem to vary a substantial amount. The likely cause of this is the fact that different passages of BSCs are used (between 0 and 4) in each experiment. This is, as mentioned earlier, due to a limitation of cells available.

When examining the performance of the different APMs a new trend, compared to API, seem to have formed; that the hydrolysate from X1 seem to boost cell proliferation the most. APM from X2 do not indicate to have a serum replacement effect through any of the tested concentrations and APM from Y seem to enhance cell growth of BSCs but not to the same extent as X1. Comparing concentration from X1 and Y a pattern can be observed; the lower concentration, the higher live cell counts which would hint towards that an even lower concentration of APM is optimal for boosting cell proliferation of BSCs.

Considering the lowest concentration of APM from X1, a substantial difference in live cell count between replicates is shown. While the highest measured value exceeds the mean for the two replicates of the 20% FBS control the other replicate is significantly lower, closer to the 4% FBS control. This large variation is suspected to be caused by inconsistent concentrations of APM between the two replicates. Working in such low concentration ranges introduces difficulties in precise execution and a small variation in e.g. mixing have a substantial effect on the result. While it is not possible to draw any conclusions, this inconsistent result, it highly motivates further testing and optimization.

*Reduced Concentration of APM*

Comparing results of the initial test of the APM and the test with reduced concentration of APM in **Figure 19,** a similar pattern regarding the relationship between the 20% and 4% FBS control is shown, meaning that the increase in live cell count from 4% to 20% is relatively small. The same passage (4) of BSCs for both experiments were used which explain the similarities and unlocks a somewhat reasonable comparison between the two experiments. However, the 20% FBS control in the later experiment showed a massive difference between replicates. This variation had thus far not yet been observed in any prior experiment and lays a suspicion towards an experimental error. Due to this large variation between the control replicates, a clear picture of how well the different APMs perform is hard to obtain.

Keeping the uncertainty of the 20% FBS control in mind, a similar result is revealed to the initial APM experiment where APM from X2 seem to perform the worst out of the three micro algae, just barely presenting live cell counts above the 4% control for all concentrations tested. APM from Y seem to boost cell proliferation further and X1 seem to perform the best. A significantly smaller variation between replicates is observed for X1 in this experiment which strengthens the assumption that the APM seem to have a serum replacement effect on BSCs. The second lowest, 0.010 [mg/ml], X1 APM concentration seem to perform the best, indicating that an optimal concentration have been found. The mean of the two replicates in this concentration are on level with the mean of replicates of the 20% FBS control.

Unfortunately, with large uncertainty in the 20% FBS it remains difficult to truly assess the performance of the APM. The optimal concentration range seem to have been found and further testing in this concentration range have the potential of revealing answers closer to the truth.

## Comments on The Performance of the Three Micro Algae and API vs APM

*Differences in Protein Concentration*

Throughout the different protein extraction methods presented in **Figure 6**, a trend can be seen where X1 generally yielded the highest protein concentration followed by X2 and that Y generally yielded the lowest amount of protein. It is expected that X1 and X2 should yield similar amounts of protein, since they origin from the same strain of micro algae and are made as a high protein food ingredient. Y on the other hand,

with its suspected high lipid content and already ruptured cell wall performed better in a media formulation (API) when attempting to preserve the functional proteins. It is suspected that due to the freeze drying, proteins are made more available and thus being more easily utilized by the BSCs. Though, when comparing the before and after appearance of the algae treated with ultrasonication in **Figure 7**, the cell wall of all algae samples seems to be effectively degraded, which contradicts this suspicion. Because of this it is instead suspected that the difference in cell proliferation boosting effect from the different APIs is due biomolecular differences and not the availability of proteins.

*X1 vs X2*

The singular difference between X1 and X2 is the holding period, where X2 had increased holding period to promote the degradation of the chlorophyll making nutrients more easily available (Figueroa-Torres, Pittman and Theodoropoulos, 2021). It is intuitive to assume that this effect would benefit the boost cell proliferation in a media formulation, but somehow it seems to have the opposite effect. While it is not clear why this is, one reason could be that during chlorophyll degradation, the algae cell is put in a state of stress (Mikami et al., 2021). When microalgae experience stress, they activate various adaptive mechanisms. Since X2 generally perform subpar to X1 throughout this study, some kind of stress response from X2 can be seen as likely explanation.

# CONCLUSION

This master's thesis successfully addressed the three primary objectives by developing an effective method for extracting functional proteins from various microalgae raw materials, assessing the proliferation of bovine satellite cells (BSCs) under varying fetal bovine serum (FBS) concentrations, and exploring the use of algae protein isolates (APIs) as a potential replacement for FBS in cultivating BSCs. The findings provide insights into the optimization of protein extraction processes and their application in cell culture, demonstrating the feasibility and potential of using algae-derived proteins in biotechnological applications.

