Developing Serum-Free Media Via Bioprocessing For Cultivated Seafood Products

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Abstract

Global food production management has become a challenge with an anticipated population of 10 billion people by 2050 and the ongoing COVID-19 epidemic. Seafood is a vital food source due to its widespread consumption, excellent nutrient profile, and low feed conversion ratio, rendering its sustainable production quintessential. Cellular agriculture or cultured meat can increase seafood production; however, the conventional use of Fetal Bovine Serum (FBS) in culture media restricts its utilization at an industrial level. FBS is effective but has many limitations: unethical animal extraction, high demand and low supply, poorly defined ingredients, variable performance, and high cost that impedes the feasibility and commercial viability of cellular agriculture. Thus, employing serum-free media becomes a quintessential need for cellular agriculture. This project aims to replace or reduce the typical 10% serum usage in Zebrafish embryonic stem cell (ESC) production media with protein hydrolysates derived from low-cost natural sources with high protein content. Enzymatic hydrolysis was performed on nine sources: insects (black army fly and cricket), plants (pea), fungi (mushroom and yeast), algae, and marine invertebrates (oyster, mussel, and lugworm). The resulting hydrolysates were evaluated for serum replacement in zebrafish ESCs. All hydrolysates were used at five different concentrations (10, 1 0.1, 0.01 and 0.001 mg/mL) in serum concentrations of 10%, 5%, and 0% with four biological replicates. The best hydrolysate sources and concentrations were selected for further testing at 2.5% and 1% serum concentrations. All hydrolysates, except for cricket, could restore or significantly increase cell growth with 50% less serum at a concentration of 0.1-0.001mg/mL. Protein hydrolysate concentration of 10 and 1mg/mL was toxic for cells. Additionally, the eight hydrolysates could reduce serum concentrations up to 75–90%. However, no protein hydrolysate could completely replace serum, as cells using only protein hydrolysates exhibited morphological aberrations and decreased growth. Replacing serum with protein hydrolysates lowers cellular agriculture's overall cost, thus enabling the commercialization of cultured meat and the development of a sustainable food system. In the future, blending various protein hydrolysate sources with or without the addition of conventional growth factors could be done to create the ideal serum-free media.

Acknowledgment: This research is funded by USDA NIFA 2021-69012-35978.

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General Audience Abstract

With a predicted population of 10 billion by 2050 and the ongoing COVID-19 outbreak, the management of global food production has become a dilemma. However, due to its widespread consumption and good nutrient profile, seafood is an essential food supply, making its sustainable production indispensable. Both capture fisheries and aquaculture are conventional ways to produce seafood. However, they are under tremendous pressure and require alternatives that can alleviate this demand and contribute to the sustainable growth of seafood. In-vitro cultured meat, also known as lab-grown meat, is a novel technique with the potential to supplement the traditional fish sector. It appears a great option, as it completely imitates meat and offers numerous environmental, financial, and health advantages. A culture medium supports the existence, survival, growth, and multiplication of meat-producing cells and tissues in cell-based meat. However, the culture medium uses a Fetal Bovine Serum (FBS) supplement, which dramatically increases the cost and raises many ethical concerns as it is derived from a cow's fetus. In this thesis, we substitute FBS with protein hydrolysates derived from nine distinct sources. Hydrolysing proteins with enzymes produce protein hydrolysates, rich in nutrients and peptides that promote cell development. Enzymes were used to hydrolyse nine unique and protein-rich sources, including insects (black army fly and cricket), plants (pea), fungi (mushroom and yeast), algae, and marine creatures (oyster, mussel, and lugworm). The resultant hydrolysates were investigated for replacement of serum in cell culture. Eight protein hydrolysates successfully replaced 90% of serum without impairing cell growth and structure but could not replace serum entirely. In the future, serum-free media could be created by combining these various protein hydrolysates with or without adding other growth-promoting components.

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Chapter 1 - Introduction

With covering over 70 % of the earth surface which earned it the name "blue planet", the sea encompasses a large amount of biodiversity and surface which is not well recognized till-date. As the time has progressed, human population has grown to a great extent which exhibited itself by imparting many deleterious effects on sea, namely: deterioration of natural environment of the oceans, profound atmospheric related changes (climatic changes), immediate and delayed effects of overfishing and overexploitation, eutrophication, pollution resulting in marine debris, deep sea extraction related problems, watershed variations, restricted understanding and inclination to conserve marine diversity and complications related to legal issues in marine problems (Solan et al., 2012). As population soaring year by year with a prediction of reaching 9 billion by 2050, the need for safeguarding food security while conserving the earth and sea became a pressing issue (Guillou & Matheron, 2014). Overall increase in food demand is the function of various factors like: elevated economic and population growth, life-style alterations, evolution of food production, globalization, marketing, urbanization, entry of private sector domain etc. (Von Braun, 2007). Although the consumption of animal-derived foods varies based on population distribution and geographic location, its consumption will undoubtedly increase in the coming years, giving rise to numerous problems. Consumption of seafood has increased rapidly over the past few years and is projected to grow even more in the future, primarily due to its improved food safety (Hosomi et al., 2012; Kearney, 2010). The fact that seafood comprises 20 percent of animal-based protein supplied, is the most widely traded global commodity, and serves as the social and economic fulcrum of numerous nations amplifies its significance.

However, as the demand for seafood has increased, countless negative environmental consequences have emerged, impacting the overall sustainability of oceans and food security (Gephart, 2019). The majority of seafood is obtained from the ocean or aquaculture. However, capture fisheries are not expected to expand further because they have already been fished to their maximum sustainable level, giving aquaculture an advantage. In addition, aquaculture, as opposed to fisheries capture, has greater control over environmental conditions and utilizes emerging technologies, such as water quality improvement, sensors, etc., that have increased productivity (Cole et al. 2009). However, the aquaculture industry faces numerous challenges, such as genetically modified fish that can spread disease resistance, chemical residue from pesticide use, expensive feed ingredients, reliance on wild fish for Fish Meal or Fish Oil (FMFO), legal issues and permits, and negative consumer perception (Cole et al. 2009). Traceability and mislabeling issues are prevalent problems in both fisheries and aquaculture industries (Stiles et al. 2013). In addition, during seafood processing, between 50 and 80 percent of the seafood is converted into by-products and wastes that are highly nutritious and contain many nutrients that could have been consumed (Pal and Suresh 2016). Several deficiencies in the fisheries and aquaculture industries necessitate the development of more efficient and less wasteful alternatives to seafood production to alleviate pressure on the ocean and aquaculture.

Numerous innovative technologies for seafood alternatives, such as insect-based proteins, cultured meat, and plant-based protein alternatives, have emerged to address these imminent issues. However, the problem with insect and plant-based alternatives is consumers' devotion to meat, i.e., they are unwilling to consume alternatives to meat and

demand authentic meat (Graça et al., 2015). *In-vitro* cultured meat appears to be an excellent choice as it mimics meat completely and would have many environmental, financial and health benefits over conventional methods. Another factor supporting it is a study conducted via Reflexive integrative Comparative Heuristics (RICH) framework was applied to all of the above meat alternatives and advocated that cultured meat would require less socio-institutional and technological change (van der Weele et al., 2019). This approach involves culturing of cells or tissues in a synthetic environment which promotes cell proliferation and growth (Bhatia et al. 2018). One of the important factors which restricts the cell culture technology formulation of culture media. A culture medium sustains the cell or tissues existence, survival, growth, proliferation and precisely affects the analysis outcomes and yield (Yao & Asayama, 2017). Although Fetal Bovine Serum (FBS) is the most used media supplement, many factors discourage its use, including cost, poor formulation, high demand-low supply, batch-to-batch variation, and the inability to grow specific cells. All of these constraints compel us to search for a serum-free medium that will reduce costs and increase cell yield and phenotypic similarity. In addition, establishing the effect of media components on cells and deciding its concentration would counteract the adverse effects of serum for an effective media formulation.

However, the most detrimental factor to serum-free media formulation is the high cost of serum substitutes. Growth factors such as insulin, Insulin-Like Growth Factor (IGF), Fibroblast-Like Growth Factor (FGF), Epidermal-Like Growth Factor (EGF), and Leukemia Inhibiting Factor (LIF) account for over 80 percent of the total cost (Specht, 2020). Since cell culture media accounts for more than 99 percent of the total cost of the

cultivated meat process (Stout et al. 2021), developing a less expensive media would significantly reduce the cost of cultivated meat. According to a GFI study, serum-free medium contain growth factors and hormones, which account for more than 95% of the cost. Additionally, these factors and hormones are critical for cell proliferation and viability (Specht 2020). Thus, developing a serum-free medium with less expensive sources of growth factors and hormones would be an efficient way to industrialize cultivated meat.

The purpose of this thesis is to develop a serum-free media by applying bioprocessing and valorization of plants, algae, yeast, marine invertebrates and insects. In this study, we use ESCs from the model organism Zebrafish to evaluate the impact of multiple protein hydrolysates as serum substitute. Protein hydrolysates have a history of being used as substitutes or supplement to serum (J. Y. Kim et al., 2011; Logarušić et al., 2021; Taylor et al., 1972, 1974; van der Valk et al., 2010). This study aims to evaluate the effect of different concentrations of each protein hydrolysate on cell performance. Overall, we reduced 90% of serum from the media; however, the complete replacement of the media was unsuccessful.

Chapter 2 – Literature review

2.1 Seafood as an important global resource

Fish, shellfish, and mollusks are among the most important food sources and the most traded commodity globally. Recently, the global production of fish, crustaceans, and mollusks has steadily risen, primarily due to the robust expansion of the aquaculture industry.

Fish is the most important type of seafood, as its international trade increased from $8 billion in 1980 to $102.5 billion in 2010, a significant increase. According to the Food and Agriculture Organization of the United Nations, sixteen percent of all animal protein ingested by humans is sourced from fish, making it one of the most important sources of protein. In addition, as shown in Figure 1, the trend of rising seafood consumption has increased faster than the population.

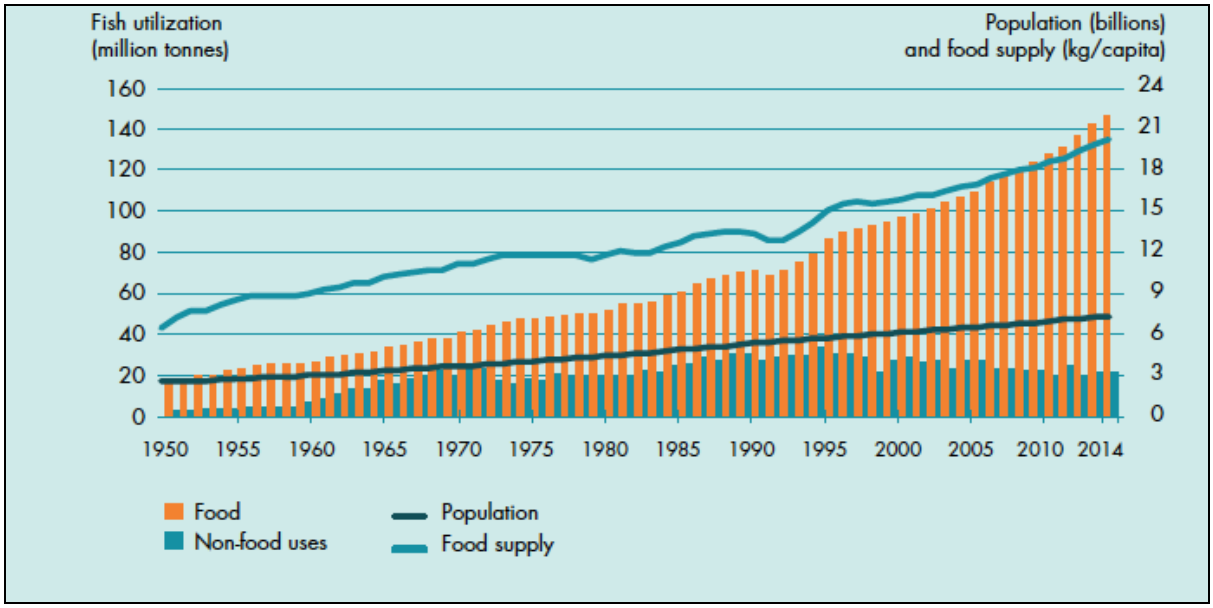

Figure 1: Overall consumption of fish and population increase from 1950-2014

FAO estimates that the annual value of the global fish trade is USD 51 billion. Over 36 million people are employed in fishing and aquaculture, and up to 200 million people

derive direct and indirect income from fish. Fish has substantial social and economic significance as well. Food fish consumption is rising, having increased from 40 million tonnes in 1970 to 86 a million tonnes in 1998 (FAO, 2000) and is projected to reach 110 million tonnes by 2010. (FAO, 1999). Seafood is now one of the most heavily traded commodities, accounting for approximately 10 percent of all food trade (Asche et al., 2015). Seafood is an important source of protein, amino acids, vital fatty acids, minerals, and vitamins for many people around the world. Recently, the nutritional benefits of aquatic organisms have been predominantly connected to their polyunsaturated fatty acid (PUFA) composition, particularly the omega-3s fatty acids. Comparable to other animal-based foods, 100 grams of some seafoods contain an average of 25 grams of protein. These well-known nutritional benefits, combined with its exceptional gastronomic value and species diversity, make this food category appealing to consumers worldwide. According to the FAO, approximately one billion people worldwide rely on fish as their primary source of animal protein. (FAO, 2018). The global increase in seafood supply has been unevenly distributed. In recent decades, global seafood production growth has outpaced global population growth, despite stagnation in global capture fishery catch due to overexploitation, and the rapid expansion of aquaculture. Both fisheries and aquaculture are the main sources of seafood products. Aquaculture and fisheries are rapidly growing industries, and their production has almost doubled in the last ten years, which 171 million tons of seafood was produced in 2016 with additional increases in production anticipated. Global data compiled by the United Nations' Food and Agriculture Organization (FAO) indicate that approximately 47 percent of fish stocks are already exploited to their maximum sustainable limits. In comparison, 18 percent are

reported as overexploited, and 10 percent are depleted (FAO, 2002), establishing a clear need to reduce fishing pressure on many of the world's fisheries. With many fish stocks already exploited to their limits, additional stressors could have important repercussions. Some of the important additional factors that can hinder the seafood from marine sources are factors like, algal blooms, presence of microplastics, acidification of ocean and ghost fishing gear.

Algal blooms are an increase in the production of algae that can reduce the concentration of oxygen in the water and produce a variety of toxins that can cause diseases in fish that can be transmitted to humans through the consumption of seafood contaminated with these toxins. Increasing instances of excessive nutrient enrichment or eutrophication caused by fertilizer and other nutrient runoff into the ocean are the primary cause of algal blooms. Consequently, algal blooms threaten aquatic biodiversity, ecosystem resilience, recreational and fishing activities, water quality deterioration, and ultimately a water shortage. In addition, the overabundance of nutrients has resulted in harmful algal bloom (HAB) outbreaks in upstream freshwaters, estuaries, coastal systems, and seas, posing a threat to all water bodies and an important human food source (Anderson et al., 2012).

Plastic pollution is pervasive in the ocean environment. The most alarming prediction is that by 2050, the weight of plastics in the ocean will exceed that of fish (Yang et al., 2021). Microplastics (MPs) are plastic fragments or particles with a diameter of less than 5 mm formed by the fragmentation of larger plastics. Due to their small size, marine organisms can accidentally ingest MPs, including fish, mussels, zooplankton, seabirds, and worms. Microplastics negatively affects the various marine organism at different trophic levels by affecting their development at embryo stage and alters behaviour and

can be fatal as well (Yang et al., 2021). Additionally, a recent study showed that microplastics can enter the bloodstream of humans which opens a new pandora's box of potential problems. The microplastics have been found in human placenta (Ragusa et al., 2021), human feces (Schwabl et al., 2019) and most recently in human blood (Leslie et al., 2022). The effect of microplastics on human health is poorly understood, but numerous scientific papers have proposed a variety of potential impacts. Potentially harmful effects of plastics on humans include developmental abnormalities, disruption of the endocrine system, and associations with respiratory and cardiovascular diseases. Thus, producing seafood without the presence of microplastics becomes a quintessential step (Rist et al., 2018).

Due to the absorption of carbon dioxide (CO_2) from the atmosphere, ocean acidification is the ongoing decrease in the pH value of Earth's oceans. Ocean acidification is often referred to as the "osteoporosis of the sea" for a valid reason. Ocean acidification can produce conditions that erode the minerals used by oysters, clams, lobsters, shrimp, coral reefs, and other marine organisms to construct their shells and skeletons, thereby reducing their production. Other fishes that feed on these shellfish also suffer the destruction of their food sources, resulting in an overall ecosystem disruption. Ocean acidification also promotes harmful algal blooms, further destabilizing the sea's ecosystem balance (Gao et al., 2019).

Fishing bycatch is a significant threat to marine mammals, resulting in the extinction of approximately 65,000 species worldwide. Bycatch occurs when commercial fishers capture unwanted or unintended fish and aquatic organisms, as well as seabirds, in their nets while fishing for a specific species, size, or sex of fish. The World Wildlife Fund

(WWF) estimates that 40 percent of all fish caught worldwide are incidental catches, which disrupts the ecosystem (Keledjian et al., 2014). Bycatch has a devastating effect on marine populations because it reduces the number of marine animals, thereby diminishing the overall sustainable production of seafood. This problem affects not only marine animals but also the marine ecosystem. Other issues, such as climate change, ocean acidification, etc., in addition to bycatch problem poses an enormous threat to global fisheries.

After the 1970's aquaculture has boomed and overtaken capture fisheries which not only supported the global consumption of seafood but also escalated it (Asche et al., 2015). The Figure 2 shows the overall growth of aquaculture which was slow earlier escalated quickly and overtook capture fisheries.

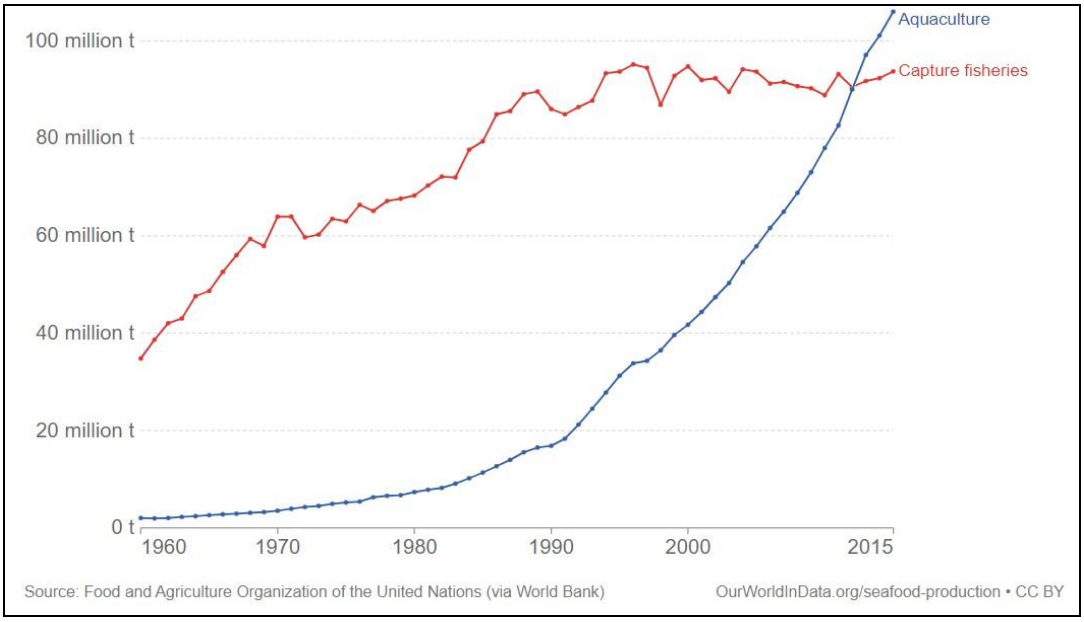

Figure 2: Increasing trend of aquaculture production overtaking capture fisheries from 1980 - 2015

Despite its unmatched success, aquaculture conferred multiple environmental and well-being issues. Environmental issues constitute -

1) Aquaculture involves genetically manipulating fish which if released in the environment will transmit diseases resistance and unwanted genes.

2) Risk of physical injuries, atypical microbial exposure and toxic chemicals to people working in aquaculture industry.

3) Environmental dangers pertaining to eutrophication, chemical pollution especially high usage of antibiotics that imparted antibiotic resistance to many water-based microbes and overall dependence on capture fishery for fish stock and feeding aquaculture fishes.

4) Lowering of nutritional value of aquaculture fish as compared to wild fish

5) Profound complexity of legal issues related to aquaculture. (Cole et al., 2009)

Some common problems that affect both capture and aquaculture industry are mislabelling of fish and traceability issues. Mislabelling of fish or fish fraud has become a common problem which usually occurs for fish that apparently look similar. In most cases the swapped fish is either more easily available or has lower price, for example there are 58 species of fish that look similar and can be sold as grouper some of which are not grouper at all. Due to lack of information on the fished cannot be tracked back to its original selling place which gives rise to traceability issues (Stiles et al., 2013).

Due to all these issues, there is a dire need of seafood alternatives that can be produced sustainably which can run parallel to aquaculture and marine fisheries as a support system as well as reducing pressure on them.

2.2 Cultured seafood – an effective alternative to traditional fisheries practices

The cell-based seafood industry has an exceptional capacity to adapt to the current difficulties impacting the capture and aquaculture industries and alleviate a portion of the

load on traditional fisheries. Utilizing animal-free and morally acceptable in-vitro cell and tissue culture techniques is fundamental to meat production (Bhatia et al. 2018). Cultivated meat has the potential to provide excellent control over crucial aspects of meat, resulting in favourable characteristics such as flavour, texture, and colour. (Ben-Arye and Levenberg 2019). Figure 3 demonstrates an overall process of cell-based seafood from extracting cells to production of meat.