The study revealed that the duration of ultrasonication influences protein yield, with different microalgae samples responding optimally at specific sonication times. While longer sonication durations generally enhanced protein extraction efficiency for samples X1 and X2, a ten-minute sonication was optimal for Y samples. However, balancing yield with protein quality is crucial, as prolonged ultrasonication may lead to protein denaturation. Alkaline extraction, combined with ultrasonication, emerged as the most effective method for API development, aligning with existing literature and proving superior to acidic hydrolysis and enzymatic methods. Furthermore, reducing the osmolality of APIs through raw material washing significantly improved BSC growth, indicating that high osmolality was a contributing factor to previously observed toxic effects.

When testing the APIs as a serum replacement, initial high concentrations exhibited toxicity, but lowering the concentration mitigated this effect, allowing for some cell growth. The API developed through ultrasonication, and alkaline extraction demonstrated potential growth-enhancing effects, especially when paired with reduced FBS concentrations. The replication of results with API 2.0 confirmed its consistent performance, highlighting its potential in reducing FBS dependency in BSC cultivation. Additionally, the development of algae peptide mixtures (APMs) offered promising results, particularly with the X1 hydrolysate, which showed cell proliferation enhancement at optimal concentrations. Despite some experimental variations and uncertainties, the overall findings suggest that with further optimization, algae-derived protein and peptide extracts could become viable alternatives to FBS, supporting sustainable and ethical advancements in cell culture technologies.

# FUTURE PERSPECTIVES

The current study highlights significant findings in the development and optimization of Algae Protein Isolate (API) and Algae Peptide Mixture (APM) for potential use as serum replacements in cell culture media. However, several avenues remain unexplored and merit further investigation to refine these protein isolates and enhance their efficacy.

Firstly, replicating the experiments, particularly those involving APM, is crucial. The initial results indicate promising trends but also exhibit considerable variability, suggesting that reproducibility and consistency need to be established. Conducting additional replicates with tighter control over experimental conditions will help confirm the observed effects and provide more robust conclusions.

Accurate determination of protein concentration in APM is essential for understanding its performance. The BCA assay, while widely used, showed significant variation in this study. Employing alternative protein quantification methods such as the Lowry protein assay or Bradford assay can provide a cross-validation of results and potentially lower the observed variability. These methods might offer a more precise assessment of protein content, leading to better optimization of APM concentrations for cell culture applications.

To further purify the API and reduce potential toxic effects, acid precipitation could be employed. This technique can help isolate proteins by precipitating them at their isoelectric point, potentially removing impurities and toxic components. A purer API could lead to improved cell viability and growth, enhancing its utility as a serum replacement.

Finally, developing an artificial FBS using recombinant growth factors while excluding transferrin is a forward-looking strategy to investigate if the API exhibit similar properties to transferrin.

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# APPENDIX

## List of Abbreviations

| Abbreviation | Definition |
|---|---|
| API | Algae protein isolate |
| APM | Algae peptide mixture |
| BCA | Bicinchoninic acid assay |
| DMEM | Dulbecco's modified eagle's medium |
| FBS | Fetal bovine serum |
| PS | Penicillin-Streptomycin |
| PBS | Phosphate buffered saline |
| BSC | Bovine satellite cell |
| DAPI | (4',6-diamidino-2-phenylindole) |
| NaOH | Sodium Hydroxide |
| HCL | Hydrochloric Acid |

## List of Reagents

**Table 3. List of reagents used in cultivation and staining experiments.** Catalogue number, manufacturer and vendor included.

| Reagent | Catalogue number | Manufacturer | Vendor |
|---|---|---|---|
| PBS | #10010023 | Gibco™ | ThermoFisher Scientific |
| DMEM | #10566016 | Gibco™ | ThermoFisher Scientific |
| Trypsin | #25200072 | Gibco™ | ThermoFisher Scientific |
| FBS | #A5256701 | Gibco™ | ThermoFisher Scientific |
| PS | #15140163 | Gibco™ | ThermoFisher Scientific |
| Paraformaldehyde | #J61899.AP | Thermo Scientific™ | ThermoFisher Scientific |
| Triton 100-X | #HFH10 | Invitrogen™ | ThermoFisher Scientific |
| DAPI | #62247 | Thermo Scientific™ | ThermoFisher Scientific |

# POPULAR SCIENCE SUMMARY

## Cultured Meat: A Potential Sustainable and Ethical Solution

**Cultured meat production has emerged as a promising solution to address the environmental and ethical concerns associated with traditional meat production. As the global population increases, so does the demand for animal products, necessitating the exploration of sustainable meat production methods. The role of cell culture media in cultivating meat is crucial, emphasizing environmental benefits and ethical implications.** Conventional meat production is resource-intensive, contributing significantly to deforestation, greenhouse gas emissions, and water pollution. Cultured meat offers a sustainable alternative that can drastically reduce these environmental impacts. Ethically, cultured meat presents a more humane option by eliminating the need for mass-scale animal slaughter.

To meet the increasing demand for cultured meat, scalable and cost-effective production processes are essential. Cell culture media, a nutrient-rich solution that provides essential support for growing and maintaining cells, play a pivotal role in this, and exploring sustainable sources for its components is vital to mitigate environmental impact. A major challenge is the use of fetal bovine serum (FBS) in cell culture media, which raises ethical concerns and is expensive. FBS is made by collecting blood from unborn calves and processing it to extract the nutrient-rich serum used in cell culture. Investigating alternatives to FBS, with a focus on developing serum-free media, enhances the sustainability and ethicality of cultured meat production.