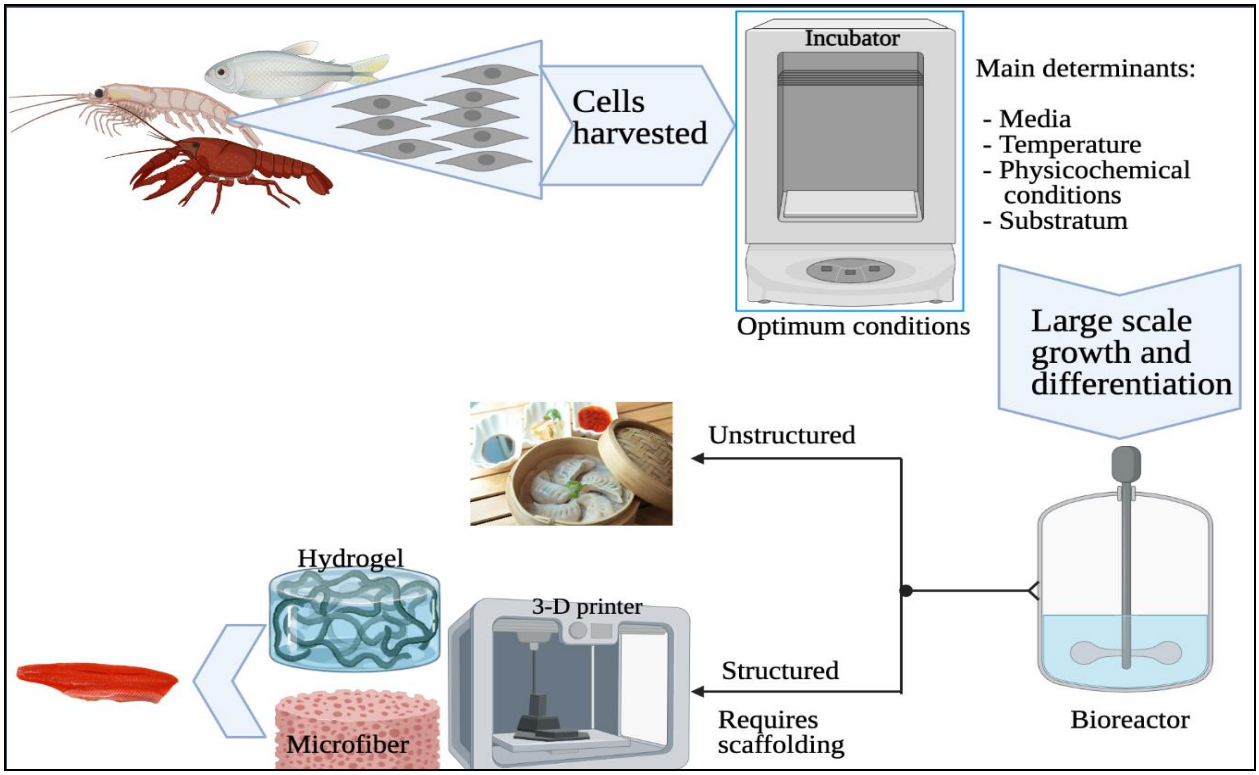

Figure 3: Overall production of cell-based meat from extracting the cell to bioreactor

In contrast to the production of meat from animals, the cell culture process is extremely rapid and effective, as it eliminates energy or nutrients requirements for unnecessary biological functions such as locomotion, reproduction, etc. (Bhat et al. 2017). The carbon footprint of cultured meat is very small when compared to aquaculture, as it does not require the same amount of space and resources (Whitford et al. 2018). Overall,

cultivating cell-based seafood can be more effective than traditional methods. However, its efficacy is highly dependent on the development of its fundamental subject of animal cell culture, which has been well-known to humans for decades. Despite the development of animal cell culture research in the 1800s, it was primarily concerned with mammalian cell culture's pharmacological applications, and little attention was paid to developing cultured meat items (Yao and Asayama 2017). Highly expensive cultivated beef patties were introduced in 2013 and have proved to be the most popular implementation of cultivated meat to date. (Post and Hocquette 2017). Consequently, an interest in processing cell-based seafood was generated because of its valuable nutritional profile and high human consumption. Aquatic animal cells have still not been extensively studied in vitro as compared to their terrestrial counterparts (Krueger et al. 2019). Most of the *in vitro* fish cell culture has been focused on toxicological studies (Zurita et al. 2019) and biomedical research, with great emphasis on Zebrafish (Geisler et al. 2017). A muscle-based protein system for goldfish is the one of reported successful instance of cell-based fish developed by NASA funding (Benjaminson et al. 2002). Recently, embryonic fish stem cells were developed by fish wastes (Tsuruwaka & Shimada, 2022) and several companies have patented fish cell lines. However, there is still a lack of well-defined research in fish and other aquatic animal cell cultures, making it crucial to explore and develop this field with modern technology to fill these knowledge gaps for producing cell-based seafood.

Although aquatic cell culture is less elucidated than mammalian cell culture, there are many advantages of fish-based cell culture, which will expedite the path of cultured meat for fish. Fish cell culture can grow and proliferate aptly in low oxygen or hypoxic

conditions due to specific and conserved transcription factors (Vaquer-Sunyer and Duarte 2008). Since oxygen is essential for the growth of cells in a bioreactor system, the ability to grow in hypoxic conditions gives fish cells an additional advantage (Potter et al. 2020). Fish cell cultures are more resilient to alterations in the systems' pH as they have superior intracellular buffering capacity than mammalian cell cultures (Castellini and Somero 1981; Castellini et al. 1981). The temperature requirement for fish cell culture is also very flexible since it mostly mimics the fish's environment (Potter et al. 2020).

Although producing fish - based food without animals requires elevated progress but it has the potential to solve many problems posed by modern day world. *In-vitro* cell and tissue culture techniques make chief foundation for production of seafood without animals. Fabricating and optimizing cell culture for an animal requires basic information about their genetics, biochemistry, cellular and molecular biology, and growth requirements. Starting with Zebrafish (*Danio rerio*) for cultivated meat is ideal since it is a model organism with elucidated growth details. (Schartl 2014). Quintessential information of zebrafish such as embryo formation, pluripotency markers, etc. is well elucidated, that aids development of cultivated meat (Potter et al. 2020). Additionally, Zebrafish is a naturally lean fish with approx. 1-2 % fat, which makes it more straightforward since culturing two types of cells becomes rather challenging (Potter et al. 2020).

The main techniques and subject that is used for creating cell-based meat is animal cell culture technology. It is a diverse subject that allows us to grow animal cells invitro with great efficiency and control. Thus, we will discuss a little bit about the **animal cell culture history and basics** involved that can help augment the cell-based meat industry.

2.3 Basics of animal cell culture

The onset of the twentieth century saw the uprising and development of a relatively new technology which is animal cell culture. It emerged from excrescence of a nerve from frog to isolation of specific cells to manipulating them for precise applications. Although the term "tissue or cell culture" restricts the augmentation it has seen in the recent years but still remains the widely generic term used till day. A favourable boost to this technique was given by the culturing of warm blooded animals that have homology to humans thus opening many doors to *in-vitro* modelling and testing (Freshney, 2015; Nema & Khare, 2012). Many riveting biological processes and their manipulation for fabrication of various products resulted in the development of this field further. Some of these processes are: intracellular activity, their transfer and fate (biomolecular synthesis and their resultant fate), impacts of extracellular responses on cells (lack of nutrients, stress etc), cell-cell interplay, effect of genetics in cells and most importantly, cell by-products of interest (Freshney, 2015).

Definition, initiation and types of animal cell cultures:

The non-specific and collective definition of animal cell culture would be "removal and transfer of cells from its genesis & propagating it in a fabricated environment which would aid its existence" (Butler, 2004; Freshney, 2015). There are many ways to commence animal cell cultures, they are listed as follows:

1) Organ culture:

These cultures retain their composite "architectures" as, a whole organ or a part of adult organ is utilized. Due to their nature, they exhibit certain characteristics such as, their differentiated phenotype and organization, functional operation, limited growth activity

and therefore restricted life span. They are excellent for studying various effects of internal and external factors on the organ and elucidation of their physiological role *ex-vivo* (Freshney, 2015; Verma, 2014). Various successful hair follicle (HFs) organ cultures were done that delineated the hormonal regulation of epithelial HF progenitor cells, function of HF stem cells, factors affecting hair shaft elongation and providing an opportunity for studying and manipulating them according to need (Langan et al., 2015). Many other organ culture studies will augment the existing information about them allow their manipulation for future applications.

2) Primary explant culture:

Disintegrated animal tissue is used for the initiation of the culture on a nutrient medium which is referred to as the explant. The desired excised animal tissue is supported on a biological matrix like extracellular matrix components, and cells start to grow and resettle on the matrix provided from the perimeter of the explant. Fundamentally we are allowing outgrowth of cell on a provided substratum. This permits us to investigate various aspects of cells like their morphology, growth patterns and effect of genetics or other external variables (like drugs) on their growth and phenotype etc (Freshney, 2015; Verma, 2014). In a study, non-small cell lung cancer (NSCLC) tumours were garnered and grown as explant cultures. Effects of cisplastin TNF-related apoptosis-inducing Ligand (TRAIL) and analysis of Tumour Protein 53 (TP53) expression were investigated. Many correlations were seen, like negative correlation of cisplastin with advanced tumour stage, mutated TP53 induces elevated cell apoptosis in response to cisplastin and no relationship between TRAIL and cisplastin effects on tumours. This demonstrates

efficacious nature of primary explants as preclinical models for testing of new drugs (Karekla et al., 2017).

3) Dissociated cell culture:

As the name insinuates, the instigation of culture is at the level of cell rather than an organ or a small fragment. The cells are produced by explants or cell suspensions, this requires mechanical or enzymatic degradation of the tissue or fragment (Verma, 2014). As established above, the cell culture will require explant or tissue to initiate it in the first place but it has many advantages over the other culture initiations: requires less effort for maintenance, variety of characterization techniques can be used for qualitative purposes, can be comfortably propagated and differentiated with low variability (Freshney, 2015). These qualities enables the researchers to exploit and utilize cell cultures in many fields like: studying experimental models for prediction (Karekla et al., 2017), analyzing physiological requirements of cells (Jordaens et al., 2015), investigation of cell development and differentiation (Mitchell et al., 2010), effects of genetic manipulation (Lahti et al., 2012), biotechnological aspects (like production of recombinant proteins, vaccines etc) (Takeuchi et al., 2014). The key for our study would be the "biotechnological aspect" as the generation of cells effectively would be the key to cellular agriculture or cultured meat.

Types of cell culture:

1) Primary cell culture:

These cultures are comprised of cells which directly obtained from its source i.e. tissue, explant etc. via mechanical, chemical or enzymatic disintegration. Depending on their origin, they can be differentiated or undifferentiated. They have relatively stagnant

growth rate and divergent nature but give highly favourable description of its source from which they are obtained. Their morphology and phenotype will depend on the source from which it is derived. Its advantage include generating a population similar to the source genotypically and phenotypically but are hard to obtain and have small lifespan(Honegger, 1999; Verma, 2014).

2) Secondary cell culture:

The subculturing (passage) of primary cell cultures will produce secondary cell cultures if cultivated for a prolonged period with a fresh supply of medium at periodic intervals. Their growth is established, straightforward and at one's disposal. They have multiple applications in many research areas as they are able to produce substantial populations almost indistinguishable in nature which are amenable to transformation. One core flaw is genetic instability of cells as they tend to differentiate over a period of time and generate a anomalous population. These cell cultures can have limited number of generations and proliferations which would make them finite cell lines. Also, they can grow limitlessly which is possible due to their transformation into infinite dividing state as they underwent a steady genetic change (Honegger, 1999; Verma, 2014).

Based on the cell types, many different cultures can be constructed which would be discussed later.

Advantages of cell cultures

1) Effectively delineates the physiological responses with variation in physicochemical environment (pH, temperature, oxygen presence etc). Cell cultures have a superior control over these conditions which could be varied minutely and precisely.

2) Quantitative and qualitative uniformity and reproducibility can be achieved.

Disadvantages of cell cultures

1) Requirement of highly proficient employees, thus immense endeavours and expenses are required.

2) High chance of contamination, thus maintenance of highly sterilized conditions is a must due to sluggish growth of animal cells as compared to possible contaminants (bacteria, virus etc).

3) Dependence of animal cells on composite environment as they are not self-sufficient when solitary.

4) Cell or tissue composition is mostly heterogeneous and inconsistent from their mother cultures. Selective pressure has to be maintained in order to attain homogeneity. (Anaya et al., 2013)

Principal determinants in animal cell culture

1) Culture medium:

Animal cells require an effectively nourished and well-supplemented media for their sustenance and growth as they depend on their surroundings. Animal cell culture media is a complex mixture of biomolecules and other chemical components. Different cells have additional requirements, so the medium needs to be adjusted accordingly. Since life, growth, and yield depend on media, it becomes the paramount factor in animal cell culture.

2) Optimum physicochemical conditions:

These constitute of some highly important factors like pH, temperature, oxygen and osmolarity, they will exert a tremendous effect on growth and survival of animal cells. A few other factors to take into consideration are viscosity, surface tension and foaming

3) Substratum for growth:

Most of the animal cells are incapable of living without a support, so they require a solid foundation, these cells are called "anchorage dependant cells". These cells grow as a monolayer on the substratum (solid material). The few left can grow without support as a suspension, therefore called "anchorage independent cells". This dependant nature of majority of cells will decide whether attachment layer is required or not and make this a chief factor in success of the animal cell culture. (Bhatia et al., 2019; Freshney, 2015; Lodish et al., 2000)

2.4 An important ingredient of the cell culture: The medium

Without any doubt, culture medium is paramount to the cell culture process and its veracious selection decides the success of the approach (Bhatia et al. 2018). As already established the culture medium sustains the cell or tissues existence, survival, proliferation and precisely affects the analysis outcomes, yield and narrowing down quintessential media components that causes prime effect on various aspects of cell culture. Predominantly, media can be differentiated in two forms: natural media and artificial media. Following are elaborated versions of two forms of culture medium (Yao & Asayama, 2017)

Table 1: Various types of media, their origin, and examples

S.No.	Designation of the media and their definition	Different types associated and their description	Examples
1.	Natural Media This type of media is composed only of constituents that occur naturally and is sufficient to allow growth and proliferation of cells	• Clots • Tissue extracts	• Plasma, serum • could be extracts from various sources like embryos, spleen, liver etc

		• Fluids of biological origin	• Serum, lymph etc
2.	Artificial Media This type of media does not occur naturally and is fabricated according to the need of cells. This would mostly contain basal medium and required additives	• Serum comprising medium - With addition to basal media, serum is added as a supplement • Serum free medium - This medium has no component of serum as supplement, so uses other alternatives with basal medium • Xeno-free medium- Utilizes constituents of human origin but any component derived from animal is not used • Protein free media - will have no protein constituents, but utilizes peptides, protein hydrolysates etc • Chemically defined medium - All the components	• Serum from any origin can be used as an additive • Additives like extracts, protein fractions etc can be used • Human derived serum or supplements etc can be used • Any form of protein hydrolysate like peptides, protein factions etc can be utilized • Any constituents which can be defined and quantified can be used, for example -

		added would have known components, therefore components like hydrolysates, serum, various extracts cannot be included	purified recombinant proteins

(Yao & Asayama, 2017)

Basal medium is fundamentally a defined medium that supports the growth of cells that mostly constitutes of salts, amino acids, vitamins, and minerals. These mediums have a wide array of constituents that supports cell growth based on the type of cells. Some examples are Eagle's basal medium, Eagles minimum essential medium, Dulbecco's modification of Eagle's medium etc. In most cases, addition of serum is necessary for appropriate growth conditions to produce required cells (Butler, 2015). Pioneering works of many scientists led to the elucidation of major components of medium and how it affects cell culture. Since the function of medium is to mimic the body fluid composition which *in-vivo* is regulated by complex organs, tissues and bimolecular entities, each component is highly influential and necessary for optimum growth of cell *in-vitro*. Although depending on cell-type nutritional requirements may vary, following are the chief constituents of media which affect the cell culture process:

1. Basal salt solution:

Mostly constitutes inorganic salts which regulates pH and osmolarity which would be mimic the body fluid composition. Usually bicarbonate buffer system that follows Henderson-Hasselbalch equation would be ideal (Bhatia et al., 2018). In addition to various minerals and vitamins, commercially available basal salt solutions contain a variety of amino acids that support nutrient quality. Usually, between 260 and 320

mOsm/kg, the ideal osmotic pressure that promotes cell proliferation is created by basal salt solution.

2. Amino acids:

All essential and non-essential amino acids are indispensable components of any medium. It is necessary for cell growth, serves as a transporter for amino acids, synthesizes proteins and nucleotides, and provides an alternative energy source when the primary source is depleted. In addition, amino acids especially glutamine are connected to the TCA cycle, the production of nitrogen-containing compounds, and most importantly, the production of ketone bodies, which are utilized by numerous biochemical pathways such as fat synthesis, glycolysis, the urea cycle, etc (Nelson et al., 2008).

3. Vitamins:

These organic compounds are essential because enzymes need them as cofactors in order for them to function correctly; without them, enzymes are inactive. Since most in vitro animal cells cannot synthesize most vitamins, they must be entirely acquired by media supplementation. (Bhatia et al., 2018; van der Valk et al., 2010)

6. Trace elements/Micronutrients:

Despite their requirements in miniscule quantities, they play important roles which is directly proportional to viability of cells. They form active centre of enzymes which aids electron transfer, assist protein synthesis, signal transduction and DNA metabolism. This includes various ions, vitamins and minerals (Bhatia et al. 2018; Yao and Asayama 2017).

7. Glucose:

Most important direct energy source for the cell which is responsible for sustenance of the cell. Glucose is converted into pyruvate or lactate depending on availability of oxygen, then it will enter citric acid cycle for complete oxidation to CO_2 and thus in turn provides energy in form of ATP (Bhatia et al. 2018).

8. Hormones:

They are bimolecular regulatory entities that influence several characteristics of cells in response to stimuli in the culture media. Growth hormones directly affect the growth and multiplication of cells; for instance, hydrocortisone aids in the effective cloning of numerous animal cells, estrogen promotes growth and proliferation of certain cell types, lowers inflammation, stimulates lipid synthesis, etc.

9. Growth factors:

They affect many aspects of cell cycle like instigating proliferation, differentiation, maturation etc and also decide import or export of a particular molecule. All growth factors are usually very specific to the type of cells they act on, for example: Epidermal Growth factor (EGF) will induce proliferation of many epithelial cells and Fibroblast growth factor will induce growth of fibroblasts etc (Yao and Asayama 2017).

11. Other proteins:

They are vital biomolecules that support cell adhesion, acts as alternative source of energy when it is scarce and act as transporter for vitamins, lipids, amino acids and trace elements (Yao and Asayama 2017).

12. Lipids and similar components:

They serve multiple roles: main component of biological membranes which imparts them fluidity, universal energy source and also helps to deposit energy in a condensed form, also essentially performs signal transduction. In addition, lipid-based components are insoluble in water-based environments, necessitating various chemical dissolving agents to make them accessible to cells.

13. Polyamines:

It is a dynamic regulator of normal cell activities that aids protein and nucleotide synthesis and, by extension, the health and metabolism of the cell as a whole. Their absence can have a disastrous effect on cell growth and proliferation and, consequently, cell culture yield. Spermidine and putrescine are the two most essential polyamines for cell proliferation and growth.

14. Reductants:

These agents help in the maintenance of the cellular REDOX environment, which helps extensively if cell is under oxidative stress. They help to import and export cysteine to the required destinations. Some examples are: 2-mercaptoethanol and reduced glutathione

15. Additives:

They are for multipurpose usage and therefore are different bimolecular or chemical identities. One class of these are shear force protection agents like, carboxymethyl cellulose (CMC), tween 80 etc which being lipophilic in nature also aids solubilisation of lipid components of media. Another class constitutes of protease inhibitors which halts unnecessary protease activity thus acting as a stabilizing and detoxifying agent. There are many which would be designed according to the cell or tissue in question.

16. Adhesion factors:

These most important part of cell culture as they advocate adhesion of anchorage-dependent cells which improves the yield of cell culture. Most of the cells are anchorage dependant and thus require a substratum support to grow and proliferate (Bhatia et al. 2018; van der Valk et al. 2010; Yao and Asayama 2017).

2.5 Fetal Bovine Serum (FBS) – the most important component of media

Nutrient composition in cell culture media dramatically influences the cell's growth-supporting microenvironment. Standard basal media formulations include buffered solutions of salts, sugars, vitamins, and amino acids (e.g., Dulbecco's Modified Eagle's Medium, DMEM). Proteins, peptides, growth factors and hormones are not found in basal media but are vital for cell proliferation because they serve as a source of building blocks, energy, and signalling molecules and they also play an essential role in media preparation (Sinacore et al. 2000; Freshney 2016). The most used media supplement for cell culture that contains these important proteins is Fetal Bovine Serum (FBS), also called serum. Serum is a clear biological fluid, a complex mixture of macromolecules derived from fetal calves' blood and is the most common source of proteins and peptides in media (Logarušić et al. 2021). The serum is an excellent media supplement that consists of various constituents with optimum concentrations that promote cell proliferation and development. The serum contains polypeptides and proteins, growth factors, amino acids, lipids, carbohydrates, polyamine, urea, inorganic molecules, hormones, and vitamins; essentially, every essential cellular component outlined in the preceding section (Freshney 2015). Fetal bovine serum has a substantial production globally with approximately 500,000 liters harvested per year (Brunner et al. 2010). However, it has a fair share of disadvantages: ethically questionable extraction, high

demand - low supply, poorly defined components, batch to batch variation, high probability of inherent contamination, and inability to grow many specialized cells (Galbraith et al. 2018). These challenges give rise to the need of fabricating a novel media, free of serum that can counter the disadvantages posed by serum. Serum-free and chemically defined media are available options for substituting serum (Kuo et al. 2020).

Serum-free media are devoid of serum and other unprocessed biological fluids of human or animal origin, but highly pure animal and human supplements can be added. Some examples are animal and plant hydrolysates, plate lysates, serum fractions, purified recombinant proteins, etc., are utilized (Karnieli et al. 2017). However, serum-free media have shown inconsistencies in the growth of various cells and may contain components that are cannot be defined which impart variability (Bhat et al. 2021).

Chemically defined media (CDM) is a subset of serum-free media in which all of the constituents added are extremely pure and precisely defined at the molecular level (Freshney 2016). Even though chemically defined media (CDM) is preferred, it often inhibits cell growth compared to serum-containing media because different cells have complex and variable nutritional requirements. As a result, CDM fabrication is a time-consuming and challenging task, and its supplementation with hydrolysates is a good alternative (Ling et al. 2015).

Protein hydrolysates are defined as a composite blend of many bioactive entities that is composed of varying size of oligopeptides, peptides and amino acids and produced by complete and partial hydrolysis of different protein sources (Nasri 2017). As depicted in Figure 4, protein hydrolysates are a source of numerous essential biomolecules, including oleic acid, linoleic acid, linolenic acid, phospholipids, carbohydrates, vitamins, etc., that

help cell growth and function. Due to the presence of many bioactive compounds, peptides, proteins, and lipids, protein hydrolysates would construct an appropriate and efficacious substitute for serum. Some advantages of protein hydrolysates are: 1) Analogous to serum, the hydrolysate is also a concoction of many bioactive moieties like lipids, carbohydrates, vitamins etc. that augment the growth, survival, and proliferation of cells; 2) Protein hydrolysates economically and ethically are viable; 3) Hydrolysates have already been used and successfully implemented in animal cell culture. Despite all the advantages offered by it, hydrolysate also has some disadvantages: 1) similar to serum, the composition is not clearly defined as well as may have batch to batch variability; 2) May also have high risk of contamination (Yao and Asayama 2017). Despite these issues, protein hydrolysates had been used by many researchers as growth media and serum substitutes for cell culture. These include plant-based like soy, wheat, rice (Lobo-Alfonso et al. 2008), and rapeseed (Farges-Haddani et al. 2006); animal-based such as Primatone RL (Schlaeger 1996), lactoalbumin hydrolysate (Mendonça et al. 2007), Casein hydrolysates (Phelan et al. 2009; Lahart et al. 2011) and microbial-based as yeast hydrolysate (Lobo-Alfonso et al. 2008; Mosser et al. 2013).

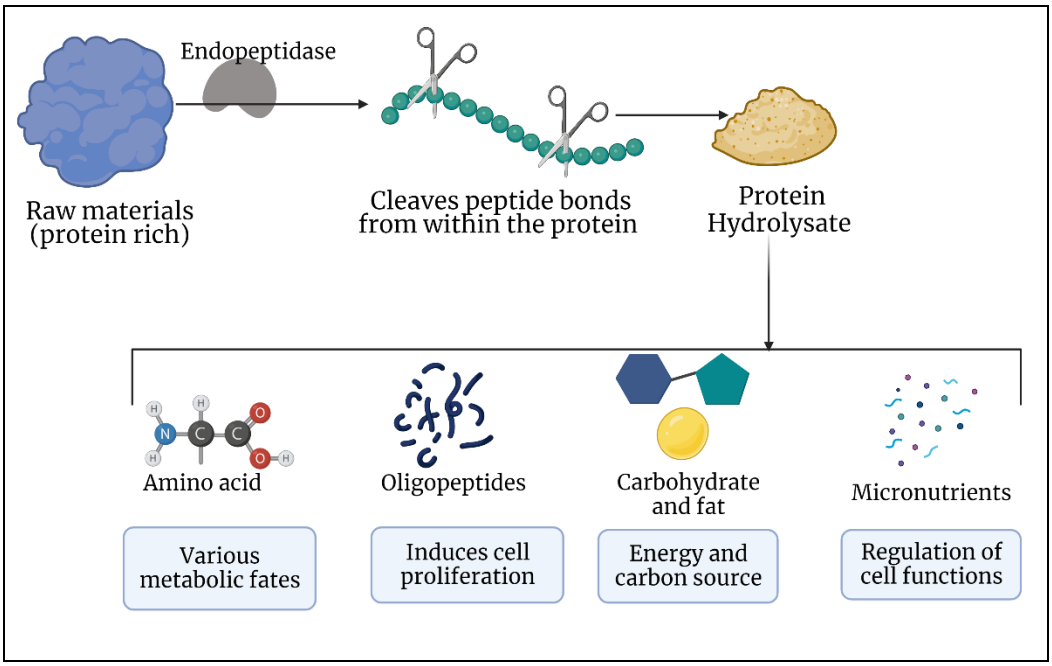

Figure 4: Enzymatic production of protein hydrolysates with their nutritional and cell augmenting properties

Following are certain studies in which protein hydrolysates were able to positively impact cell growth and proliferation:

1) Importance of defined plant protein hydrolysates was confirmed in a study that utilized a predefined medium optimum for cultivation of mouse hybridoma cells (ME-750) which is used to produce specific antibodies. This medium had a mixture of DMEM, Hams F-12 medium and Roswell Park Memorial Institute Medium - 1640 (RPMI-1640) in the ratio of 2:1:1 respectively. This media was recognised from another study conducted by Franěk and Šrámková 1996. In this study soy protein and wheat gluten hydrolysates were prepared from scratch by using soy powder and wheat flour and their composition was determined by Gas chromatography. Many fractions of both soy and wheat hydrolysates were prepared via small-pore size-exclusion chromatography including the unfractionated hydrolysate. When added to culture media of mouse hybridoma cells, both of them showed increased cell viability and product yield (antibody production). A specific wheat hydrolysate peptide

faction named HY-TRIT a 21, showed highest cell viability and product yield. An intelligent guesswork suggested that presence of high glutamine constituting peptides conferred the desired effect to wheat hydrolysate peptide fraction. Significant difference of viability and product yield between unfractionated and fractioned peptides led to a conclusion the nature of peptides have distinct effects on culture. This contradicted the previously developed notion which stated that peptides only have "nutritional value" (Franěk et al., 2000).

2) Effects of multiple hydrolysates on two different recombinant Chinese Hamster Ovary's (rCHO) cell line's growth and product yield was studied and using design of experiments (DOE) software optimum concentrations of hydrolysates were established. In this study, utilization of an already established basal SFM was done. Using a simplex lattice design, ten mixtures were generated that had variable concentrations of hydrolysates which were yeastolate ultrafiltrate, soy hydrolysate and wheat gluten hydrolysate respectively. In general, soy hydrolysate showed favourable effect on cell growth and proliferation whereas yeastolate showed negative effect on growth but significant increase in product formation. Based on these finding, all the data was analyzed using DOE to generate ideal mixture concentration of hydrolysates. Three mixtures showed high potential were: 1) one mixture having high concentration of soy hydrolysate which enhanced cell growth and viability and 2) two different mixtures having more concentration of yeastolate which increased the product yield. The effect of yeastolate was different on two different rCHO cell lines, signifies the importance of specific media optimization on different cell lines even if they have same origin. However, the DOE optimized media was not able to mimic the high antibody production that was observed in media formulations by simplex lattice design. Overall, soy and wheat hydrolysates showed positive effect on growth of cells whereas yeastolate was able to increase the product formation. The reason was

ascribed to high osmolarity conferred by yeastolate which was observed during cultivation. This study also advocated the previously stated study which established that specific peptide has specific roles and is not limited to providing nutrition. This investigation was powerful because this approach generated a optimized media in less than a month with relative accuracy (S. H. Kim & Lee, 2009).

3) Productive replacement of Bovine Serum Albumin (BSA) with a plant protein (PP) was accomplished by supplementing an already known chemically defined medium North Carolina State University - 23 (NCSU) for *in-vitro* cultivation of porcine embryos. The inference derived the notion that embryos can be cultivated with plant protein supplementation, but optimization is required. This strategy did not utilize stepwise serum replacement strategy, rather the embryos were directly cultivated on NCSU-23 supplemented with plant protein. The exact composition of plant protein was not revealed but stated to be a concoction of many plant proteins and soya lecithin prepared via homogenization at high pressure. The NCSU-23 was one of the many mediums which were chemically defined medium which were recognized for cultivation of porcine embryos. The driving cognition of this study utilized the fact that animal proteins can be replaced with plant protein and this fact was previously known. Plant proteins have been recognized to contain several free amino acids, peptides of various degrees, carbon source and micro-nutrients. (Grad et al., 2010).

6) Successful adaptation of Chanel catfish ovary (CCO) cell line was achieved via complete elimination of FBS by wheat gluten hydrolysate in UltraCulture Serum free media (UC SFM). This study also followed the approach of "stepwise serum reduction strategy" in which systematic serum reduction over a period of done. This study utilized a pre-established basal medium optimum for the growth of the cell lines. During earlier studies their proliferation and nutrient profiles were determined. Two other alternatives for serum were tested and they

were: Soy and yeast hydrolysates, although these two had inhibitory effects. The reason for impedance for both the hydrolysates was attributed to presence high quantity of nutrients namely amino acids and peptides. This study also utilized the step wise serum reduction strategy from 10 to 0% with three hydrolysates mentioned. The statistical analysis between serum free media and serum free media supplemented with wheat gluten hydrolysate however revealed that its addition didn't exhibit any advocating effect either. However, the reason for this was ascribed to unknown composition of UC SFM with a possibility that the medium already had components of hydrolysates (Radošević et al., 2016).

2.6 Non-mammalian based protein hydrolysate in animal cell culture (ACC)

Historically, mammalian derived hydrolysates were used extensively to replace serum. They are excellent source of proteins, peptides having bioactive properties and nourishment which makes it an ideal for serum substitution (Martínez-Alvarez et al., 2015). However, due to their taxonomic similarity to humans (Van Huis, 2013), the risk of disease transmission is relatively high, which leads to their avoidance.

An animal tissue extract known as Primatone RL has been used as an inexpensive serum replacement in animal and murine cell cultures. This extract has a complex of many proteins, lipids, micronutrients and amino acids etc. Utilization of Primatone RL had been known to promote the growth of cells so that they achieve high cell densities, extended the life span of cells and elevated production of cell based products (Schlaeger, 1996).

Lactalbumin is a milk-based protein (Buttriss, 2003) that yields lactalbumin hydrolysate, which has been used in animal cell culture and cell line maintenance. A mitogen named LH-FI was isolated from it that was substituted for 10% FBS in Swiss 3T3 cells

(Chou et al., 1979). Another major milk based protein hydrolysate, casein which is produced via pepsin and pancreatic digestion had also been used in animal based cell cultures (Nielsen et al., 2019). Jurkat T cells exhibited varying effects when supplemented with casein hydrolysate on its proliferation and viability (Phelan et al., 2009).

Despite established nutritional and growth inducing properties, mammalian-based hydrolysates have their limitations. Contamination introduced by mammalian-derived hydrolysates, which ultimately becomes part of the terminal product, is one of the main shortcomings that led the direction of animal-free media. Viral contamination is the primary concern posed by these hydrolysates restricting their use. (Merten, 2002).

Certain novel sources may be able to overcome the disadvantages associated with conventional mammal-based hydrolysates. Among these lesser-known and used sources are protein hydrolysates derived from insects and marine invertebrates. Both of these non-mammalian sources are diverse, abundant, and high in protein (Gomez et al., 2019; Oonincx et al., 2010).

Insects belong to the largest class of animals, covering 95% of the biodiversity and were historically consumed at various stages of life (Anaya et al., 2013). There are many reasons insects make a good source of protein hydrolysate which assists in sustainability as well: 1) Insects have an excellent nutritional profile which consists high quantity of protein (50-71%), fats (13.4 – 33.4), and fibers (5.1 – 13.6) (Rumpold & Schlüter, 2013) which validates them as a potential candidate for serum substitution.. 2) Insects produce less amount of Green House Gases (GHGs) and ammonia compared to other animals, which leads to climate change and global warming (Oonincx et al., 2010) making their hydrolysate production more environmentally friendly in comparison to other animals. 3)

Since humans and mammalian animals have a close taxonomic relationship, the risk of zoonotic diseases such as bovine spongiform encephalopathy (BSE) and avian influenza (H5N1) is high, rendering mammalian-based hydrolysate unsuitable for use, making insects an ideal protein hydrolysate source (Van Huis, 2013). Black soldier fly (*Hermetia illucens*) and cricket (*Gryllidae*) are two widely accepted edible insects with high nutritional qualities (Bessa et al., 2020) that can produce high-quality protein hydrolysate that can be used in animal cell culture.

The marine world, which has a similar spectacular biodiversity, is a rich natural resource for a variety of biologically active compounds that have the potential to generate high-quality protein hydrolysates. Marine invertebrates are an excellent source of proteins and peptides, which can act as hormones, growth factors, and amino acids (Hamed et al., 2015). The oyster (*Crassostrea virginica*) is a vital marine bivalve, accounting for 33% of global mollusk production (Wijsman et al., 2019). It is a good source of protein, containing up to 80 percent protein on a dry weight basis (Gomez et al., 2019), making it an ideal candidate for the production of protein hydrolysates. Mussels (*Mytilus edulis*), another protein-rich bivalve, have been used to synthesize protein hydrolysate with bioactive properties such as antioxidant and cell-protective qualities (Oh et al., 2019). Another highly novel, protein-rich (Hirabayashi et al., 1998) and unexplored protein source is a marine invertebrate called the lugworm (*Arenicola marina*). Until now, the only successful application of lugworm protein hydrolysate has been documented in the treatment of damaged hair (Shin et al., 2015).

2.7 Plant-based protein hydrolysates in ACC

Hydrolysates derived from plants have been routinely used to reduce or eliminate serum from traditional basal media formulations, frequently in combination with a variety of other supplements (Babcock & Antosh, 2012). Some successful examples of plant hydrolysates as serum substitutes are soy (*Glycine max*), wheat (*Triticum*), rice (*Oryza sativa*), and rapeseed (*Brassica napus*).

Soy hydrolysate is produced from soybeans that have high protein content of 40 - 50% with lipids and carbohydrates. Soybean has many storage proteins like albumins, glycinin and β-conglycinin. Production of soy hydrolysate can be done using enzymatic digestion by various enzymes like pepsin, trypsin, papain and pancreatin (L. Zhang et al., 2010). Soy hydrolysates are known to increase cell proliferation, viability and protein production which makes it an effective replacement of serum. Apart from being a source of many macronutrients, soy hydrolysate has many micronutrients that can affect cell cultures like lactate, succinate and citrate (Gupta et al., 2015). Soy hydrolysate has been used in mammalian cell culture especially in Chinese Hamster Ovary (CHO) cells for production of recombinant protein production. In one study involving CHO-320 cells, soy hydrolysate exhibited marked elevation in cell growth and slight increase in interferon γ (γ - IFN) production. It was established that increase in γ - IFN was a result of rise in protein synthesis which was compromised via lowering of cell growth as well as escalation in secretion (Michiels et al., 2011).

Wheat hydrolysate is one of the most cost-effective proteins produced by the wheat industry that is rich source of many proteins and polypeptides. Production of wheat hydrolysate requires enzymatic action of various proteases namely alcalase, protamex, flavouzyme etc (Wang et al., 2007). One paper reported high cell growth and viability in

mouse hybridoma (ME-750) cells and rise in monoclonal antibody (mAb) production when serum was substituted with wheat hydrolysate (Franěk, 2004).

Rice hydrolysate is produced from rice bran in copious amounts by the agriculture industry. It has various macromolecules like carbohydrates, proteins, fibers and lipids (Tsigie et al., 2012). Rice is rich in many proteins like Albumins, globulins, prolamins and glutelins. Depending on the type of and part of the rice used, the biomolecular concentrations may vary. Many methods ranging from alkali extraction to enzymatic degradation have been applied in the production of rice hydrolysates (Hoogenkamp et al., 2017). One report which used serum-free media for CHO-320 cells described ameliorated production of γ - IFN as well as cell growth and proliferation (Bare et al., 2001).

Rapeseed is one of the most important crops used to produce oil. Rapeseed has decent proportion of amino acids as well as many other bioactive moieties like phenols, phylates and glucosinolates (Yoshie-Stark et al., 2008). Apart from these, rapeseed has cruciferin and napin that imparts it emulsifying and oil binding properties which is required in animal cell culture. These molecules under pressure are also known to produce textures similar to meat (Kyriakopoulou et al., 2019). Rapeseed has only been recently suggested as an analogue to serum as compared to its other counterparts. One study that used CHO-C5 cell line for production of γ-IFN utilized serum free media with rapeseed peptide factions as a supplementIt demonstrated an increase in cell growth that was not statistically significant to the positive control, but cell death was significantly reduced; the explanation for this is unknown. Another factor they found was correlation between concentration of rapeseed peptide factions and cell growth, a higher

concentration of these fractions negatively impacted cell growth (Farges-Haddani et al., 2006).

Pea (*Pisum sativum*) is another highly promising and underutilized plant-based protein source. As a high-quality protein with a balanced amino acid ratio and all essential amino acids, pea protein could meet FAO/WHO recommendations (Joint & Organization, 2007). In addition, the lower allergenicity, non-GMO nature, higher nutritional value, and overall economic benefits have made its hydrolysate a potentially good option for serum substitution. (J. Ding et al., 2020)

2.8 Fungi and Algae based protein hydrolysates in ACC

Numerous protein hydrolysates derived from fungi and algae have been used to replace serum in media, with yeast (*Saccharomyces cerevisiae*) being the most successful. These sources are naturally high in protein (typically greater than 30%), making them excellent candidates for protein hydrolysates. Additionally, their rapid growth rates and ability to grow on novel substrates make them commercially viable (Ritala et al., 2017).

Yeast hydrolysate is the most commonly used serum replacement in animal cell culture (Sung et al., 2004). Yeast hydrolysates are a concoction of many biomolecules like nucleic acids, carbohydrates, proteins, fats and other cellular components which makes it an excellent nutrient source (S. H. Kim & Lee, 2009). Yeast extracts and yeast peptones had been used as media additive for IgG production for CHO-AMW cell line. These two were able to ameliorate cell growth and viability but exerted two different effects. Yeast extract was able to induce high cell growth which led to establishment of high cell density whereas yeast peptone enhanced IgG production. A future incentive for their

further characterization was also given to fully understand these effects (Mosser et al., 2013).

Microalgae, specifically *Chlorella vulgaris*, is a single-celled organism that is highly nutritious due to its high protein content (>55% dry weight) and lipid-accumulating properties, earning it a place in the health supplement industry (Safi et al., 2014). In addition, it has recently been demonstrated to be an adequate serum substitute due to its cell proliferative properties (Ng et al., 2020; Song et al., 2012). However, most research has focused on extracts rather than protein hydrolysates, making it an ideal candidate for testing.

Similarly, the button mushroom, or *Agaricus bisporus*, which is the most widely cultivated mushroom in the United States, has an excellent nutritional profile with an extremely high protein content comparable to animal sources. Apart from proteins, they are excellent source of fatty acids, phenolic compounds, micro- and macronutrients (Atila et al., 2021). However, its potential for use as a serum substitute has not been investigated.

Even though all these novel sources have immensely beneficial chemical and biological composition, none of them have been intensively applied in animal cell culture systems. Despite of the fact that these sources show great promise as serum substitute, extensive research and evidence is required for their active utilization.

2.9 Effect of Protein hydrolysates on growth and biomass

Apart from their role of providing nourishment to the cells for growth, protein hydrolysates have shown many outcomes that suggest that their role is not limited to providing nutrients.

1) Due to absence of serum in serum free media, cells are exposed to additional stresses that leads to programmed cell death or apoptosis. This affects not only cell growth but production of cell-based products like monoclonal antibody. Hydrolysates are known to enhance cell viability by extending life span of cell by subduing apoptosis. Although the exact reason for this is not known, but it is proposed that peptides in the hydrolysate function as survival factor that are able to somehow suppress programmed cell death and induce cell growth as well as production of by-products (Franěk, 2004).

2) Many growth factors are required for proliferation of cells like insulin and insulin like growth factors, epidermal growth factor etc. They are known to have "bioactive" properties which imparts them growth factor like properties. It is proposed that hydrolysates provide these growth factor like properties to the cell which in turn improves cell metabolic efficiency and resultantly improves cell growth (Burteau et al., 2003; Mosser et al., 2013).

3) As hydrolysates are source of many micronutrients like minerals, vitamins etc., these are well known to enhance oxidative metabolism. These micronutrients are required for functioning of many enzymes that take part in basal functioning of cells and thus increase cell viability and growth (Luo et al., 2012).

4) Some peptides in hydrolysates are known to increase the cell-based products by inhibiting cell growth. This shift is usually associated with the termination of exponential

phase and onset of stationary phase that induces production of cell-based products. This process is usually related to the capacity of cell to consume lactate as an alternative source thus suppressing its toxic effect and maintaining overall productivity (Burky et al., 2007; Mosser et al., 2013). Figure 5 summarizes how protein hydrolysates drive cell growth and survival.

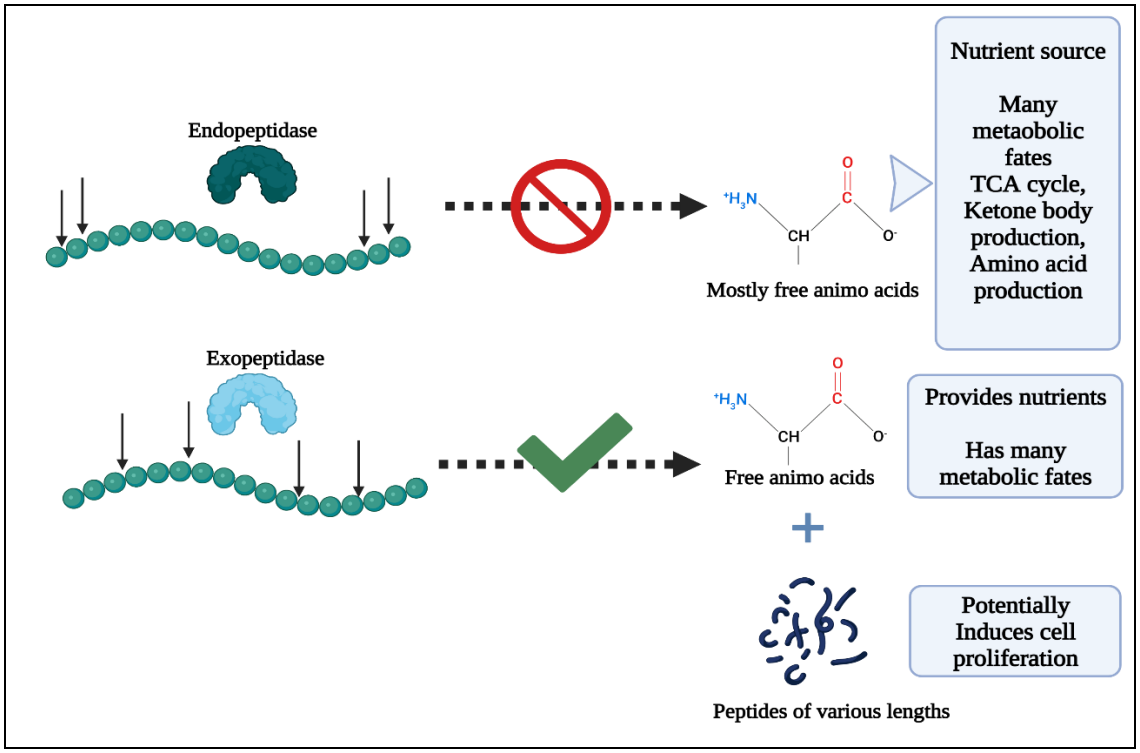

Figure 5: The overall rationale for how protein hydrolysates might provide cell-enhancing qualities

In the Table 2, a brief account of protein hydrolysate utilization and the effects it exerted in animal cell culture is given below:

Table 2 - Different protein hydrolysates used for animal cell cultures in different studies

Protein	Type (s) of cell	Resultant products of	References

hydrolysate (s) used	**cultivated**	**the animal cell culture**	
Plant protein hydrolysate	Mouse hybridoma cells (ME-750)	Production of specific antibodies	(Franěk et al., 2000)
Yeastolate ultrafiltrate, soy hydrolysate, wheat gluten hydrolysate and their blends	Two recombinant Chinese hamster ovary cells (rCHO)	Biomass and antibody production	(S. H. Kim & Lee, 2009)
Yeast hydrolysate; wheat gluten hydrolysate; Soy hydrolysate	Channel catfish ovary (CCO) cell line	Biomass production	(Radošević et al., 2016)
Soy protein hydrolysate	Chinese hamster ovary cells (CHO)	Production of specific antibodies	(Gupta et al., 2015)
Peptide faction of rapeseed hydrolysate	Chinese hamster ovary cells (CHO)	Production of specific antibodies	(Farges-Haddani et al., 2006)
Bacto-soytone and soy hydrolysate	Human keratinocytes	Biomass production and maximum cell density	(Y. K. Lee et al., 2008)
Soybean and rice grain protein hydrolysate	Human skin fibroblast (HSF) 1184 cells	Biomass production and maximum cell density	(Farizhandi et al., 2014)

As established from the table above, protein hydrolysates have been used successfully in many animals' cell cultures for achieving high density biomass production as well as producing cell-based commodities. To successfully commercialize and industrialize cultured meat, a media that boosts cell growth and proliferation without the use of serum is essential. This thesis brings us one step closer to that goal by incorporating a variety of protein hydrolysate sources.

2.10 Production of protein hydrolysates

Protein hydrolysates can be synthesized in a variety of ways, including chemical processing, enzymatic hydrolysis, or microbial fermentation (M. Nasri, 2017). Chemical processing method utilizes strong acid and alkali treatment. Acid hydrolysis typically involves boiling the protein-rich substrate at 110° C, followed by treatment with 6N HCl or other potent acids. This method is the gold standard for amino acid analysis on various substrates and is highly effective at cleaving peptide bonds and generating protein hydrolysates (Kechaou et al., 2009; TSUGITA & SCHEFFLER, 1982). However, the chemical method of production necessitates strong acids and bases, is not environmentally friendly, inexpensive, and may impair the bioactive potential of protein hydrolysate. Fermentation of substrate to create protein hydrolysate with bioactive characteristics is the most cost-effective approach compared to chemical and enzymatic methods, but it is also the most time-consuming and inefficient (R. Nasri et al., 2022). Among all methods and according to the literature, in vitro hydrolysis of protein substrates using appropriate exogenous proteolytic enzymes is the widely used process to produce protein hydrolysates and peptides with desirable biological properties (Kristinsson & Rasco, 2000). Enzymatic proteolysis is better for producing protein hydrolysates than chemical treatments because the process conditions are milder (pH 6.0–8.0; temperature 40–60°C), and the enzymatic hydrolysis is highly controlled. Furthermore, unlike chemical processes, the overall amino acid composition of enzymatic protein hydrolysates is nearly identical to that of the protein substrate, with slight modifications depending on the enzyme used. Enzymatic digestion does not use organic solvents or toxic chemicals, making it suitable for the food and pharmaceutical industries,

relatively economical, and highly efficient (Choi et al., 2010). Enzymes from animal sources are more specific to their site of action compared to plant enzymes, which are broader in their action. Endo and exopeptidases are the two primary classes of enzymes that can be utilized to create protein hydrolysates. Endopeptidases recognize specific amino acids in the middle of the peptide, whereas exopeptidases recognize one or two terminal amino acids. Endopeptidases, specifically Alcalase, are the most prevalent and effective enzymes used for the synthesis of protein hydrolysates that are known to have bioactive properties, according to various studies. (Marson et al., 2020)

2.11 Conclusion

Seafood is one of the most important food sources as it is the most consumed and traded commodity which is rich in various primary nutrients. The traditional source of obtaining seafood is via capturing fish from the sea and aquaculture both of which have shortcomings and is not sustainable. Thus, there is a dire need of alternatives that can support and take the pressure of the traditional seafood sources. Cell based seafood and cellular agriculture is a potent tool that can increase seafood production to meet the demands of a growing world population. However, since not a lot is known about aquatic cells, use of zebrafish embryonic stem cells seems a good way to go since it's a modal organism with well elucidated biochemistry and genetics. Cell culture technology is a powerful tool for cellular meat production, but the conventional use of Fetal Bovine Serum (FBS) in culture media restricts its utilization at an industrial level. FBS is effective but has many limitations: unethical extraction, animal origin, high demand and low supply, poorly specified ingredients, variable performance, and high cost. Such limitations impede the use of serum-based media in cellular agriculture on a commercial

scale. Thus, employing serum-free media becomes a quintessential step for cellular agriculture's overall development. Chemically defined media is great option for serum free media as it exerts a great control and efficiency, however it come with some limitations. Protein hydrolysate are an effective serum free media supplement with a rich literature of success. Various insect, plant, algae and fungi-based protein hydrolysate are novel, protein rich that have a good potential for producing bioactive protein hydrolysates that can have cell augmenting properties. Endopeptidase enzymatic production of various protein rich substrate to produce protein hydrolysates is the most effective and environmentally friendly method that can produce peptides that can increase cell growth and proliferation. Thus, for this study, we chose various protein-rich substrates derived from non-mammalian sources and hydrolyzed them with Alcalase. This endopeptidase has the potential to yield a mixture of amino acids and various bioactive peptides. We utilized these protein hydrolysates in a variety of serum conditions (10, 5, 2.5, 1 and 0%) at multiple concentrations (10, 1, 0.1, 0.01 and 0.001 mg/mL). This was performed to comprehend the effect of serum, its interaction with protein hydrolysate, and the potential for protein hydrolysates to reduce or replace serum.