This project explored algae protein isolates (APIs) as potential serum replacements in cultured meat production and revealed promising avenues. Various methods for extracting proteins from microalgae were tested, emphasizing the balance between optimizing yield and maintaining protein quality. Ultrasonication and alkaline extraction emerged as effective methods. While longer ultrasonication durations generally increased protein yield, they could also degrade protein quality. Balancing yield and quality were crucial for the successful extraction of growth factors needed for cell cultivation.

API development showed promising results, particularly when combining ultrasonication and alkaline extraction. Lowering the concentration of APIs reduced their toxicity and supported better cell growth compared to initial tests. Washing the raw materials to reduce salt content improved cell growth, indicating that high salt content contributed to toxicity. The findings confirmed that algae-derived proteins have the potential to reduce FBS dependency, supporting the development of more sustainable and ethical cell culture media.

Additionally, algae peptide mixtures (APMs) were developed and tested. APMs showed potential in boosting cell growth at optimal concentrations. However, variations in results highlighted the need for further optimization and testing.

Overall, the study suggests that with further refinement, algae-derived protein and peptide extracts could become viable alternatives to FBS, paving the way for more sustainable and ethical advancements in cultured meat production. By reducing reliance on FBS and utilizing sustainable sources for cell culture media, the cultured meat industry can make significant strides toward a more sustainable and humane food production system.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

## Labbodlat kött: En potentiellt hållbar och etisk lösning

**Produktion av labbodlat kött har framträtt som en lovande lösning för att hantera de miljömässiga och etiska problem som är förknippade med traditionell köttproduktion. I takt med att världens befolkning ökar, ökar också efterfrågan på animaliska produkter, vilket nödvändiggör utveckling av hållbara metoder för köttproduktion. Cellodlingsmediets roll i framställning av labbodlat kött är avgörande, med betoning på miljöfördelar och etiska implikationer**. Konventionell köttproduktion är resursintensiv och bidrar avsevärt till avskogning, utsläpp av växthusgaser och vattenföroreningar. Labbodlat kött erbjuder ett hållbart alternativ som kan minska dessa miljöpåverkan drastiskt. Etiskt sett presenterar labbodlat kött ett mer humant alternativ genom att eliminera behovet av masslakt av djur.

För att möta den ökande efterfrågan på labbodlat kött är skalbara och kostnadseffektiva produktionsprocesser väsentliga. Cellodlingsmediet, en näringsrik lösning som ger nödvändigt stöd för att odla och underhålla celler, spelar en avgörande roll i detta, och att utforska hållbara källor för dess komponenter är viktigt för att minska miljöpåverkan. En stor utmaning är användningen av fetalt bovint serum (FBS) i cellodlingsmedier, vilket väcker etiska bekymmer och är dyrt. FBS tillverkas genom att samla blod från ofödda kalvar och bearbeta det för att extrahera det näringsrika serumet som används i cellodling. Att undersöka alternativ till FBS, med fokus på att utveckla serumfria medier, förbättrar hållbarheten och etiken i produktionen av labbodlat kött.

Detta projekt undersökte algproteinisolat (API) som potentiella serumersättningar i produktionen av labbodlat kött och avslöjade lovande resultat. Olika metoder för att extrahera proteiner från mikroalger testades, med betoning på balansen mellan att optimera proteinutbyte och bibehålla proteinkvalitet. Ultraljudsbehandling och basisk extraktion framstod som effektiva metoder. Medan längre ultraljudsbehandlingar generellt ökade proteinutbytet, kunde de också degradera proteinkvaliteten. Att balansera utbyte och kvalitet var avgörande för en lyckad extraktion av tillväxtfaktorer som behövs för cellodling.

Utvecklingen av API visade lovande resultat, särskilt när ultraljudsbehandling kombinerades med basisk extraktion. Att sänka koncentrationen av API minskade dess toxicitet och stödde bättre celltillväxt jämfört med initiala tester. Att tvätta råvarorna för att minska saltinnehållet förbättrade celltillväxten, vilket indikerade att högt saltinnehåll bidrog till toxiciteten. Resultaten bekräftade att alge-baserade proteiner har potential att minska beroendet av FBS, vilket stödjer utvecklingen av mer hållbara och etiska cellodlingsmedier.

Dessutom utvecklades och testades algpeptidblandningar (APM). APM visade potential att öka celltillväxten vid optimala koncentrationer. Variationer i resultaten betonade dock behovet av ytterligare optimering och testning.

Sammanfattningsvis tyder studien på att med ytterligare förfining kan alg-baserade protein- och peptidextrakt bli livskraftiga alternativ till FBS, vilket banar väg för mer hållbara och etiska framsteg inom produktionen av odlat kött. Genom att minska beroendet av FBS och använda hållbara källor för cellodlingsmedier kan industrin för odlat kött göra betydande framsteg mot ett mer hållbart och humant livsmedelsproduktionssystem.