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Chapter 3

Effects of enzymatic hydrolysis on the functional properties, antioxidant activity and protein structure of black soldier fly (*Hermetia illucens*) protein

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Abstract

The effects of chemical protein extraction (through conventional methods), and enzymatic hydrolysis with Alcalase, Papain and Pepsin, on the functional properties, antioxidant activity, amino acid composition and protein structure of black soldier fly (*H. illucens*) larval protein were examined. Alcalase hydrolysates had the highest degree of hydrolysis ($P < 0.05$), with the highest hydrolysate and oil fraction yield ($P < 0.05$). Pepsin hydrolysates showed the lowest oil holding capacity, as compared with that of other enzymes and protein concentrate ($P < 0.05$), whereas no significant differences were observed among other enzymes and protein concentrate ($P > 0.05$), thus indicating that Alcalase and Papain produced peptides with more hydrophobic amino acids. The emulsifying stability and foam capacity were significantly lower in protein hydrolysates than protein concentrate ($P < 0.05$). The antioxidant activity of protein hydrolysates from protein concentrate and Alcalase was higher than that with Papain and Pepsin ($P < 0.05$), owing to the higher hydrophobic amino acid content. Raman spectroscopy indicated structural changes in protein α-helices and β-sheets after enzymatic hydrolysis. These results demonstrated that enzymatic hydrolysis under moderate conditions and short periods of time could potentially provide a method to separate proteins with high nutritional value and bioactivity from insects, thus potentially enabling sustainable development of food and feed.

Keywords: Insect proteins, black soldier fly, enzymatic hydrolysis, functional properties, antioxidant activity, amino acids composition, Raman spectroscopy.

3.1 Introduction

The FAO has estimated that by 2050, the world's population will reach 9 billion people, thus requiring food production to increase by 70% and meat production to increase 100% to meet global demands (FAO, 2020). However, current agricultural practices are not sufficiently sustainable to address food insecurity concerns, and despite efforts to address this concern, one in eight people globally are food insecure, and one in six American children may not know where their next meal will come from. The importance of food security has been highlighted in the COVID-19 pandemic, during which many food processors and food supply chain stakeholders were shut down, thus creating a meat shortage and increasing food insecurity concerns. In addition, meat accounts for only 15% of the total energy in the global human diet, whereas approximately 80% of agricultural land is used for animal grazing or the production of livestock feed and fodder (Herrero et al., 2015, 2016). Meat consumption must be reduced by 70% to achieve sustainable food production systems and meet food security requirements (Kanianska, 2016). Furthermore, food loss is another challenge in the sustainability, economics and the nutritional status of food. Despite considerable progress in agricultural production, post-harvest practices and supply chain management, approximately 30–40% of the total food produced is lost annually in the US (Ziolkowska, 2017). Thus, there is an urgent need to develop novel and smart food production systems to reduce food waste, increase production yield and provide sustainable alternative proteins with minimum impact on the environment.

One sustainable food system is entomophagy, or eating insects, as a part of a diet widely followed in Asia, Africa and Latin America (Liceaga, 2019). Insects represent the

largest sector of fauna, accounting for 95% of biodiversity, and have historically been consumed at various stages of life (Anaya et al., 2013). In African countries such as Zambia and Nigeria, where the meat supply is insufficient, insects are a valuable source of protein (Omotoso, 2006). Because of their sustainability, excellent nutritional value (protein 50–71%, fats 13.4–33.4% and fiber 5.1–13.6%), low emissions and greenhouse gas production, excellent feed conversion ratios, low water consumption, inexpensive feed sources and low concerns regarding zoonotic diseases, insects are a favorable candidate alternative protein that may be developed for food and feed products (Van Huis, 2013). Furthermore, entomophagy can be considered a control step to protect crops from infestation, and insects can be easily converted to products such as protein isolates or protein bars, with enhanced flavor and appearance (Tan et al., 2016).

The most important hurdle to the use of insects as food in Western countries is consumer acceptance. Studies have indicated that in developed countries, insects may be accepted by consumers when they are fragmented and included in a food as a protein powder or ingredient (Borremans et al., 2020). By applying enzymatic hydrolysis technology for protein recovery, a broad spectrum of food and feed ingredients might be produced with improved and upgraded functional properties and protein nutritional value (Ovissipour et al., 2013) .

From a food science and technology perspective, studies have developed protein hydrolysates from different insects including crickets (*Gryllodes sigillatus*) (F. G. Hall et al., 2017), migratory locusts (*Locusta migratoria* L.) (Purschke et al., 2018), mealworms (*Tenebrio molitor*) (Y. Tang et al., 2018) and black soldier flies (BSF) (*H. illucens*) (Caligiani et al., 2018; Firmansyah & Abduh, 2019; Mintah et al., 2020; Zhu et al., 2020).

Few studies have been conducted on BSF hydrolysis, including the antioxidant properties of BSF hydrolysates (Firmansyah & Abduh, 2019; Zhu et al., 2020), and chemical and enzymatic hydrolysis of BSF for extraction and characterization of different fractions (Caligiani et al., 2018; Mintah et al., 2020). BSF enzymatic hydrolysis, functional properties, antioxidant activity, nutritional value and protein structure have not been evaluated through a systematic approach. This study aimed to process novel functional proteins from BSF larvae by using enzymatic hydrolysis to provide an alternative protein for developing food and feed.

3.2 Materials and methods

2.1. One-step chemical extraction of protein

BSF larva meal was provided by Fluker Farms (Baton Rouge, LA, USA) and stored in a refrigerator until use in experiments (1 week). BSF meal was first defatted by mixing one-part BSF meal and two parts of petroleum ether (w/v) at room temperature in a shaking incubator for 1 h. The solvent containing lipids was removed, and the procedure was again repeated for the residues. Then lipids were recovered by solvent evaporation under a vacuum drier at 40°C. The defatted pellet was washed three times with deionized water to remove solvent residue. The defatted BSF pellet was treated with 40 ml of 1 M NaOH at 40°C for 1 h. The supernatant was collected with a centrifuge, and protein was recovered by precipitation with 10% trichloroacetic acid solution in acetone. Samples were kept at -20°C overnight, centrifuged at 4450×*g* for 30 min, and then washed three times with acetone and dried with a freeze drier to obtain BSF protein concentrate.

2.2. Enzymatic hydrolysis of BSF meal

The commercial proteolytic enzymes used in this study—Alcalase®, an endoprotease enzyme (2.4 AU/g) from *Bacillus licheniformis,* crude powder Papain, a cysteine-protease from *Carica papaya* latex (1.5 units/mg) and Pepsin, an endoprotease from porcine gastric mucosa (250 units/mg)—were provided by Sigma-Aldrich Inc. (St. Louis, MO, USA).

The BSF larva meal (50 g) and distilled water (150 ml) (1:3 w/v) were mixed in a shaking incubator at room temperature for 2 h for hydration. Then the mixture temperature was increased to 60°C with constant stirring in a mini shaker for 20 min at 220 rpm. Each enzyme was added at a ratio of 2% of the BSF meal. To enhance the enzymatic hydrolysis efficacy, 1% of each enzyme was added at the beginning of the process, and the other 1% was added after 60 min (two-step hydrolysis). Enzymatic hydrolysis was performed for 120 min. Hydrolysis was conducted at a pH of 6.85 for Alcalase and Papain, and a pH of 3 for Pepsin. The enzyme for each mixture was inactivated at the end of the hydrolysis by heating the solution to 90°C for 10 min in a water bath. The heated suspension was centrifuged (2500 *g,* 5 min, room temperature; Eppendorf 5417-R) thus resulting in three distinct phases including a semisolid phase at the bottom containing insoluble protein and chitin, an intermediate supernatant liquid phase containing protein hydrolysates and a light liquid phase at the top containing the lipid fraction. All samples were frozen at -20°C, and the intermediate supernatant phase was separated and freeze-dried. The resulting freeze-dried protein hydrolysates were placed in sealed polystyrene conical tubes and stored at -20°C until further use. The yield of hydrolysate fractions (wet weight basis) was determined according to the weight of the

oil, hydrolysates and solid layers. All experiments were performed in six replicates (N=3).

2.3. Degree of hydrolysis

The degree of hydrolysis was measured according to formol titration as the proportion of α-amino N with respect to total N in the sample (Taylor, 1957), in triplicate.

2.4. Analysis of functional properties

The functional properties of BSF protein concentrate and hydrolysates were determined in triplicate. The fat adsorption capacity of the BSF protein concentrate and hydrolysates was determined by mixing 500 mg of each sample with 10 ml canola oil, according to Sathivel et al., 2005 and Shahidi et al., 1995; this was followed by mixing and incubating samples for 30 min at room temperature with intermediate mixing every 10 min. The samples were then centrifuged at 2500 g for 30 min. Free oil was removed, and oil adsorption was evaluated according to weight differences and was expressed as ml of oil adsorbed by 1 g protein of BSF protein concentrate and hydrolysates.

Emulsifying stability (ES) was determined according to Yasumatsu et al., 1972 by placing 500 mg of each protein sample into a 250 ml beaker and mixing with 50 ml 0.1 M NaCl. Then 50 ml of pure canola oil was added to the mixture. A highspeed hand-held homogenizer was immersed in the mixture and operated for 2 min at maximum output to create an emulsion. From each emulsion, three 25 ml portions were placed in graduated

cylinders and kept for 15 min at room temperature. Then the aqueous volume and total volume were measured. ES (%) was calculated as follows:

$$\text{ES}\ (\%) = \frac{\text{Total volume} - \text{aqueous volume}}{\text{total volume}} \times 100$$

Foam capacity and foam stability were determined with the aeration method according to Pacheco-Aguilar et al., 2008. Briefly, 750 mg of protein sample was added to 25 ml of deionized water with a final pH of 6.8 and mixed with a stir bar for 10 min at room temperature. Protein mixtures were aerated with a homogenizer. Foam capacity was determined according to the percentage increase after aeration according to the following equation:

$$\text{Foam capacity}\ (\%) = \frac{\text{volume after aeration} - \text{volume before aeration}}{\text{volume before aeration}} \times 100$$

Foam stability (%FS) was determined according to the percentage of foam remaining after 10, 30, 60 and 90 min.

2.5. Antioxidant activity

The antioxidant activity of the hydrolysates was determined with tt DPPH free-radical scavenging capacity. DPPH• solution was prepared by dissolving DPPH in 75% DMSO and dilution to a final concentration of 0.2 mM DPPH•. The hydrolysates (1.0 ml) were mixed with 1.0 ml of fresh DPPH• solution, incubated in the dark for 1 h, then measured at 515 nm with an Evolution 60S spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA) against a 75% DMSO blank. DPPH• radical scavenging activity

was calculated with the following equation:

$$\text{DPPH} \bullet \text{ radical scavenging activity (\%)} = (1 - \frac{As}{Ac}) * 100$$

where A_S is the sample absorbance, and A_C is the absorbance of a blank control. The total protein in the hydrolysates was quantified with a colorimetric method with Thermo Scientific™ Pierce™ 660 nm protein assays. The samples were mixed with the assay reagent at the recommended 1:15 ratio. Pre-packaged, prediluted bovine serum albumin was used as the protein standard, with concentrations ranging from 125 µg/ml to 2000 µg/ml. Protein concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Pittsburgh, PA, USA) with reference to the absorbances obtained for a series of standard proteins.

2.6. Amino acid composition

The samples were hydrolyzed for 16 h at 130°C in HCl (vapor phase), and this was followed by derivatization with Waters AccQTag derivatization reagents. Derivatized amino acids were quantified with RP UPLC, with a C18 analytical column (1.7 µm, 2.1×100 mm) and acetonitrile/water as buffers.

2.7. Raman spectroscopy

Raman spectra were collected with a DXR2 microscopy Raman spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with a 785 nm diode laser source. Spectra were collected from 1700 to 1500 cm^{-1} (protein amide I and amide II regions) with a spectral resolution of 5 cm^{-1} under fixed parameters including a laser

power of 20 mW, an average of five measurements and 100 scans. Spectra were collected from 10 mg/ml protein solutions.

2.8. Data analysis

Each experiment was conducted with at least with three replicates (n = 3) to ensure reproducibility. The results are expressed as the mean of the replicates ± standard deviation. The significance of differences among the biofilm removal treatments was determined with one-way analysis of variance, and differences were considered significant at $P < 0.05$. Raman spectra were pre-processed by using baseline correction, and this was followed by normalization and smoothing to flatten the baseline and remove noise. Second derivative transforms with the Savitzky-Golay filter with a gap value of 11 cm^{-1} were applied to reduce the spectral overlap and enhance discrimination of the spectral signature. Supervised chemometric models for the 1700–1500 cm^{-1} region of the protein concentrate and protein hydrolysate spectra associated with the amide I and II regions of proteins were developed to perform principal component analysis (PCA) and obtain loading plots, with Unscrambler® X software (version 10.5) (CAMO Software, Oslo, Norway).

3.3 Results and discussion

3.3.1. Degree of hydrolysis and yield

The degree of hydrolysis (DH) of BSF proteins hydrolyzed by Alcalase, Papain and Pepsin in a two-step enzymatic hydrolysis process in 120 min is presented in Table 1. The highest DH was achieved by Alcalase (18.4%), followed by Papain (15.34%) and

Pepsin (9.8%), thus suggesting that Alcalase is the most suitable enzyme for the BSF protein hydrolysis process. These results are similar to those from previous studies on the enzymatic hydrolysis of migratory locusts (*Locusta migratoria*) (Purschke et al., 2017), tropical banded crickets (*Gryllodes sigillatus*) (F. G. Hall et al., 2017), BSF (*H. illucens*) (Zhu et al., 2020) and lesser mealworms (*Alphitobius diaperinus*, LM) (Leni et al., 2020). In contrast, another study has shown that the lowest DH for BSF hydrolysates is associated with Alcalase (6%), as compared with Papain (25%), Pepsin (17%) and Pancreatin (25%) (Caligiani et al., 2018). This difference may be explained by two reasons: the difference in the enzyme to substrate ratio and a reduction in the enzymatic reaction rate due to limitation of the enzyme activity because of formation of inhibitory products, enzyme inhibition and enzyme deactivation (Ovissipour et al., 2009; Valencia et al., 2014). Caligiani et al., (2018) have used a 1% enzyme to substrate ratio and hydrolyzed BSF for 24 h. However, in this study, we applied a 2% enzyme to substrate ratio in a two-step enzymatic hydrolysis to improve the enzymatic reaction and increase the degree of hydrolysis by adding 1% enzyme to substrate at the beginning and 1% after 60 min of enzymatic hydrolysis. Moreover, A strong relationship between the enzyme to substrate ratio and DH has been shown during enzymatic hydrolysis of tropical banded crickets (*Gryllodes sigillatus*) (F. G. Hall et al., 2017) and BSF (*H. illucens*) (Firmansyah & Abduh, 2019).

Table 1

Details of enzymes used, DH and yield[1]

Enzyme	pH	Temperature (°C)	DH (%)	Yield (wet weight basis%)		
				Hydrolysates	Oil	Solid layer

Alcalase L	2.4	6.85	60	18.4 ± 1.5 a	51.4 ± 1.7 a	7.1 ± 1.6 a	41.6 ± 2.9 a
Papain		6.85	60	15.34 ± 1.1 b	37.8 ± 1.1 b	4.6 ± 0.3 b	57.5 ± 1.1 b
Pepsin		3	37	9.8 ± 2.3 c	44 ± 3.2 c	3.2 ± 0.6 c	52.8 ± 3.8 c

[1]Values are means ± SE (n=6). Values in columns with different letters are significantly different ($P < 0.05$).

The yield of different hydrolysis fractions including BSF hydrolysate, oil and insoluble solid are presented in Table 1. The results illustrated that the highest BSF-H and oil fraction yields were achieved by Alcalase, with the lowest solid fraction. In contrast, enzymatic hydrolysis with Papain resulted in the lowest hydrolysate and oil fraction yield, with the highest amount of solid layer. Higher oil and hydrolysate recovery with Alcalase than Protamex and Neutrase during hydrolysis of salmon (*Salmo salar*) heads (Gbogouri et al., 2006), Alcalase than Protamex and Flavourzyme during hydrolysis of sardines (*Sardina pilchardus*) (Kechaou et al., 2009), and Alcalase than several commercial enzymes during hydrolysis of anchovies (*C. engrauliformis*) (Ovissipour et al., 2013) have been reported. Caligiani et al. (2018) have indicated that BSF-H with Alcalase, as compared with Papain and Pepsin, shows the highest protein yield during a 24 h hydrolysis process, with 10% oil recovery for all enzymes; this value is significantly higher than that in our study. This difference may be explained by the difference in BSF meal compositions and the longer enzymatic hydrolysis, which might have increased the oil recovery from the intact protein.

3.3.2. Functional properties

The results of oil adsorption are presented in Fig. 1a-c as a function of hydrolytic enzymes. The oil adsorption of BSF protein concentrate and protein hydrolysate results indicated that the Pepsin-hydrolysate oil adsorption was significantly lower than that of other protein hydrolysates and protein concentrate ($P < 0.05$), whereas there was no significant difference among Alcalase, Papain and protein concentrate. These results indicated that Pepsin did not enhance peptide functional properties, did not develop peptides with proper hydrophobic residues (CH_3) and had significantly lower aromatic amino acid content (phenylalanine and histidine, tyrosine) (Vioque et al., 2000). The amino acid composition results from this study illustrated that the hydrophobic amino acid (HAA) content was significantly lower in Pepsin hydrolysates than Alcalase and Papain hydrolysates and protein concentrate (Table 2). The results from this study are consistent with those from another study that has reported the potential of Alcalase to produce the highest number of hydrophobic amino acids from white shrimp (*Litopenaeus vannamei*), thereby increasing the protein hydrolysate oil holding capacity (Latorres et al., 2018). Migratory locust (*Locusta migratoria* L.) protein hydrolysate oil adsorption results have shown improved oil adsorption with enzymatic hydrolysis with Neutrase, Flavourzyme and a mixture of enzymes, as compared with protein concentrate (Purschke et al., 2018); these results are in line with our findings in this study.

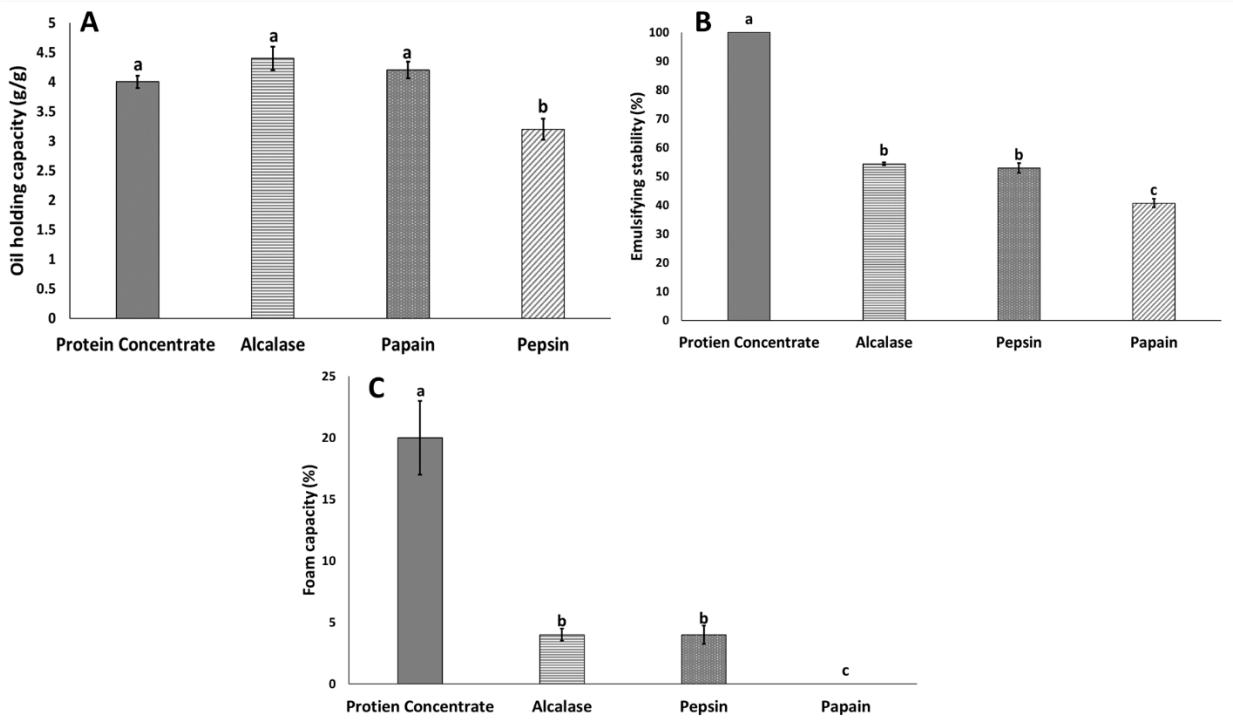

Fig. 1. Functional properties of BSF protein concentrate and BSF hydrolysates. (A) Oil holding capacity; (b) ES; (c) foam capacity

The ES of proteins can be defined as a protein's ability to form and stabilize emulsions. The ability to form emulsions is an essential characteristic required by any protein based moiety to be used in food-based applications, such as imparting functionality (Zayas, 1997). Enzymatic hydrolysis of BSF protein concentrate significantly decreased the emulsification properties from 100% to 40% in hydrolysates. Among the hydrolysates, Papain had the lowest ES value (40%), which was significantly lower than those of Alcalase and Pepsin. Only a few studies have demonstrated the emulsifying properties of insect protein (Adebowal et al., 2005; Omotoso 2015; Hall et al., 2017; Purschke et al., 2018). Strong emulsification properties have been reported for intact insect protein including that from African crickets (*Acanthoplus discoidalis*) (Adebowal et al., 2005), moth (*Cirina forda*) larvae (Omotoso, 2006), silkworms (*Bombyx mori*) (Omotoso,

2015), mealworms (*Tenebrio molitor*) (Bußler et al., 2016), migratory locusts (*Locusta migratoria* L.) (Purschke et al., 2018), *Gryllodes sigillatus, Schistocerca gregaria* and *Tenebrio molitor* (Zielińska et al., 2018).

Our study showed that the ES for protein concentrate was significantly higher than that of the hydrolysates, thus indicating that enzymatic modification of BSF larva protein did not improve the ES. A strong correlation between peptide size and emulsifying properties has been demonstrated. For example, salmon protein hydrolysates with a higher degree of hydrolysis show poorer emulsifying properties (Sathivel et al., 2005), a finding associated with larger peptides enhancing emulsifying properties (Lee et al., 1987). In addition, the presence of some polysaccharides can elevate the viscosity and in turn increase the stability of emulsions (Dickinson 1994; Wani et al., 2013; Zielińska et al. 2018). Zielińska et al. (2018) have also found a strong positive correlation between insect polysaccharide content and ES. In this study, we used a chemical method for extracting protein from BSF larvae according to previous studies (Caligiani et al., 2018). Poor chitin extraction from BSF protein was observed with the chemical method, in contrast to high chitin yield during enzymatic hydrolysis of BSF larvae (Caligiani et al., 2018). In this study, we used both chemical and enzymatic methods for separating BSF larval protein. More research is needed to explain the emulsifying properties and potential links to chitin content, which may affect emulsifying properties.

The results of the foaming capacity (%) of BSF protein concentrate and protein hydrolysates illustrated that the highest foaming capacity was associated with BSF protein concentrate, with 20% foaming capacity, followed by Alcalase and Papain hydrolysates, with 4% foaming capacity. The lowest foaming capacity was observed in

proteins hydrolyzed with Pepsin. Overall, all proteins showed unstable foam stability (< 2 min). Poor to no foaming capacity and stability have been reported for several edible insects. For example, 6% foaming capacity has been reported for whole giant African cricket (*A. discoidalis*) powder, with only 3% foam stability after 2 h (Adebowale et al., 2006), and pallid emperor moth (*Cirina forda*) powder, which has low foam capacity and foam stability (Omotoso, 2006). In addition, Yi et al. (2013) have reported poor foaming capacity over a range of pH values for five different insect proteins extracted with an acidic method. Improved foam capacity and foam stability after moderate enzymatic hydrolysis, as compared with that of insect powder, has been reported (Hall et al., 2017; Zielińska et al., 2018; Leni et al., 2020). Intensive enzymatic hydrolysis results in a higher degree of hydrolysis and smaller peptides with poor foam capacity. Leni et al. (2020) have shown that intensive enzymatic hydrolysis of protein from lesser mealworms decreases the foaming capacity, whereas protein hydrolysates with 5 to 10% DH, show 5 to 73% foaming capacity, and protein hydrolysates with 15% DH show no foaming capacity. In this study, we used two step enzymatic hydrolysis, which resulted in protein hydrolysates with high DH and poor foaming capacity. Moreover, these previous studies have compared the foaming capacity of protein hydrolysates with that of intact protein. However, the control group in our study was alkali extracted protein concentrate, which had less than 3% DH with high foaming capacity. Extracted protein from three edible insects, mealworms (*Tenebrio molitor*), tropical house crickets (*Gryllodes sigillatus*) and desert locusts (*Schistocerca gregaria*), has shown strong foaming capacity and foam stability, as compared with that of the intact insect protein (Zielińska et al., 2018). Caligiani et al. (2018) have extracted protein from BSF with three methods and reported

that one-step chemical protein extraction, as used in the current study, resulted in protein with strong foaming capacity.

3.3.3. Antioxidant activity

The ability of various protein hydrolysates to scavenge DPPH radicals is shown in Fig. 2. All protein hydrolysates were able to scavenge of DPPH radicals, in accordance with other findings including those in housefly larvae (Wang et al., 2013), mealworm (*T. molitor*) larvae protein hydrolysate (Y. Tang et al., 2018) and BSF (*H. illucens* L.) (Zhu et al., 2020). In this study, Alcalase hydrolysate showed higher antioxidant activity than protein concentrate and other hydrolysates produced by Papain and Pepsin. This finding may be explained by the higher HAA content in protein hydrolysates produced by Alcalase (Chen et al., 1991). Wang et al. (2013) have reported high antioxidant properties in housefly larvae protein hydrolysates, owing to the high amount of HAA. Zhu et al. (2020) have also demonstrated that peptide fractions with more than 50% HAA have the highest antioxidant properties. An increase in the presence of peptides with hydrophobic amino acids located at the water-oil interface act as electron donors that augment the scavenging of DPPH radicals (Dong et al., 2008). The results of amino acid composition analysis in this study showed significantly higher HAA content in protein concentrate and protein hydrolysates produced by Alcalase rather than Papain and Pepsin (Table 2). Higher antioxidant properties in hydrolysates produced by Alcalase have also been reported for other protein sources, such as anchovy sprat (*C. engrauliformis*) fish (Ovissipour et al., 2013). Hydrophobic amino acid residues can increase the presence of peptides at the water/lipid interface and therefore facilitate the scavenging of free radicals (Ovissipour et al., 2013).

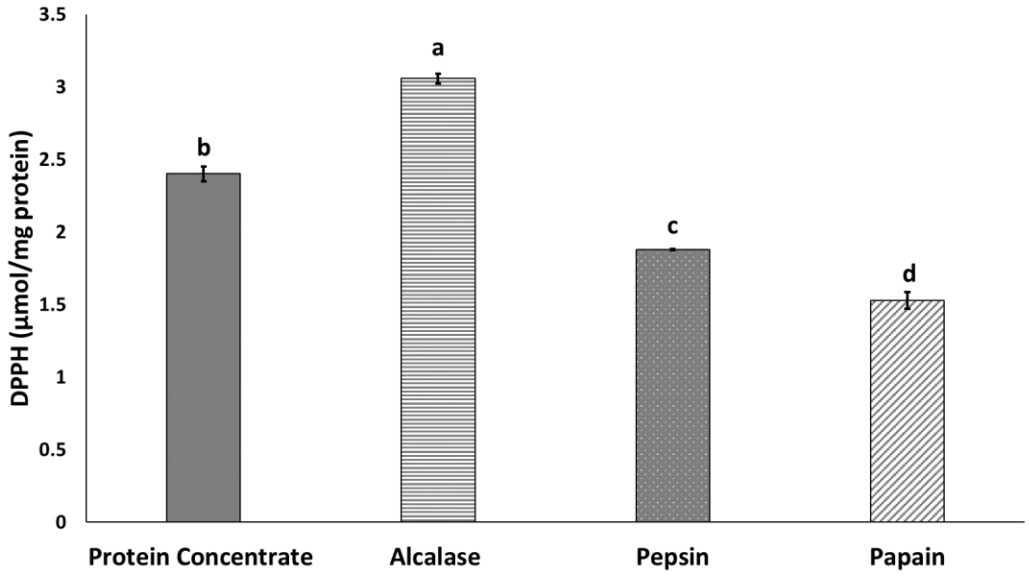

Fig. 2. Antioxidant properties of BSF protein hydrolysates

3.3.4. Amino acid composition

The amino acid compositions of BSF meal, BSF protein isolate and BSF protein hydrolysates are presented in Table 2. The most dominant amino acid in the protein hydrolysates and intact protein was glutamic acid. Similar results have been reported by other researchers for enzymatic hydrolyzed BSF meal (Caligiani et al., 2018; Firmansyah & Abduh, 2019; Janssen et al., 2017; Zhu et al., 2020). The most dominant amino acid in protein hydrolysates from other insect species such as tropical banded crickets (*Gryllodes sigillatus*) (F. G. Hall et al., 2017) and housefly larvae (Wang et al., 2013) is also glutamic acid. HAAx (Ala, Ile, Leu, Phe, Pro, Tyr and Val), which are associated with peptides' bioactive properties, such as antioxidant ability (Saadi et al., 2015), were higher in BSF protein concentrate, followed by Alcalase and Papain hydrolysates, and the lowest HAA values were associated with Pepsin hydrolysates and intact protein. Similar results were observed for essential amino acids (EAA), positively and negatively charged amino

acids (PCAA and NCAA) and aromatic amino acids (AAA). The results illustrated that protein concentration and enzymatic hydrolysis increased the quality and functional properties of the protein from BSF. However, depending on the enzymatic hydrolysis process and the type of enzyme, the functional properties and bioactivity of the peptides may be negatively affected. Other researchers have also indicated that enzymatic hydrolysis with Alcalase increases the quality of the amino acid composition of insect protein hydrolysates (Firmansyah & Abduh, 2019; F. G. Hall et al., 2017; Zhu et al., 2020). Leni et al. (2020) have compared the effects of different commercial enzymes on BSF protein hydrolysate free amino acid composition and found that Pepsin and Papain assisted hydrolysis result in the lowest and highest free amino acid content, respectively.

Table 2

Amino acid composition of BSF intact protein, protein isolate and hydrolysates

Amino acid	Quantity (mg/g)					
	Intact protein	Protein concentrate	Alcalase-hydrolysate	Pepsin-hydrolysates	Papain-hydrolysates	Reference protein FAO/WHO 1985
ALA	10.95	38.94	41.90	11.95	34.71	
ARG	6.34	31.81	28.86	9.46	22.86	
ASP	13.97	82.42	58.51	18.71	46.29	
GLU	16.75	95.48	74.86	35.19	68.31	
GLY	7.82	31.67	33.15	11.61	32.16	
HIS	5.09	19.35	20.71	10.39	18.69	15
ILE	6.91	35.97	25.44	5.17	16.94	30
LEU	9.86	50.63	37.43	5.54	24.97	59

LYS	8.64	54.08	36.12	7.52	27.04	45
PHE	6.06	34.43	20.21	3.78	12.31	38
PRO	9.15	31.11	38.47	14.51	34.30	
SER	3.84	13.74	14.41	4.11	11.99	
THR	4.42	16.63	18.14	4.45	14.14	23
TYR	7.10	27.62	30.51	7.01	23.85	
VAL	9.79	43.40	38.60	7.75	30.31	39
HAA[1]	59.83	262.11	232.56	55.70	177.37	
PCAA	20.06	105.23	85.68	27.37	68.59	
NCAA	30.72	177.90	133.37	53.91	114.60	
TEAA	50.78	254.49	196.65	44.60	144.39	
EAAI	0.3	1.1	0.85	0.27	0.7	
AAA	19.31	79.38	77.45	22.59	63.14	

[1] HAA: hydrophobic amino acids; PCAA: positively charged amino acids; NCAA: negatively charged amino acids; TEAA: total essential amino acids; EAAI: essential amino acid index; AAA: aromatic amino acids.

3.5. Raman spectroscopy and chemometrics

Fig. 3 indicates a comparison among chemically (protein concentrate) and enzymatically (protein hydrolysates) extracted protein structure. The amide II (1500–1600 cm^{-1}) and amide I (1600–1700 cm^{-1}) regions provide secondary structural information about proteins, including C=O stretching, C–N stretching and N–H in plane bending of backbone peptide groups (Ovissipour et al., 2018). The amide II region showed several peaks including those at 1555 and 1587 cm^{-1} which were assigned to amide II and phenylalanine, respectively. The peak intensity decreased after enzymatic hydrolysis. Protein concentrates showed the highest peak intensity, followed by Alcalase,

Papain and Pepsin hydrolysates. In the amide I region, several peaks were observed, among which 1645, 1655, 1658 and 1667 cm^{-1} were assigned to the α-helical structure of the amide I (Movasaghi et al., 2007). With enzymatic hydrolysis with Alcalase and Papain, the peaks became more defined than those of protein concentrate, whereas weak bands were observed for Pepsin protein hydrolysates, thus indicting strong protein denaturation. The reduction and absence of α-helical structure in heat and acid treated protein has also been reported for fish protein, such as salmon (Ovissipour et al., 2018), and for cricket microwave-assisted hydrolysis (F. Hall & Liceaga, 2020). Bands around 1618 and 1622 cm^{-1} were assigned to tryptophan (F. Hall & Liceaga, 2020; Movasaghi et al., 2007) and disappeared after enzymatic hydrolysis, is in agreement with other research findings (F. Hall & Liceaga, 2020). The peak intensity around 1622, 1676 and 1680 cm^{-1} significantly decreased during hydrolysis, thus indicating an absence of β-sheet structure and protein aggregation.

The PCA results illustrated that the spectral changes in the amide I and II regions of the BSF proteins were dependent on processing and enzymes, and the PCA model discriminated spectral changes in protein hydrolysates processed with different enzymes. In the PCA model for BSF proteins, the PC1 and PC2 components explained 47% and 36% of the variation, respectively, in the spectral band corresponding to the protein region.

Loading plots were prepared to identify the contributions of key wavenumbers to the PC1 and PC2 analysis. The key wavenumbers identified with loading plots can aid in understanding the biochemical and structural transformation induced in BSF protein after processing with different enzymes. The loading plot for BSF proteins illustrated that the

major peak with the greatest contribution to differences in PC1 was around 1602 cm^{-1}, thus illustrating phenylalanine changes and conformation in the amide I region. The other larger peaks were 1676, 1643, 1550 and 1583 cm^{-1}, which were assigned to amide I (β-sheet), amide I (α-helix), tryptophan and the C=C bending mode of phenylalanine, respectively (Movasaghi et al., 2007). The major peaks with the greatest contribution to differences in PC2 were around 1594, 1533, 1603 and 1626 cm^{-1} from highest to lowest contributions. These wavenumbers have been assigned to phenylalanine amide carbonyl group vibrations and aromatic hydrogens, and the C=C in-plane bending mode of phenylalanine, tyrosine and tryptophan, respectively, in prior studies (Movasaghi et al., 2007). PC1 and PC2 indicated protein denaturation, aggregation and free amino acid formation as a result of enzymatic hydrolysis of BSF protein.

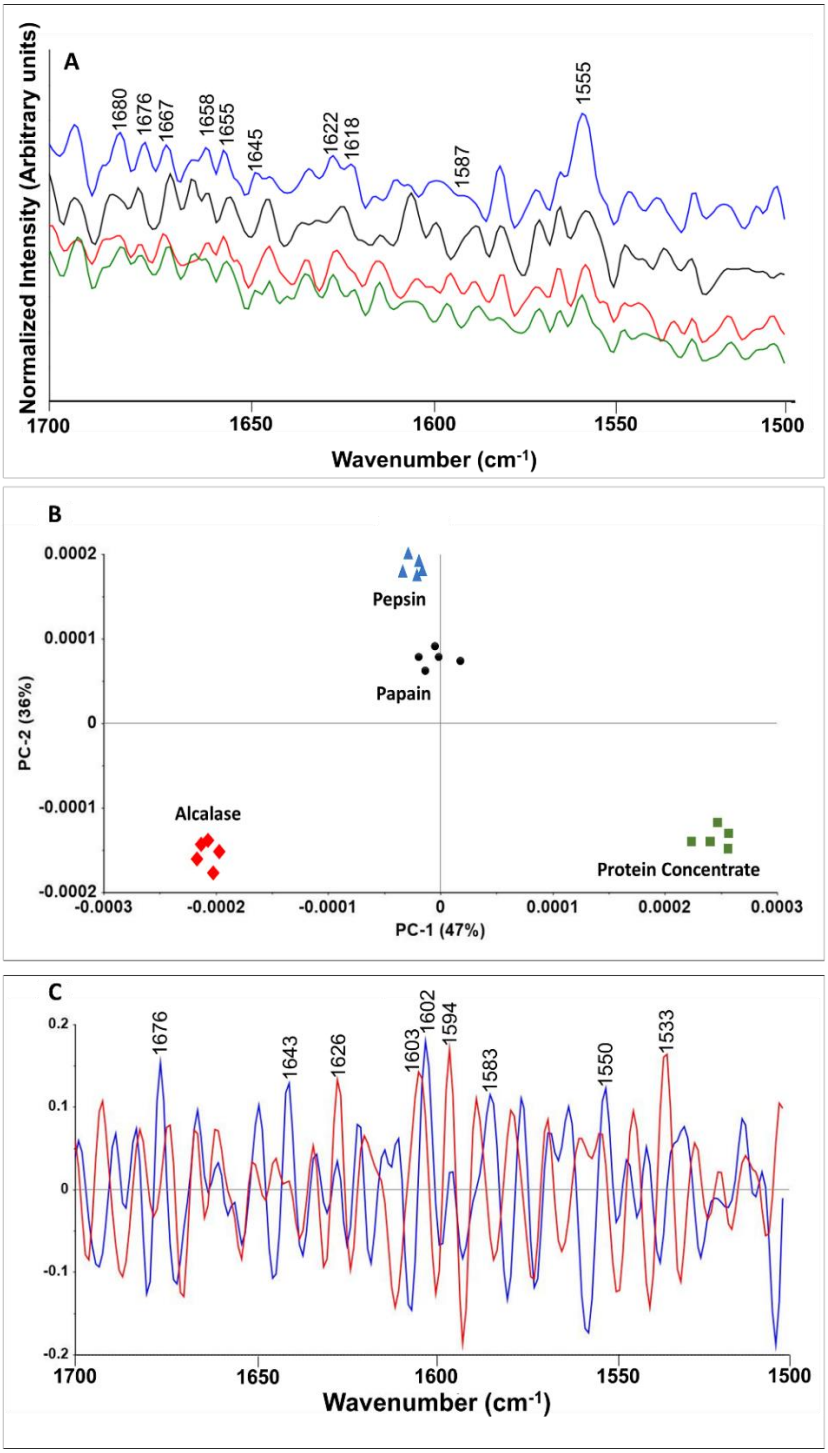

Fig. 3. (A) Effect of enzymatic hydrolysis on Raman spectra (1700–1500 cm^{-1}) of BSF protein: protein concentrate (blue); Alcalase hydrolysate (black); Papain (green), Pepsin

(red); (B) PCA model of protein concentrate and protein hydrolysates; (C) loading plot of BSF proteins: PC1 (blue), PC2 (red).

4. Conclusion

This study evaluated the effects of three commercial enzymes—Alcalase, Papain and Pepsin—on the degree of hydrolysis, protein and oil fraction yields, functional properties, antioxidant activity, amino acid composition and protein structure of BSF larvae. The results showed that, under two-step hydrolysis, Alcalase produced protein hydrolysates with a higher degree of hydrolysis, better functional properties, greater antioxidant activity and amino acid compositions with higher levels of HAA. Compared with the conventionally extracted protein (protein fraction), enzymatic hydrolysis reduced the functional properties in BSF hydrolysates; the lowest measured parameters were associated with Pepsin enzyme, mainly because of the poor amino acid composition of peptides. Enzymatic hydrolysis of BSF protein with Alcalase and Papain offered a sustainable processing method, which may result in protein hydrolysates with a higher content of amino acids. The results from this study provide a baseline for developing sustainable alternative feed and foods from insects.

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Chapter 4
Evaluating the potential of various protein hydrolysates to reduce fetal bovine serum in cell culture media for cultivated seafood production

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Abstract

One of the restrictions in cellular agriculture is the culture media price. Fetal Bovine Serum (FBS) is the most used and expensive media supplement. This study aims to replace or reduce the typical 10% serum application in Zebrafish embryonic stem cell (ESC) production media with protein hydrolysates derived from low-cost natural biomass including insects (black army fly and cricket), plants (pea), fungi (mushroom and yeast), algae, and marine invertebrates (oyster, mussel, and lugworm). All the hydrolysates exhibited sufficient yield and productivity for scaling purposes. The black soldier fly possessed the highest protein content and quality, as well as effective functional properties that have the potential to be utilized under large reactor conditions. The black soldier fly had the highest protein content and quality and functional properties that could be utilized under extensive reactor conditions, including a higher oil and emulsification capacity and a lower foaming capacity. All protein hydrolysates with concentrations between 1 and 10 mg/mL were found to be toxic to cells. However, except for cricket, all hydrolysates were able to restore or significantly increase cell growth and viability with 50% less serum at a concentration of 0.001-0.1mg/mL. However, no protein hydrolysate could completely replace serum, as cells using only protein hydrolysates exhibited morphological aberrations and significantly decreased growth ($p<0.005$). We used even more reduced serum-based condition 2.5% and 1% and narrowed the protein hydrolysate concentration ratios 0.1-0.001 mg/mL which were found to be effective previously without having a cytotoxic effect. To ensure cell health and membrane integrity, we analysed the Lactate Dehydrogenase Activity under the specified protein hydrolysate conditions (LDH). All protein hydrolysates enhanced cell growth and viability under serum-reduced conditions of 75 to 90 percent at 0.1 and 0.01 mg/mL concentrations. In addition, light and fluorescence imaging revealed cell morphological features comparable to the gold standard (10% serum). However, cells treated with protein hydrolysates exhibited significantly decreased cell membrane integrity ($p<0.05$); only lugworm and black soldier fly hydrolysates (0.01 mg/mL) at concentrations of 2.5% and 1% reduced LDH release and had a favourable effect on cell health. Overall, lugworm and black soldier fly hydrolysate was able to lower serum by up to 90% while preserving excellent cell health. Incorporating these eight protein hydrolysates into cell culture media has the potential to reduce the overall cost of cultivated fish.

Keywords: Protein hydrolysate, serum-free, bioprocessing, cultured meat, cellular agriculture.

Introduction

The increase in the human population leads to many challenges, such as food shortages and hunger (Otsuka 2013). The world population will reach 10 billion by 2050, requiring a 70% increase in meat production to meet global demand (FAO 2020). Animal-based products, especially meats, are the primary food sources globally (Whitnall and Pitts 2019). It has been estimated that the requirement for animal-based meat will rise to 500 million tons to feed people by the year 2050 (Costello et al. 2020). Among animal-based meat, seafood is an essential commodity that constitutes 20% of animal protein eaten, and its consumption has increased rapidly from previous years (FAO 2016). Conventionally, seafood is obtained from the sea or cultivated in aquaculture. Capturing fish from the oceans is not sustainable, as it has already been exploited due to overfishing, fraud, by-catch, microplastic concerns, and pollution (Smith et al. 2010; Asche et al. 2015). Aquaculture has emerged as a viable alternative to capture fisheries, but it faces several challenges, including adding nutrients to the water, reliance on fisheries, antibiotics, emerging diseases, and permit issues. (Cole et al. 2009). With all of these setbacks in the seafood industry, novel alternative meat sources are required to sustain the fisheries & aquaculture industry and environment in their current state. Many innovative meat substitutes have emerged, namely, insect-based proteins, cultivated meat, and plant-based protein alternatives. The problem with insect and plant-based alternatives is consumers' devotion to real meat, resulting in an unwillingness to consume non-authentic meat (Graça et al. 2015). *In-vitro* cultivated meat appears to be an excellent choice as it produces traditional meat with many environmental, economic, and health benefits over other alternatives (van der Weele et al. 2019). Cultured meat involves culturing cells or

tissues in-vitro that promote its proliferation, metabolism, and growth (Bhatia et al. 2018).

The first step in producing cultivated meat is generating a source of stable, self-renewing cells that possess a high proliferation rate and differentiation potential. Cells that can be considered for this purpose are Embryonic Stem Cells (ESC's), induced Pluripotent Stem Cells (iPSC's), Adult Stem Cells (ASC's), satellite cells, and specific dedifferentiated cells. Unlike other cell lines and sources, ESC's have higher pluripotency and the capacity to differentiate, making them perfect starting cells for the cultivation of meat and forming a cell repository (Fish et al. 2020).

The second critical factor in cell culture technology is the formulation of culture media. A culture medium sustains the cell or tissue's proliferation and growth (Yao and Asayama 2017). Fetal Bovine Serum (FBS) is the most commonly used media component that aids cell proliferation and metabolism. However, many factors discourage its use, such as high cost, ill-defined formulation, high demand-less supply, high variability, inability to grow specific cells, and ethical source issues (Freshney 2015). Cell culture media accounts for more than 99 percent of the total cost of the cultivated meat process (Stout et al. 2021), developing a less expensive media would significantly reduce the cost of cultivated meat. According to GFI's study, serum-free media contain growth factors and hormones, which contribute over 95% to the cost. Additionally, these factors and hormones are critical for cell proliferation and viability (Specht 2020). Thus, developing a serum-free medium with less expensive sources of growth factors and hormones would be an efficient way to industrialize cultivated meat.

In addition to increasing their biomass, cells should also exhibit healthy characteristics. One of these characteristics is measuring lactate dehydrogenase leakage in the media to determine the membrane integrity of cells. If cell health is compromised for some reason, the media will need to be reformulated because the cells will perish slowly and may acquire undesirable mutations. Leaking of cell enzymatic contents typically indicates a certain level of cytotoxicity, indicative of future apoptosis and necrosis or the initiation of tumorigenic potential (Kumar et al., 2018; Serganova et al., 2018). During apoptosis or necrosis, the cell membrane becomes permeable, compromising its integrity, and LDH, a ubiquitous enzyme, is then released through the damaged plasma membrane and can be detected (Chan et al., 2013). Higher LDH activity is frequently associated with the presence of cancer cells, as it has been used as a cancer or tumorigenic marker in several clinical studies. In contrast, lower LDH activity has been interpreted as an absence of a tumorigenic environment. This results from the Warburg effect, in which malignant cells modify their metabolomic profile to match the requirements of continually expanding cells by boosting glucose to lactate synthesis by 10-100 times (Mishra & Banerjee, 2019). As a result of the altered plasma membrane of cancer cells, a greater quantity of LDH is released, making it an effective marker. This measurement allows us to determine if a medium allows for the proper proliferation of cells and maintains cell health over extended periods without exerting any adverse effects. It is crucial because the incorporation of immortalized cell lines with tumorigenic potential in the production of cell-based meat can have many potential negative effects and consumer feedback.

In this paper we develop a serum-free media by applying bioprocessing and valorization of plants, algae, yeast, marine invertebrates, and insects' protein as growth factor

alternatives. In this study, we will use ESCs from the modal organism Zebrafish to evaluate the impact of multiple protein hydrolysates as serum substitute. Protein hydrolysates have a history of being used as substitutes or supplement to serum (J. Y. Kim et al., 2011; Logarušić et al., 2021; Taylor et al., 1972, 1974; van der Valk et al., 2010). This study aims to evaluate the effect of different concentrations of each protein hydrolysate on cell performance as well as cell health.

Materials and Methodology

Materials and chemicals/reagents used – The black soldier fly was provided by Chapul (San Jose, California, USA), and the cricket powder was provided by Cricktone (Saint Louis, Missouri, USA). The algae and pea protein were provided by Mountain Rose Herbs (Eugene, Oregon, USA) and NorCals organic (USA) respectively. Mushroom, oyster and mussel were obtained from the local market. Lugworms were bought from Wilcox Bait and Tackle (Newport News, Virginia, USA). A pre-prepared yeast hydrolysate, Bacto™ TC Yeastolate, serum, Lebovitz 15 media, HEPES and sodium bicarbonate were provided from Thermo-Fisher scientific (City, State Country). The enzyme used in this study was commercially available—Alcalase®, an endoprotease enzyme (2.4 AU/g) from *Bacillus licheniformis* and supplied by Sigma-Aldrich Inc. (St. Louis, MO, USA). The zebrafish embryonic stem cell line – ZEM2S CRL-2147™ were obtained from The American Type Culture Collection (ATCC). The antibiotics which were initially used to cultivate zebrafish Embryonic Stem Cells (ESC's) were obtained from Cytiva (City, State Country).

Production of protein hydrolysates from various sources

The enzymatic hydrolysis process of various substrates was performed with slight modification depending on the substrate according to Batish et al. (2020) and Firmansyah and Abduh (2019). Raw material from eight sources were grinded and blended using Ninja BL480D Nutri Personal Countertop Blender and mixed with water at a specific ratio. The pH of each reaction vessel was adjusted to 6-8 using 1M NaOH and HCl. The mixture was heated and shaken for 40-50 minutes at 190 rpm to attain 60°C. Once the reaction vessel achieved the desired temperature, the Alcalase enzyme was added, and the hydrolysis process was allowed to run for a predetermined time (Table 1). After hydrolysis, the reaction was halted by placing the reaction vessel in the oven and allowing it to reach a temperature of 90°C for 10 minutes for inactivation of enzyme. Next, the heated slurry was cooled down for 30 minutes and centrifuged at 7000g for 10 minutes at room temperature in a 50 mL falcon tube. The centrifugation resulted in two or three phases: the sludge having an insoluble and unhydrolyzed source, the liquid layer having the protein hydrolysate, and the lipid layer. The falcon tubes were stored at -20°C overnight, and individual layers in falcon tubes were separated using a scalpel. The liquid layer was lyophilized, and the dry yield (percentage) and productivity (mg/mL) were determined using the equations given below. Until further use, the lyophilized protein hydrolysates were kept at -20°C.

$$\textbf{Yield}\ (\%) = \frac{\textbf{Weight of lyophilized protein hydrolysate}\ (\textbf{g})}{\textbf{Weight of raw material used}\ (\textbf{g})} \times \textbf{100}$$

$$\textbf{Productivity}\ \left(\frac{\textbf{mg}}{\textbf{mL}}\right) = \frac{\textbf{Weight of lyophilized protein hydrolysate}\ (\textbf{mg})}{\textbf{Reaction volume}\ (\textbf{mL})}$$

The hydrolysates were prepared in at least six replicates, with detailed information given in the table below:

Table 1: Specific reaction conditions for enzymatic hydrolysis of all substrates

Substrate	Water to substrate ratio (g/mL)	Enzyme added (% of substrate (g))	Hydrolysis time (hour)	Sources of substrate	Reference
Black soldier fly	1:3	2%	1	Chapul	(Batish et al., 2020)
Cricket	1:3	2%	1	Cricktone	(F. G. Hall et al., 2017)
Algae	1:10	2%	4	Mountain rose herbs	(Ritala et al., 2017; Tchorbanov & Bozhkova, 1988)
Pea	1:20	4%	4	NorCal organic	(J. Ding et al., 2020)
Oyster	1:3	1%	1	Anderson's neck oyster company	(He et al., 2020)
Mussel	1:3	2%	1	Graham and Rollin's	(Normah & Nurdalila Diyana, 2018)
Lugworm	1:1	2%	1	Wilcox Bait and Tackle	(Shin et al., 2015)

Mushroom	1:10	2.5%	4	Amazon fresh	(Ang & Ismail-Fitry, 2019)

Amino acid analysis, protein content and degree of hydrolysis

The total amino acid analysis was performed according to Association of Official Agricultural Chemists (AOAC) 982.30 E(a,b,c) (AOAC, 2006). Overall, after the digestion of samples completely with 6N HCl, an ion exchange chromatography is employed with post column ninhydrin derivatization and quantitation. The crude protein content was assessed using AOAC standard method, Kjeldahl (AOAC, 2006). The protein content was determined by multiplying the crude nitrogen content with nitrogen to protein conversion factor (Kp), the Kp was determined from literature and given as follows:

Table 2: Protein conversion ratio

Source of protein	**Protein conversion ratio**	**References**
Black soldier Fly	4.43	(Smets et al., 2021)
Cricket	5.00	(Ritvanen et al., 2020)
Algae (*chlorella vulgaris*)	6.35	(Safi et al., 2013)
Pea	5.36	(Mariotti et al., 2008)
Mushroom	4.7	(Mattila et al., 2002)
Animals (all marine invertebrates) and yeast	6.25	(Mariotti et al., 2008; Steckelberg et al., 2013)

The protein quality assessment was conducted using Digestible Indispensable Amino Acid Score (DIAAS) as recommended by FAO (FAO, 2011) using the following formula:

$$\textbf{100 x lowest value } \left(\frac{\textbf{mg of DIAA in 1g of protein}}{\textbf{mg of DIAA in 1g of reference protein}}\right) = \textbf{DIAAS \%}$$

where is reference protein used being amino acid pattern identified in young children.

The degree of hydrolysis was measured using the indirect formol titration method established by Sörensen and Taylor (Rutherfurd, 2010). A sample of 1.5g was weighed and dissolved 50mL of water with pH adjusted to 7. Then formaldehyde which was pre adjusted to pH 8.5 was added to the mixture and allowed to react for 5 minutes. Then the solution was adjusted to 8.5 using 0.1M NaOH and amount of NaOH was recorded. The % free amino acid groups and degree of hydrolysis were determined using the following equation:

$$\frac{\textbf{A x B x 14.007 x 100}}{\textbf{C x 1000}} = \textbf{\%free amino acid groups}$$

$$\frac{\textbf{D x 100}}{\textbf{E}} = \textbf{Degree of hydrolysis}$$

Where A, B, C, D and E were amount of NaOH used (mL), concentration of NaOH used (M), amount of sample used (g), %free amino acid groups and %nitrogen content respectively.

Evaluation of techno-functional properties of hydrolysates produced

Three techno functional properties were analyzed for all eight protein hydrolysates produced and yeast hydrolysate. All the experiments were conducted in triplicates.

Oil absorption capacity (OHC)

The oil absorption capacity was tested for all protein hydrolysates according to Shahidi (1995). First, 500 mg of protein hydrolysate was mixed with 10mL of pure canola oil. This mixture was left at room temperature for 30 minutes and gently mixed every 10 minutes. After the oil adsorption process was done, the mixture was centrifuged at 2500g for 10 minutes at room temperature, and free oil was removed using the pipette. The formulae given below calculated the oil adsorption, and the results were expressed as 1g of protein hydrolysate.

$$\textbf{Oil Holding capacity} = \frac{\textbf{Mass of sample (with oil)} - \textbf{Mass of dry sample(g)}}{\textbf{Mass of dry sample(g)}}$$

Emulsification capacity (EC)

The emulsification capacity was tested for all protein hydrolysates according to Yamsumatsu (1972). 500mg of protein hydrolysate(s) were mixed with 50mL of 0.1M sodium chloride solution in a 250mL conical flask at room temperature. After that, 50mL of pure canola oil was added, and the mixture was homogenized for 2 minutes to create an emulsion. The 25 mL volume of the freshly prepared emulsion made was transferred into three graduated cylinders and kept at room temperature for 10 minutes. The volume of the aqueous and emulsion phase was recorded, and the emulsification capacity was calculated according to the equation given below

$$\textbf{Emulsyfying capacity}\left(\textbf{EC}(\%)\right) = \frac{\textbf{Total volume} - \textbf{Aqueous volume}}{\textbf{Total volume}} \textbf{x 100}$$

Foaming capacity (FC)

The foaming capacity was tested for all protein hydrolysates, according to Pacheco (2008). A magnetic stirrer was used to disperse 750 mg of protein hydrolysate in 25 mL

distilled water in a 100 mL beaker for 10 minutes. Then, protein hydrolysate was homogenized for two minutes to promote aeration and foam formation. The volume increase during aeration was recorded, and the foaming capacity was calculated using the following equation.

$$\textbf{Foaming capacity}\left(\textbf{FC}(\%)\right) = \frac{\textbf{Aerated volume} - \textbf{Normal volume}}{\textbf{Normal volume}} \textbf{x 100}$$

Cell culture conditions and maintenance

The zebrafish embryonic stem cell (ZEMS2) was obtained from the American Type Cell Culture (ATCC) under dry ice conditions (130°C). The media used to culture cells initially was Lebovitz-L-15 media (L-15), Dulbecco's Modified Eagle Media (DMEM) and Ham's F12 Media (F-12 media) in ratio 15:50:35 respectively with buffering agents added 20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 0.18g/L sodium bicarbonate with 10% FBS. The cell in ampoule were thawed at 28°C and resuspended in 5% FBS in 9mL media in T-25cm2 for 30 minutes and then add up more FBS to make up 10% FBS. The cells were maintained in an incubator set at 28°C and regularly subcultured when they were confluent (80-85%). After some subcultures, the media was reduced to L-15 with HEPES and sodium bicarbonate to reduce the complexity of media.

The subculturing process was done when cells reached 80-85% confluency by rinsing the cells with PBS and adding Tryple express to detach cells from the flask surface. The flasks were kept at 28°C for 5 minutes to allow complete detachment and then the cells were transferred to 15mL falcon tube. The Tryple was neutralized by serum free media (L-15 media) and centrifuged at 130g for 8.5 minutes and a white pellet was obtained.

The supernatant was removed, and the pellet was resuspended in 10% serum containing media. The cell number and viability were analyzed using automatic cell counter and cell splitting was done based on that. The cells reached confluency in a week with media change once on the third day.

Cell growth, morphology and viability assays with various protein hydrolysate concentrations

To test the power of protein hydrolysates to replace serum various concentrations of protein hydrolysates were prepared – 10mg/mL, 1mg/mL, 0.1mg/mL, 0.01mg/mL and 0.001mg/mL. The protein hydrolysate samples were prepared by mixing them in filter sterilized (0.22um) water made at concentration of 100mg/mL and then further diluted when required.

The cell growth analysis was done for all nine reconstituted hydrolysates and each hydrolysate was replicated for four biological replicates and three technological replicate each. All 12 welled plates were seeded with 50,000/mL cells per well with 10% serum. The cells were incubated with 10% serum for 24 hours for even attachment of cells. After 24 hours, the media was changed to various concentration of protein hydrolysates and serum. The basal media composed of L-15 media with serum concentrations of 10%, 5%, 2.5%, 1% and 0% serum. All nine protein hydrolysates were added to all serum concentrations, and each experiment included serum controls containing no protein hydrolysate. The cell numbers and morphology were recorded every 24 hours and continued till three days using CKX-CCSW Confluency Checker by taking three images per well. The cell numbers were counted and specific growth rate and population

doubling time was calculated according to the formulas given below. All the hydrolysate conditions were tested in four biological replicates with three technical replicates each.

$$\frac{\mathbf{Ln(Cell\ count\ at\ 3rd\ day)\ /(cells\ seeded)}}{\mathbf{Time\ (hrs)}} = \mathbf{Specific\ growth\ rate}\ (\mu\ (\mathbf{hr^{-1}}))$$

$$\frac{\mathbf{Ln\ (2)}}{\mu} = \mathbf{Population\ doubling\ time\ (PDT\ (hr))}$$

The viability of cells was tested by PrestoBlue staining. After 72 hours, the reagent 100uL of PrestoBlue was added to every well and left to incubated at 28°C for two hours. After two hours, the reading was taken on the microplate reader at 570nm and 600nm absorbance and the dye reduction was calculated according to the following formula.

$$\mathbf{\%Reagent\ reduction} = \frac{\mathbf{(117216\ X\ A1) - (80586\ X\ A2)}}{\mathbf{(155677\ X\ N2) - (14652\ X\ N1)}}\mathbf{x\ 100}$$

where A1, A2, N1 and N2 are absorbance of sample wells at 570 and 600nm and media only wells at 570 and 600nm.

Fluorescent Imaging of cells

Concentrations of protein hydrolysates with excellent cell growth characteristics were chosen, cultured, and evaluated for fluorescence imaging. The cells were fixed for 10 minutes with 4% paraformaldehyde and rinsed twice with PBS to remove the paraformaldehyde. The cells were made permeable to dyes by treating them with 0.1% Tween, incubating them for ten minutes, and then rinsing them with PBS. To visualize cell nuclei, 25uL of Hoechst dye was diluted with PBS at a ratio of 1:2000, added to the cells, and incubated in the dark for 10 minutes. After incubation, the cells were washed twice with PBS, and 1 mL of PBS was added to each well prior to the addition of the second dye for cytoskeleton visualization. Each well was given two drops of actin green

dye, incubated for thirty minutes, and then washed twice with PBS. The cells were then suspended in a live cell imaging solution to improve the image quality. The cells were subsequently observed using a fluorescent microscope utilizing UV light with excitation/emission wavelengths of 361/486 nm and blue-cyan light with excitation/emission wavelengths of 495/518 nm, both independently and simultaneously.

Lactate Dehydrogenase activity

The lactate dehydrogenase activity of the most potent protein hydrolysate concentrations was evaluated for 2.5 and 1% serum concentrations. The cells were cultivated in 12 welled plates and potent protein hydrolysate concentrations like those stated in the previous section. After three days, the supernatant was harvested and centrifuged at 13500 rpm for 15 minutes at 4°C to remove any cell-based debris. Next, the supernatant was transferred to 96 welled plates, and the test was conducted following the instructions provided by the kit. Four biological replicates and three technical replicates per biological replicate were used for statistical reproducibility.

Statistical analysis

The statistical analysis was conducted using JMP 16 software. The data was tested for normality and homoscedasticity to confirm normal distribution. The normality was tested using Shapiro-wilk test and Normal Quantile Plots (NQP) and the even distribution of variability was tested using Levene’s test. If the data was found to be normally distributed, the One – Way ANOVA and Tukey's HSD (honestly significant difference) test was performed, otherwise the Wilcoxson/ Kruskal Wallis test and Steel

Dwass/Wilcoxson pair comparisons were done with their respective controls. The differences with $p<0.05$ were considered statistically significant.

Results and Discussion

The yield and productivity of the protein hydrolysate are described in the table given below.

Table 3: Percentage dry yield and productivity (mg/mL) obtained for all protein hydrolysates

Type of substrate	**Weight of hydrolysate**	**Dry Yield (%) wt/wt**	**Productivity (mg/ml)**	**Yield and productivity from literature**	**Reference**
BSFL	8.29 ± 0.23	16.57 ± 0.46	60 ± 0.00	10.7-6.9% 21 mg/mL. 12.1-12.4%	(Firmansyah & Abduh, 2019) (Abduh et al., 2020)
Cricket	8.34 ± 0.72	16.68 ± 1.45	60 ± 0.01	9.7 – 12.1% 5.7 – 11.2%	(Trinh & Supawong, 2021) (Hall et al., 2017)
Algae	3.57 ± 0.22	23.8 ± 1.48	20 ± 0.00		
Pea	2.97 ± 0.13	39.58 ± 1.74	20 ± 0.00	19.2 – 32.1%	(Maache-Rezzoug et al., 2011)
Mussel	4.89 ± 0.20	9.78 ± 0.40	30 ± 0.00	5.27 – 8.66 %	(Normah & Asmah,

				8.34%	2016) (Mohd Rodzi, 2015)
Oyster	4.7 ± 0.47	9.39 ± 0.95	30 ± 0.00		
Lugworm	15.02 ± 0.62	30.05 ± 1.24	100.16 ± 0.00		
Mushroom	0.43 ± 0.03	5.78 ± 0.35	2.9 ± 0.00	3.2 – 3.6 %	(Ang & Ismail-Fitry, 2019)

For production of a hydrolysate that properly supports animal cell culture growth, the whole protein rich substrate was used instead of isolating protein concentrates. A complete substrate hydrolysis provides not only peptides and amino acids but other molecules such as vitamins, lipids, minerals, and inorganic acids that could support the growth of animal cells. The productivity and yield of protein hydrolysates were examined to determine their potential for large-scale manufacturing. The yield and productivity values for black soldier fly, cricket, pea, mussel, and mushroom hydrolysates were higher or comparable to those found in the literature. We could not find yield and productivity estimates for algae hydrolysates, oysters, and lugworms as they were not estimated with respect to the added raw material. As was later discovered in this investigation, the concentration of protein hydrolysate required for animal cell culture applications is 0.1-0.001 mg/ml, and the yield and productivity appear favorable for cell culture applications. Culturing meat will require the cultivation of billions of cells using limited space, time, and resources. One 2L batch of any protein hydrolysate would be sufficient to supply the maximum capacity stirred bioreactor used for animal cell culture, which has

a capacity of 2000L and is used to produce animal cells. Theoretically, a 2000L bioreactor can produce $4x10^{12}$ cells from a 2L batch of a protein hydrolysate, and this bioreactor can produce between 10 and 100 kg of meat (Bellani et al., 2020).

Amino-acid analysis, protein content and degree of hydrolysis,

The amino acid profile (g/100g of sample) with other parameters are given in table 4.

Table 4: Amino acid content, nitrogen content, protein content and quality and degree of hydrolysis of all hydrolysates

Amino acid (g/100g of sample)	Yeast	BSFL	Cricket	Algae	Pea	Oyster	Mussel	Lugworm	Mushroom
Phenylalanine	2.71	1.99	2.12	3.01	4.24	1.51	1.32	1.99	0.87
Valine	3.87	4.02	4.03	4.72	4.32	2.21	2.02	2.55	1.68
Threonine	2.78	2.41	2.91	3.69	2.94	1.91	2.04	2.16	1.62
Tryptophan	0.84	0.79	0.57	0.80	0.65	0.36	0.43	0.50	0.38
Methionine	1.05	0.85	1.25	1.56	0.75	1.07	0.88	1.13	0.31
Leucine	4.68	4.09	5.16	6.16	6.76	2.91	2.77	3.66	1.66
Isoleucine	3.42	2.72	2.84	2.81	4.03	1.98	1.84	2.34	1.36
Lysine	4.90	4.85	5.27	5.16	6.43	3.11	3.03	4.02	1.62
Histidine	1.31	1.85	1.69	1.44	2.05	0.83	0.87	1.14	0.61
Taurine §	0.02	0.10	1.20	0.03	0.03	0.87	3.50	0.78	0.03
Hydroxyproline	0.00	0.00	0.20	0.23	0.05	0.58	0.28	0.31	0.28
Aspartic Acid	**6.01**	**5.51**	**8.01**	**7.17**	**9.92**	**3.95**	**4.43**	**5.05**	**2.32**
Serine	2.46	2.64	3.81	2.87	3.99	1.79	1.94	1.90	1.23
Glutamic Acid	**11.29**	**8.51**	**10.52**	**9.73**	**15.62**	**4.81**	**5.95**	**6.71**	**2.24**
Proline	**2.29**	**3.94**	**3.98**	**3.78**	**3.44**	**1.60**	**1.77**	**2.06**	**0.74**
Lanthionine §	0.11	0.22	0.20	0.13	0.21	0.15	0.13	0.22	0.00
Glycine	**2.93**	**3.23**	**4.33**	**4.42**	**3.28**	**2.09**	**3.77**	**2.70**	**0.98**
Alanine	**4.58**	**4.43**	**5.87**	**6.59**	**3.57**	**2.25**	**2.85**	**4.62**	**1.84**
Cysteine	0.71	0.49	0.59	0.79	0.55	0.58	0.61	0.66	0.19
Tyrosine	0.82	3.53	2.65	2.53	2.84	1.68	1.48	1.61	0.57
Hydroxylysine	0.15	0.04	0.04	0.00	0.00	0.03	0.09	0.00	0.00
Ornithine §	0.49	0.08	0.16	0.08	0.07	0.60	0.11	0.22	0.88
Arginine	3.02	3.47	5.76	4.82	7.44	2.91	2.82	3.51	1.20
Total amino acid content	60.39	59.70	73.12	72.48	83.14	39.70	44.88	49.80	22.61
Nitrogen content	11.19	9.92	13.91	13.08	13.82	7.15	8.68	9.06	5.47
Protein content	69.91	43.95	69.55	83.03	74.05	44.69	54.25	56.59	25.71
DIAAS score (%)	93.25	140.84	96.42	86.72	65.02	92.31	80.18	97.85	72.03
Protein quality	Good	Excellent	Good	Good	Low	Good	Good	Good	Low
Degree of hydrolysis	40.91	19.77	18.24	20.00	15.32	33.52	27.61	31.28	46.09

All amino acids contained a high concentration of amino acids with known cell-growth promoting properties, such as alanine, glycine, proline, and aspartic acid (D. Ding et al., 2019; M. Lu & Zhao, 2018). All protein hydrolysates were rich in glutamic acid or glutamine, which is also known to play an essential role in animal cell culture. Glutamic

acid can be converted into glutamine, which is a significant source of nitrogen and a source of nitrogen for de novo amino acid synthesis, which contributes to protein and nucleic acids. In addition to providing skeletons for carbon and nitrogen biosynthesis, glutamine is a significant replenisher of metabolites in the TCA cycle. As a result, glutamine contributes to the cell's overall energetics and growth. Glutamine is the most abundant amino acid in tissue culture media and contributes to the high growth potential of cells (Hosios et al., 2016). Hydrophobic amino acids glycine, alanine, valine, and leucine were also found to be dominant amino acids, suggesting the presence of bioactive peptides that can have potential growth augmenting activities. Except for pea and mushroom protein hydrolysates, all protein hydrolysates have a high protein quality measured by their DIAAS content of essential amino acid content. However, black soldier Fly was found to have the best amino acid profile of all the hydrolysates, making its protein the highest quality.

Degree of Hydrolysis (DH) is one of the basic parameters that describe the properties of a protein hydrolysate, indicating the percentage of peptide bond cleaved. Yeast and mushroom hydrolysate were found to have similar higher DH in the range of 40-46.1%. Insect, plant and algae-based hydrolysates had lower degree of hydrolysis between 15-20%. The bivalve mollusks were shown to be in the mid-range of 25-35%. All the hydrolysates were found within the range as provided by other studies. Overall, protein hydrolysates were successfully produced, as indicated by the DH range values that aligned with those reported in the literature. Typically, the DH of protein hydrolysates can range from 5% to 100%; our protein hydrolysates fall within the range of 15% to 46%, indicating successful generation of protein hydrolysates (Bush & Taylor, 2014).

(Bush & Taylor, 2014; e Silva & Silveira, 2013). DH can influence the number of peptides released and their size, conformation, and amino acid sequence, which imparts various functional and bioactive properties to the hydrolysate product (F. G. Hall et al., 2017). (Monaya et al., 2022).

Techno-functional properties

The Fig. below summarizes various functional properties for all the protein hydrolysate produced.

Oil Holding Capacity (OHC) potentially can be tied to various positive attributes of protein hydrolysates that can be useful in media formulations for animal cell cultures. The culture medium comprises several hydrophobic components, such as insulin, growth factors, hormones, and carrier proteins, which are not water-soluble,, while, are essential for cell growth, survival, and proliferation (Bhatia et al., 2019; Freshney, 2016). Due to their rigid structure and non-polar chains, lipid-based components such as sterols, cholesterol, and fatty acids are difficult to solubilize in cell media. Lipids must directly provide them in the culture medium because they build membranes, store and transport nutrients, and play a role in signal transduction, but specific cells cannot produce them (Achouri et al., 1998; Yao & Asayama, 2017). Hydrolyzing proteins expose their network and hydrophobic amino acids, which aid in the trapping of oil and potentially oil-based components, thereby boosting oil holding capacity and aiding in the solubilization and stabilization of these oil-based components (Schartl, 2014). In addition, a high oil-holding capacity is associated with increased hydrophobicity and hydrophobic amino acids, which indicates the existence of cell-proliferating bioactive peptides in the hydrolysate (Hou et al., 2017). Produced insect-based protein hydrolysates ranged from

3.54 to 4.83 g/g, which was within the range of previously reported for black soldier fly and cricket. OHC of the black soldier fly and cricket was previously reported in the range of 0.8-5.2 g/g (Mshayisa et al., 2022; Purschke et al., 2018; Wang et al., 2021) and 1.42-3.5 g/g (Leni et al., 2020; Stone et al., 2019; Zielińska et al., 2018), respectively. Hydrolysates derived from fungi in our studies varied between 2.74 and 3.03 g/g. Han et al., 2016 and Wong & Cheung, 2005 reported more oil binding capacity than we observed for mushroom hydrolysates, however Ming et al. (2015) reported a lower oil retention capacity of 1.42 to 2.87. The range of yeast hydrolysate reported by Bertolo et al., 2019 was 4.28-8.44 g/g, which was higher than our experimental results but equivalent to or superior to hydrolysates produced by other authors (Dunuweera et al., 2021; Vaithanomsat et al., 2022). The pea hydrolysate produced by us had a higher oil holding capacity than reported in the literature, which was 1.07-2.7 g/g (Ma et al., 2022; Shen et al., 2022; Stone et al., 2015). Our algae hydrolysate fell between 0.92 and 6.00, consistent with the previously reported literature (Ben Atitallah et al., 2019; Bleakley & Hayes, 2021; Waghmare et al., 2016). The oil-holding capacity of the bivalve mollusks was also in the range of 1.5-2.48 g/g (Ismail et al., 2014; YEE & AMIN, 2020), which is consistent with other studies; however, Liu et al., 2021 reported a higher range of 5-5.2 g/g. To our knowledge, no techno-functional properties of Lugworms or any comparable annelids have been evaluated. An increase in the oil-binding capacity of our hydrolysates may result from an increase in hydrophobic amino acids exposed to enzymatic protein structure breakdown. The existence of several native non-polar side chains that were initially buried inside the protein's core may bind the hydrocarbon chains of lipids, resulting in increased oil absorption (Ashaolu, 2020). The decrease in OHC is attributable

to the varying surface hydrophobicity or hydrophilicity of native proteins, which can be easily modified by the synthesis method and/or hydrolysis conditions. In addition, excessive hydrolysis contributes to the hydrolytic destruction of protein structures and the reduction of hydrophobic interactions (Amiza et al., 2012; Wasswa et al., 2007).

Emulsifying capacity is also another attractive capability that have industrial application when it comes to producing cultured meat production. Culturing cells require many water and oil based components to ensure proper cell growth, proliferation and sustenance, however presence of these opposites groups this makes the culture media thermodynamically unstable (McClements, 2004). Now, emulsion capacity of various components can help solubilization of these opposite groups and allow their transfer to the cells when required. Hydrolysis of protein exposes many amphipathic, polar and non-polar side chains that can help solubilize oil based components and create stable emulsions so that all necessary components that can be utilized by cells when required (Padial-Domínguez et al., 2020). Insect hydrolysates produced had emulsifying capacity in the range of 57.8-60% which is within the range of values reported in literature 39-98% (Adebowalea et al., 2005; Mshayisa et al., 2022; Trinh & Supawong, 2021). Yeast hydrolysate produced in our lab were around 60.4% which was higher or equivalent to values found by other researchers (Bertolo et al., 2019; Costa et al., 2012). However, mushroom hydrolysate produced had lower emulsification capacity than reported by literature which was in the range of 51.67-71% (Han et al., 2016; Ho et al., 2020; Wong & Cheung, 2005). Algae hydrolysate had emulsification capacity in the range of 56.32-62% as found in other studies (Bashir et al., 2016; K. Lu et al., 2019; Ursu et al., 2014). The EC of bivalve mollusks fell consistently in the range of 35-65% as found by other

studies (Haldar et al., 2018; Naik et al., 2020). The pea hydrolysate produced by us had a higher EC than reported in the literature which was in the range of 31-65 (Betancur-Ancona et al., 2014; del Mar Yust et al., 2010; Periago et al., 1998). An increase in emulsifying capacity is usually related to the amphipathic nature of peptides that are produced by hydrolysis. It is widely reported that peptides higher than the size of 2 kDa have a higher emulsifying capacity as they may possess both hydrophobic and hydrophilic amino acid chains that can interact with both water and oil-based components. However, lower emulsifying capacity can be attributed to the production of extremely short peptides, which reduce the overall amphipathic nature of hydrolysate.

Foaming capacity is a significant function feature for many food-based products, adding to texture or appearance. For example, bubbles define the texture of aerated commercial products, including bread, ice cream, mousse, and meringues. (Ellis & Lazidis, 2018). Foaming in a bioreactor during processing is a typical occurrence. However, it can cause significant issues, such as inhibiting cell development by limiting the surface area contact between the growth media and the bioreactor headspace and decreasing oxygen transfer rates. In addition, foam accumulation in excess can clog filters and cause vessel overpressures that exceed allowable limits. As a result, hydrolysates should not increase the foaming that occurs spontaneously in a bioreactor when added to media. Insect based hydrolysates produced by us were in the range of 4-20% which is extremely low as compared to the ones found in literature which were range of 39-100% (F. G. Hall et al., 2017; Mshayisa et al., 2022). Algae hydrolysate by us had lower foaming capacity than reported by Waghmare et al., 2016 which was 93-96% and higher than some other reports (Bashir et al., 2016; Gereniu et al., 2017). The bivalve mollusks had lower foaming

capacity than reported in literature which is in the range of 51.1-160% (Chatterjee et al., 2020; Jin et al., 2012). Yeast hydrolysate had a higher foaming capacity than reported in literature which were in the range of 0.5-10.9, (J. M. Kim et al., 2003; H. J. Lee et al., 2015) however, the other fungal based hydrolysate i.e. mushroom had lower foaming capacity than reported in studies (Ishara et al., 2018; Kamran, 2017). Pea hydrolysate had lower FC than reported by (Ma et al., 2022) but higher some as reported by others (Ladjal Ettoumi & Mohamed, 2015; L. Tang et al., 2012). Foaming qualities are generally imparted by partially denaturing a globular protein as hydrophobic regions are exposed. These regions can efficiently adsorb into air-water interfaces and lower interfacial tension, thus enhancing foaming capability. However, extensive denaturation will reduce proteins' ability to produce foams (Mauer, 2003).

Overall, cricket, pea and algae had highest oil holding capacity and mussel and oyster had the lowest. Lugworm had the highest emulsification capacity whereas cricket had the lowest. Algae had the highest foaming capacity and insect-based hydrolysates had the lowest. Overall – black soldier fly seems to have the best techno functional properties as a media component having satisfactory oil holding and emulsifying capacity but lower foaming capacity.

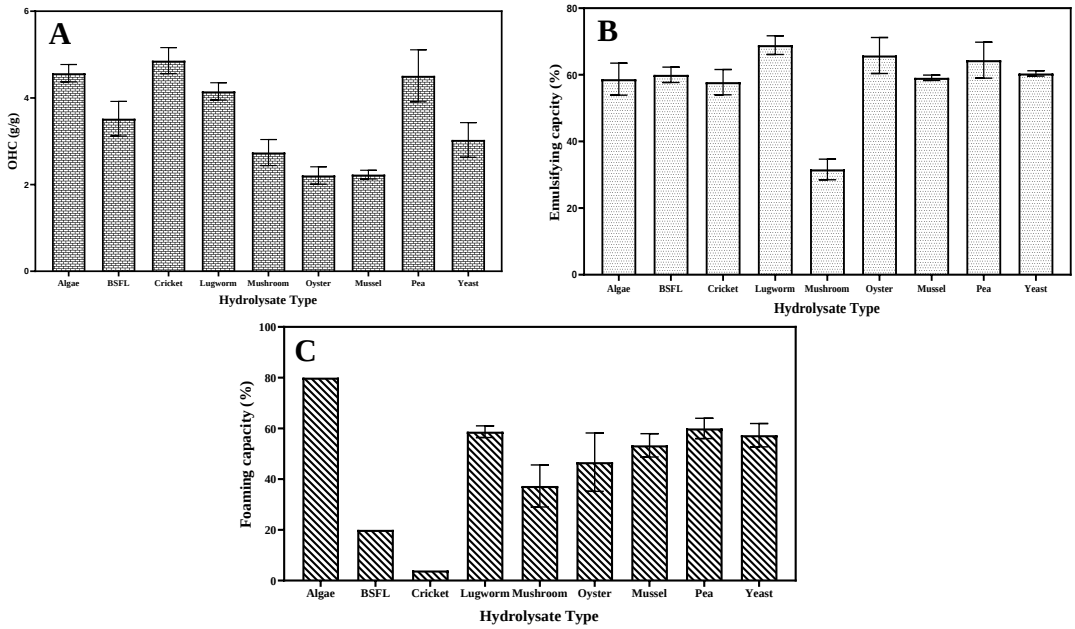


Figure 1: Techno-functional properties of all hydrolysates obtained after various tests; (A) Oil holding capacity, (B) Emulsifying capacity and (C) Foaming capacity

Cell morphology, growth, and viability

The serum is a concoction of various nutrients and growth- and survival-inducing supplements; yet many hurdles prevent its use in cellular agriculture. Due to ethical concerns, batch-to-batch variation, and the transmission of zoonotic illnesses, cellular agriculture is attempting to eliminate the use of serum. In addition, serum deficiency is known to cause cell morphological aberrations, halt growth and induce apoptosis. In this research, we attempted to reduce and replace serum with nine different protein hydrolysates, evaluating their influence on cell growth, morphology, and viability to establish whether these protein hydrolysates can reduce and potentially replace serum.

Impact of serum concentration

For zebrafish ESC's, 10% serum is the gold standard requirement for proper growth of cells and for our experiments we tried reducing the serum to 5 and 0% to see the impact on cell growth and morphology parameters. The impact of serum at various

concentrations on many parameters of cell growth and viability is demonstrated in the Figure 2 and Table 5 below.

Table 5: Comparable or superior growth rate and doubling time of protein hydrolysate conditions to the 10 % control.

Conditions	**Growth Rate (hr^{-1})**	**Population Doubling time (hrs)**
10% Serum	0.05±0.001	14.85±0.28
5% Serum	0.04±0.001	16.19±0.32
0% Serum	0.02±0.001	46.12±9.35
5% Serum + 0.001mg/mL Algae hydrolysate	0.05±0.001	14.42±0.43
5% Serum + 0.001mg/mL BSFL hydrolysate	**0.05±0.001**	**12.92±0.22**
5% Serum + 0.1mg/mL Lugworm hydrolysate	0.05±0.001	14.00 ±0.43
5% Serum + 0.001mg/mL Mushroom hydrolysate	0.05±0.001	14.68±0.38
5% Serum + 0.001mg/mL Mussel hydrolysate	0.05±0.001	14.94±0.44
5% Serum + 0.001mg/mL Oyster hydrolysate	0.05±0.001	14.76±0.45
5% Serum + 0.001mg/mL Pea hydrolysate	0.05±0.001	14.43±0.43
5% Serum + 0.001mg/mL Yeast hydrolysate	0.05±0.001	14.59±0.42

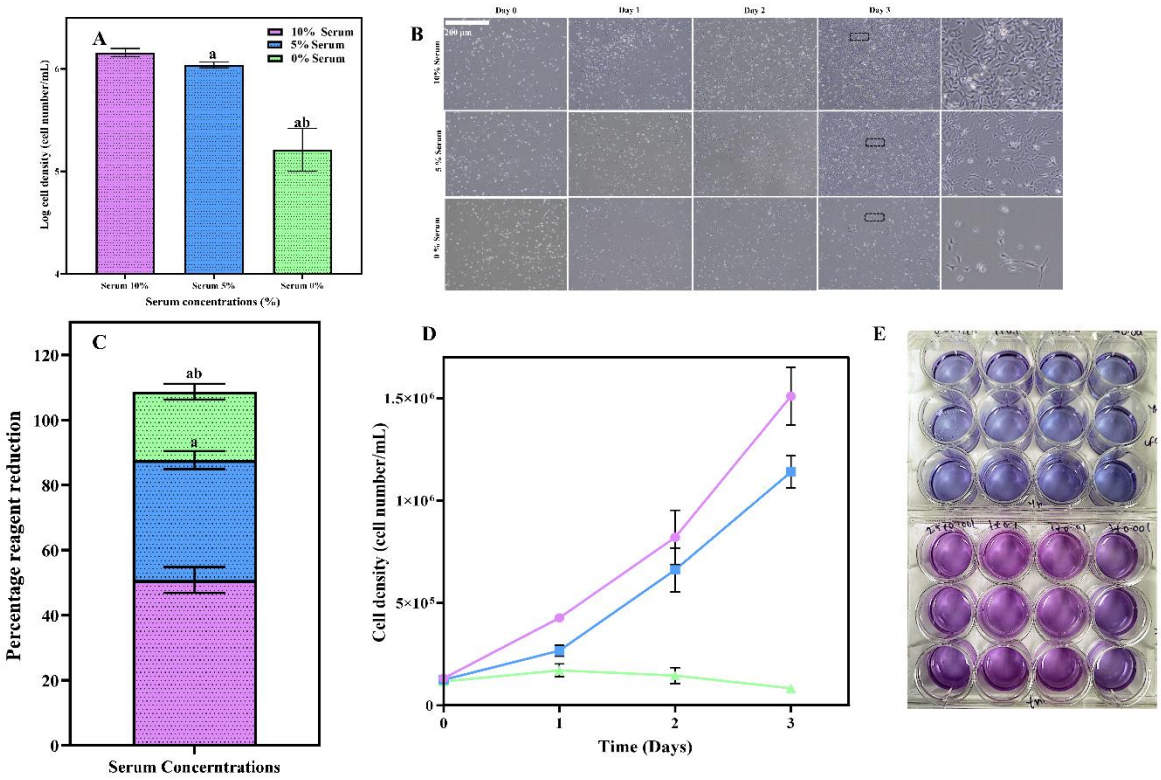


Figure 2: Cell growth parameters of ZEM2S cells at various serum concentrations obtained by imaging analysis. (A). Log transformed cell density at 3rd day for serum

concentrations at 10, 5 and 0%; a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 5% control. B) Micrographs of ZEM2S cells obtained on all four days at 10X (200μm) magnification. (C) Cell viability analysis of all protein hydrolysates for serum concentrations 0-10% using PrestoBlue dye. (D) Overall growth curve of ZEM2S cells for four days at different serum concentrations. (E) Color change of dye with presence of metabolically active cells from resazurin (indigo blue) to resorufin (magenta).

The growth and morphology of the cells were observed every day for three days using a phase-contrast microscope equipped with image analysis software. Direct image analysis is a dependable and accurate technique that has been demonstrated to be equivalent to other cell counting methods (Brinkmann et al., 2002; Farges-Haddani et al., 2006). After 24 hours of incubation in media containing 10 % serum, the test media were changed to 10, 5, and 0 % serum, respectively. As the serum concentration decreases, the percentage of cells decreases significantly from 10% to 0%. As the serum concentration decreased from 10% to 5%, cells exhibited a significant decrease, but not many morphological changes were observed. Compared to media containing 10 % serum, cell growth with 5% media decreased from approximately 1.4×10^6 to 1×10^6, representing an overall log of 0.1 reduction. There was no difference in cell morphology between the 5% and 10% positive control, demonstrating that although cells are affected by serum reduction, they retain their original morphology. At a serum concentration of 0%, there was a dramatic decrease in the number of cells, and morphological aberrations were evident. With a considerable logarithmic reduction of approx. 1, the number of cells decreases from 1.4×10^6 to 5×10^5 with an increase in rounded cells. Cell viability was measured using resazurin based assay called PrestoBlue which relies on the reducing environment produced by the cells which can be directly equated to cell viability factor. PrestoBlue is non-toxic, water soluble, fast and sensitive method for detection of viable cells. Viable

cells usually have active metabolic profile which includes that actively produces NADH creating a reducing microenvironment and reducing rezadurin to resorufin changing into from indigo blue to purple that can be quantitatively measured using absorbance and fluorescence. The cell viability analysis overall had high plate to plate variability since the measurement basis was absorbance based which is not highly accurate and sensitive. Overall, the % dye reduced trend followed similar trend as the cell growth parameters measured by image analysis. The 10% serum control had highest dye reduction of 50% indicating highest metabolic activity which reduced to approx. 37% at 5% and 21% at 0% serum concentration. As the serum concentration decreases the cell viability also significantly decreases.

ZEM2S are known to have a fibroblast or spindle-like shape; any deviation from this indicates stress-induced alterations. Overall, complete serum starvation causes an increase in dead cell debris, the loss of smooth spindle-like structure, formation of an irregular elongated spiky shape, the cytoplasm appears sparse, and the cells appear to be under nutritional stress and grow at a slower rate, as is well documented in the literature (Pirkmajer & Chibalin, 2011; Terra et al., 2011). Serum starvation is classified as an environmentally induced stress that triggers cell apoptosis and death; however, its effects vary from cell to cell. Even though serum starvation has been utilized in numerous molecular mechanistic research, its effects are not as well known or documented, providing an opportunity to fill this gap with a daily description of cell shape and growth alterations (Rashid & Coombs, 2019).

The growth rate and population doubling time of ZESC's at 10% serum concentration were observed to be 0.05/hr. and 14.85 hrs. respectively which is in accordance with

various established stem cell lines (Guan et al., 2019; Tamm et al., 2013; Zhan et al., 2019). With decrease in serum the specific growth rate decreased and population doubling time significantly increased indicating decrease in proliferation efficiency. The highest doubling time observed was that of media having 0% serum which was around 46 days.

For all our studies conducted on separate days with various protein hydrolysates, the, positive, internal, and negative controls were 10, 5, and 0%, respectively. Since there was no significant difference between the cell growth parameters for these controls on separate days, they were averaged and compared to all concentrations of protein hydrolysates at different serum concentrations.

Protein hydrolysates (10mg/mL and 1mg/mL) with various serum concentrations

The cell morphology, growth and viability parameters of zebrafish cells using the nine hydrolysates at 10 and 1mg/mL highest concentrations is shown in the figure 3 and 4 below.

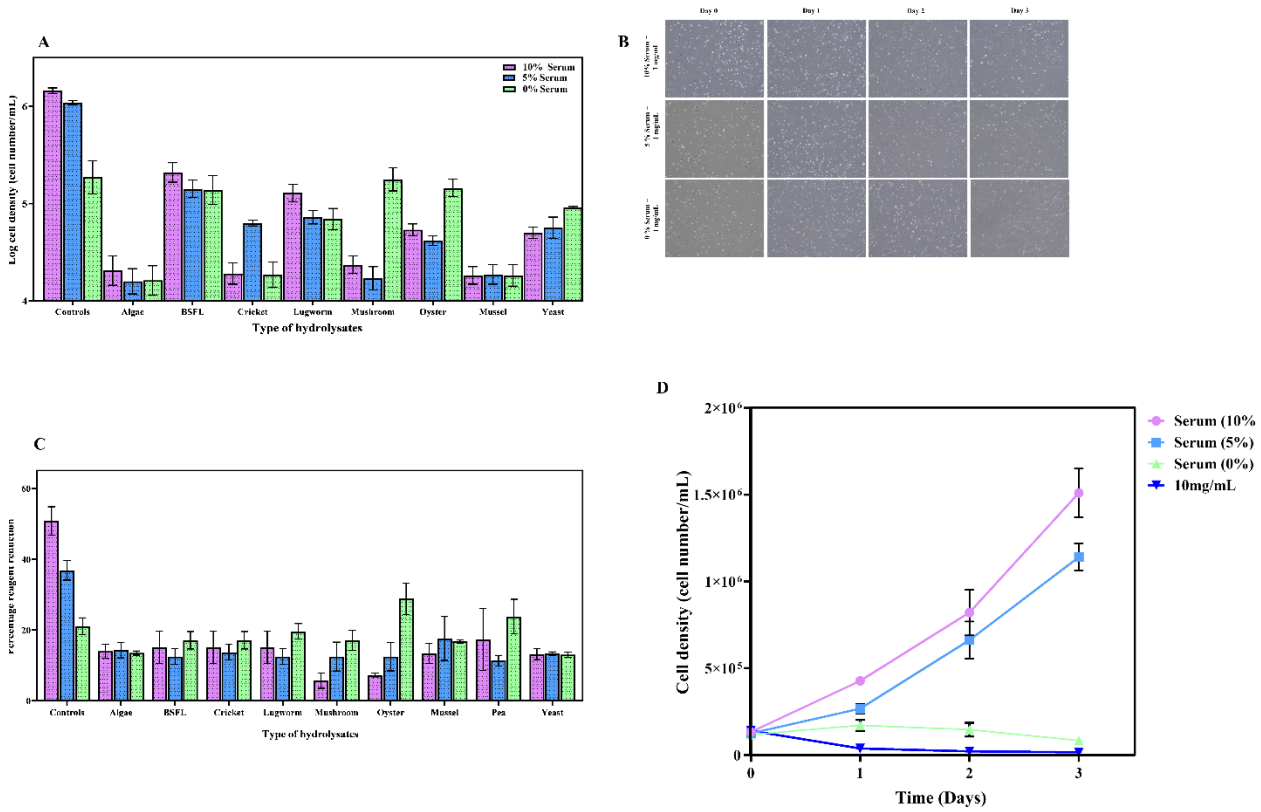


Figure 2: Cell growth parameters of ZEM2S cells at various serum concentrations with multiple protein hydrolysates at 10mg/mL concentration obtained by imaging analysis. (A) Log transformed cell density at 3rd day for all serum conditions with 10 mg/mL concentration of protein hydrolysates. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage dye reduced by cells grown with all serum conditions using all protein hydrolysates at 10 mg/mL concentration. (D) Overall growth curve of ZEM2S cells for four days for all serum conditions and 10% serum with 10 mg/mL hydrolysate (algae taken as representative).

The highest concentration of all protein hydrolysates negatively impacted cell growth at all serum concentrations. In addition, within 24 hours of adding a medium containing protein hydrolysate(s), the cells lost their fibroblast-like morphology, and cell death was induced as they assumed a rounded shape. The addition of this protein hydrolysate concentration decreased the medium's pH despite the existence of a buffering mechanism, as the oxidation of phenol red caused the yellowing of the medium. This result is

comparable to two experiments conducted with soy (Y. K. Lee et al., 2008) and fish gelatin protein hydrolysates (Hsieh et al., 2022) in which a higher dose of 4 to 6 mg/mL significantly decreased cell proliferation. Wheat gluten hydrolysate at concentrations between 6 and 12 mg/mL reduced cell proliferation, according to a study done by Radošević et al., 2016. Protein hydrolysates derived from various industrial wastes such as eggshells and carcasses at 10 mg/mL had a similar dose-dependent, cytotoxic effect on bovine stem cells in a separate study (Andreassen et al., 2020). This can be ascribed to the presence of a high concentration of nutrients, such as peptides, which disrupt the overall nutritional balance of the medium (Hsieh et al., 2022) and thus drastically alter its pH. The specific growth rate and population doubling time nearly approached negative levels as a result of the death of the cells. The 10mg/mL concentration of all hydrolysates appears to have a detrimental, almost cytotoxic effect on cells.

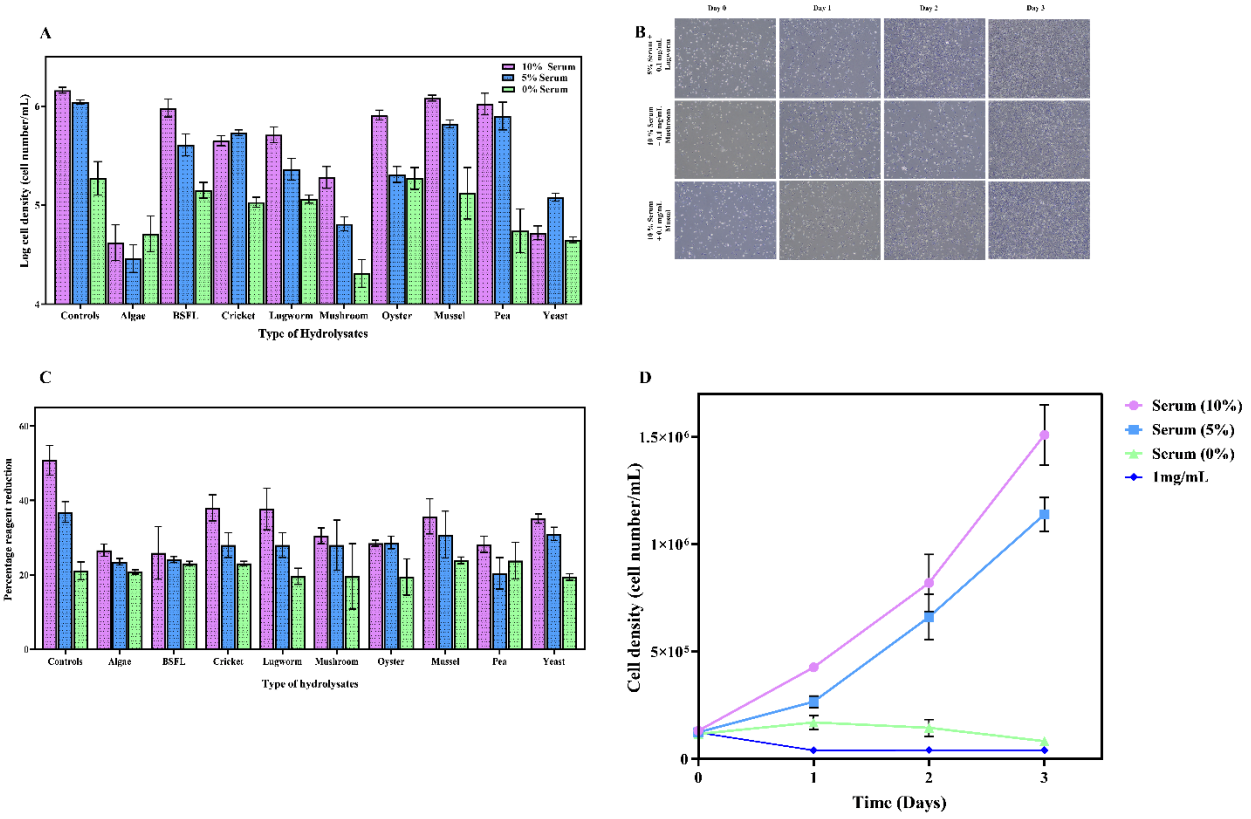


Figure 3: Cell growth parameters of ZEM2S cells at various serum concentrations with multiple protein hydrolysates at 1mg/mL concentration obtained by imaging analysis. (A) Log transformed cell density at 3rd day for all serum conditions with 1 mg/mL concentration of protein hydrolysates. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage dye reduced by cells grown with all serum conditions using all protein hydrolysates at 1 mg/mL concentration. (D) Overall growth curve of ZEM2S cells for four days for all serum conditions and 10% serum with 1 mg/mL hydrolysate (algae taken as representative).

The effects on cell growth and morphology were comparable to those observed at 10 mg/mL concentrations but emerged slightly more favorable. Aside from algae, mushrooms, and yeast, the other hydrolysates appear capable of sustaining the growth and morphology of cells at 10 and 5 percent serum concentration; however, none of them were significantly higher or comparable to the positive control, indicating once more a negative or insignificant impact on cell growth parameters. Any protein hydrolysate at a serum concentration of 0% did not significantly stimulate cell growth, even compared to the negative control. This result is comparable to Andreassen et al., 2020, in which 1 mg/mL exhibited cytotoxic effects on cells. The causes are likely dose-dependent effects on cell growth comparable to 10 mg/mL. The specific growth rate and population doubling time were still significantly lower or negative than the 10% control, indicating that a 1mg/mL concentration of any protein hydrolysate solution was unable to augment, reduce, or replace serum.

Protein hydrolysates (0.1mg/mL) with various serum concentrations

The effect of 0.1mg/mL protein hydrolysates was substantially more positive than that of 10-1mg/mL protein hydrolysates, which were cytotoxic. In some instances, the cells appear to proliferate and survive better in the presence of 0.1 mg/mL of protein hydrolysate(s) than in a 10% positive control. The figure summarizes the effects of

several protein hydrolysates at varying serum concentrations on cell growth and morphology.

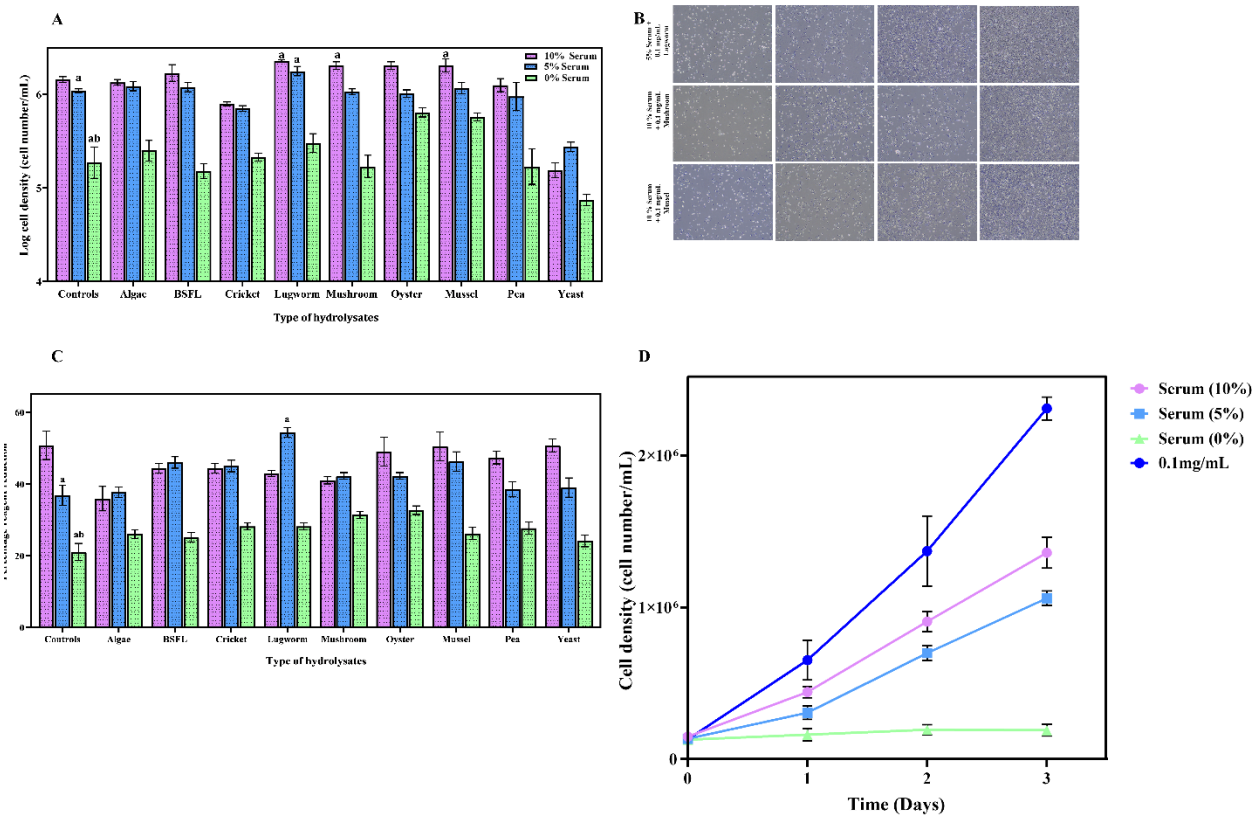


Figure 4: Cell growth parameters of ZEM2S cells at various serum concentrations with multiple protein hydrolysates at 0.1mg/mL concentration obtained by imaging analysis. (A) Log transformed cell density at 3rd day for all serum conditions with 0.1 mg/mL concentration of protein hydrolysates. a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 5% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage dye reduced by cells grown with all serum conditions using all protein hydrolysates at 0.1 mg/mL concentration. (D) Overall growth curve of ZEM2S cells for four days for all serum conditions and 10% serum with 0.1 mg/mL hydrolysate (Lugworm taken as representative).

At 10 percent serum concentration, lugworm, mussel, and mushroom exhibited much greater cell proliferation and stable morphology than 10 % control, demonstrating that the presence of hydrolysates boosts the efficacy of serum itself. Compared to the 10 % control, the specific growth rate and population doubling time were dramatically

improved. On CHO-K1 cell lines with cottonseed hydrolysate, this synergistic impact of protein hydrolysates with other medium components was demonstrated by increased cell biomass (Babcock & Antosh, 2012). Protein hydrolysate has been demonstrated to have more than a nutritional effect on cells, including protective, antiapoptotic, and growth-promoting properties. This conclusion was reinforced by a study by Burteau et al., 2003 on CHO cell lines using several plant peptones, which revealed that adding these hydrolysates did not increase the nutritive value of proteins, but dramatically boosted cell growth. Similar effects were reported when CHO cells were grown with 10% serum and algal extracts (Ng et al., 2020). The hydrolysates of pea, yeast, and cricket appear to have a detrimental effect on cell growth in the presence of 10 percent serum, as cell growth, specific growth rate, and population doubling time were all reduced. The addition of oyster, black soldier fly, and algae hydrolysate with 10% serum did not significantly affect the growth parameters. Overall, the three protein hydrolysates appear to positively influence and augment the cell growth function of 10% serum, indicating that they contain growth-stimulating compounds that can work synergistically with the serum to improve its function.

At 5 % serum concentration, lugworm hydrolysate demonstrated significantly higher growth characteristics than the 10 % control, demonstrating that the hydrolysate can augment and restore up to 50 percent of serum's function. The hydrolysate of mussels and algae replenished the lost serum, as the growth parameters were not significantly different from the 10% control. A similar concentration range of wheat hydrolysate (0.01 – 0.5) boosted osteoblastic cell line proliferation, viability, and alkaline phosphatase activity (Jo et al., 2020). The other five hydrolysates exhibited less cell proliferation than

the 10% control and had a detrimental effect on serum, demonstrating antagonistic activity with serum.

At 0% serum concentration, every hydrolysate showed a lower cell growth parameter than the 10% and 5% controls, indicating that none of the protein hydrolysates could not substitute serum at 0.1mg/mL concentration. However, the cell growth of five of the hydrolysates (algae, cricket, lugworm, mussel, and oyster) was greater than that of the 0% serum control, demonstrating that these hydrolysates contain growth-promoting properties, although not as potent as serum. Similar results were observed on bovine cells with algal extract and various other industrial byproduct hydrolysates; cell growth increased relative to serum-free conditions, albeit less than in serum-rich conditions (Andreassen et al., 2020; Defendi-Cho & Gould, 2021). In addition, the cells' morphology was drastically affected, as they had a thin, spiky, elongated appearance, indicating that protein hydrolysates may have growth-promoting properties but could not maintain the integrity of cells. Only lugworm hydrolysate exhibited significantly higher cell viability at 0.1 mg/mL concentration at 5% serum concentration which is in corroboration with the cell numbers and other parameters discussed above with percentage reduced dye at 54%. The lugworm hydrolysate also showed growth rate and population doubling time statistically similar to 10% control indicating successful serum replacement.

Protein hydrolysates (0.01mg/mL) with various serum concentrations

The effect of 0.01 mg/mL of protein hydrolysate seemed similar to that of 0.1 mg/mL, as the growth parameters and morphology of the cells were comparable to those of the 10% control group.

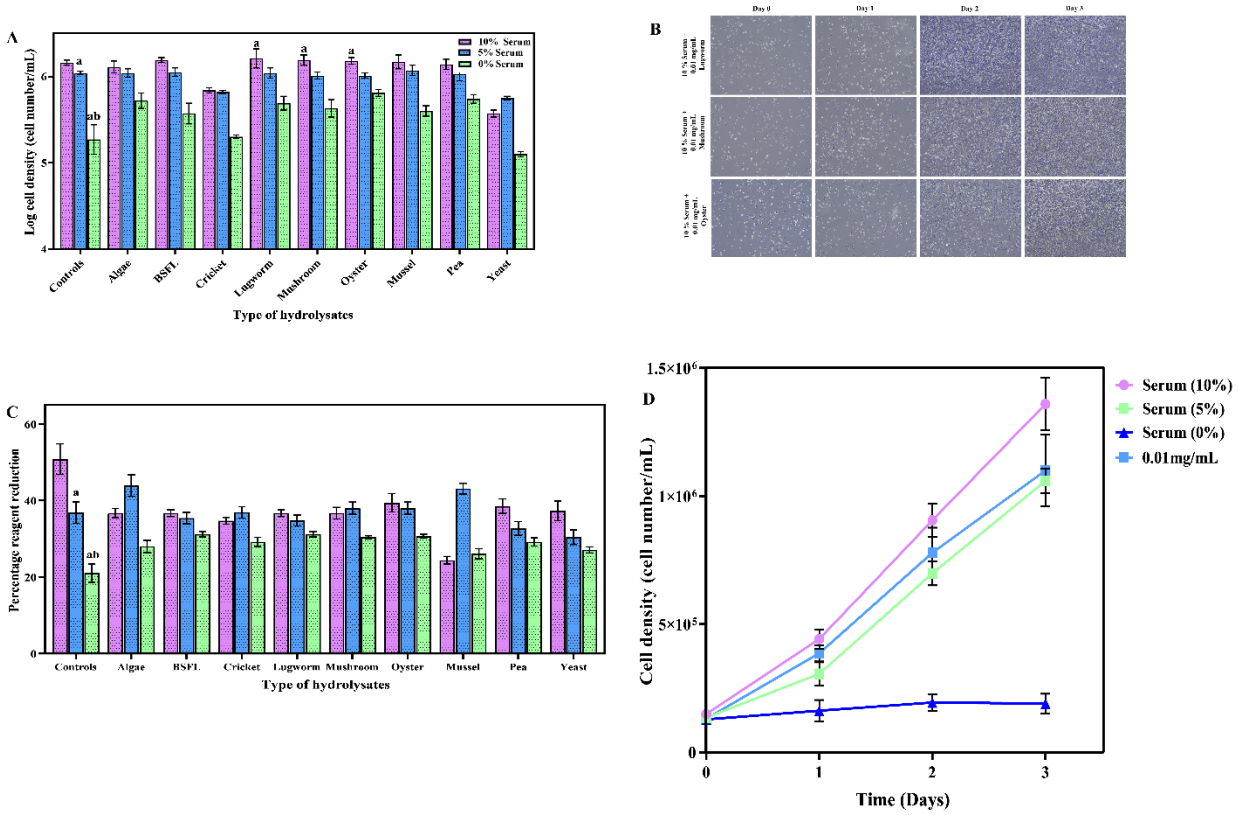


Figure 5: Cell growth parameters of ZEM2S cells at various serum concentrations with multiple protein hydrolysates at 0.01mg/mL concentration obtained by imaging analysis. (A) Log transformed cell density at 3rd day for all serum conditions with 0.01 mg/mL concentration of protein hydrolysates. a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 5% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage dye reduced by cells grown with all serum conditions using all protein hydrolysates at 0.01 mg/mL concentration. (D) Overall growth curve of ZEM2S cells for four days for all serum conditions and 10% serum with 0.01 mg/mL hydrolysate (Lugworm taken as representative).

At a 10 % serum concentration, lugworm, mushroom, and mussel demonstrated considerably higher cell growth than a 10 % control, indicating that these hydrolysates may have cell-enhancement properties that might work in conjunction with 10 % serum. Oyster and algal hydrolysates appear identical to the 10% control, indicating that these protein hydrolysates have no effect or interaction on serum. Black soldier fly, cricket,

pea, and yeast dramatically reduced cell proliferation compared to the 10 % control, indicating that these protein hydrolysates had a negative effect on serum.

At 5% serum concentration, only mussel hydrolysate was able to restore the cell growth parameters as the cell numbers were found to be significantly not different than the 10 % control. Silk sericin hydrolysate was also able to exhibit similar cell growth on the Hela cell line at 0.01 mg/mL (M. Zhang et al., 2019). However, all other hydrolysates showed significantly lower cell growth than 10% serum, indicating that at 0.01mg/mL concentration, the hydrolysates acted antagonistically with 5% serum and thus negatively impacted cell growth parameters.

At 0% serum concentration, none of the hydrolysates were able to increase the cell growth parameters as compared to 10% control; however, apart from cricket and yeast hydrolysates, all other hydrolysates were able to significantly increase the cell growth as compared to 0% control indicating some potential cell growth activity of these hydrolysates. However, the loss of cell morphology and starvation of cells indicated that the protein hydrolysates lack potential nutrients or other ingredients to sustain cell health.

At 0.01 mg/mL none of the hydrolysates showed any significantly higher cell viability. Only mussel hydrolysate should have shown higher cell viability, which can be explained due to less accurate nature of the colorimetric test.

Protein hydrolysates with 0.001 mg/mL with various serum concentrations

The effect of 0.001 mg/mL on cell growth parameters appears to be better than that of all other hydrolysate concentrations, as this concentration successfully reduced the serum concentration by up to 50 percent for most of the hydrolysates.

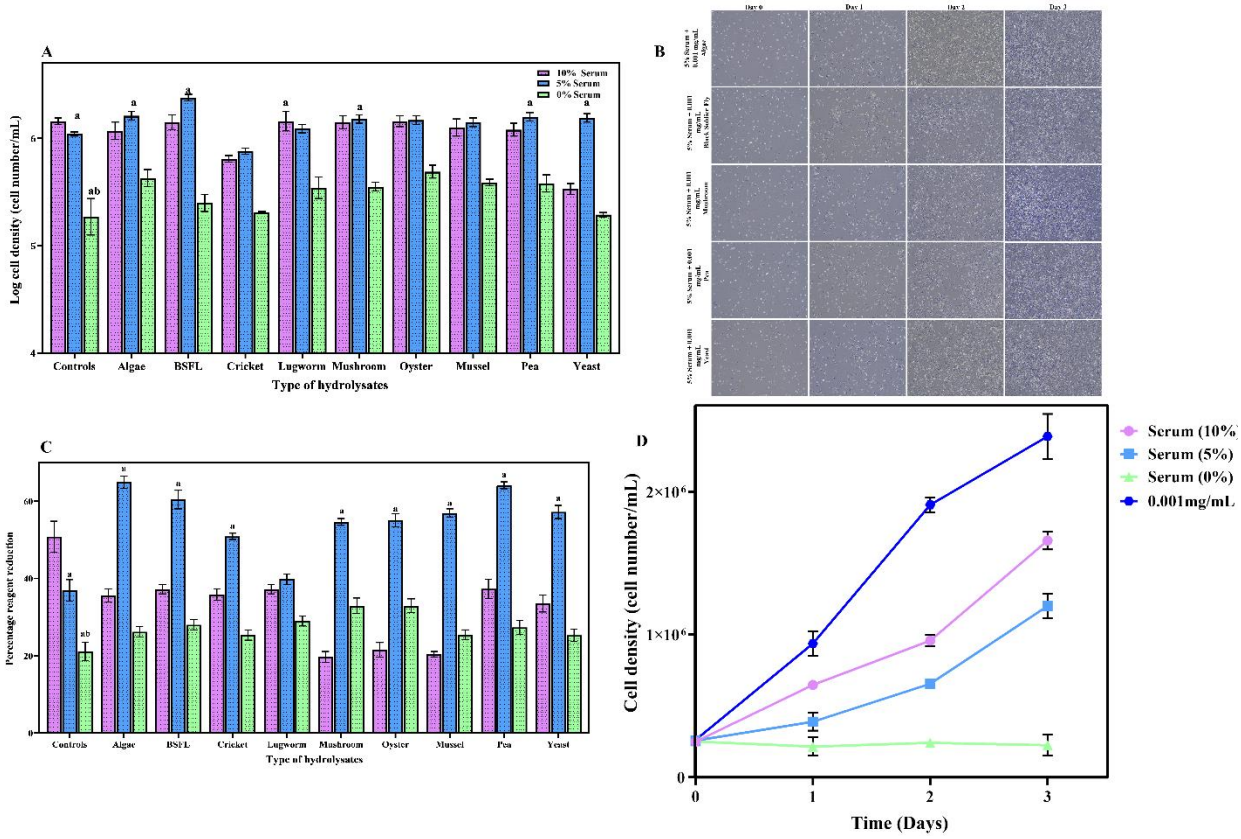


Figure 6: Cell growth parameters of ZEM2S cells at various serum concentrations with multiple protein hydrolysates at 0.001mg/mL concentration obtained by imaging analysis. (A) Log transformed cell density at 3rd day for all serum conditions with 0.001 mg/mL concentration of protein hydrolysates. a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 5% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage dye reduced by cells grown with all serum conditions using all protein hydrolysates at 0.001 mg/mL concentration. (D) Overall growth curve of ZEM2S cells for four days for all serum conditions and 10% serum with 0.001 mg/mL hydrolysate (Lugworm taken as representative).

At 10 % serum, only lugworm hydrolysate exhibited higher cell growth parameters than the 10 percent control, indicating the possible presence of cell-enhancing ingredients that can act in concert with the highest serum concentration. All other protein hydrolysates exhibited % either no significant difference or lower cell growth, indicating that these protein hydrolysates with a 10 percent serum concentration have no potential effects.

At a serum concentration of 5%, the growth of algae, black soldier fly, oyster, mushroom, and pea hydrolysates was significantly higher than at a serum concentration of 10%, indicating significant potential for reducing and enhancing the function of serum. There was no significant difference between the hydrolysate of mussels and yeast and the control level of 10%, indicating a successful reduction of serum to 50%. Another study validated algae extract's 0.001 mg/mL concentration as a 50% replacement for serum (Ng et al., 2020). Except for cricket and lugworm, all hydrolysates at a concentration of 0.001mg/mL were able to reduce and increase the cell growth parameters in animal cell cultures by up to 50 percent. In comparison to the 10% control, the black Soldier Fly hydrolysate exhibited a significantly higher growth rate and a shorter population doubling time, indicating successful replacement of the serum. All other hydrolysates showed population doubling time and growth rate statistically similar to 10% gold standard.

Similar to protein hydrolysates at concentrations of 0.1 and 0.01 mg/mL, the effect of protein hydrolysates at a concentration of 0.001 mg/mL without serum was equivalent. None of the protein hydrolysates could restore the serum function required for growth and morphology at 0% serum. In addition to losing their characteristic fibroblast-like morphology and taking on a dendritic appearance, the cells' growth was significantly lower than the gold standard 10 percent serum control. As expected, however, the cells appear to grow significantly faster than the 0% control, indicating that protein hydrolysates do possess some growth-promoting properties, albeit not to the same extent as 10% serum supplementation. Except for mushroom, cricket, black soldier fly, and pea, the growth of all other hydrolysates was significantly higher than the 0% control.

In general, all the hydrolysates, except the cricket hydrolysate, were able to effectively reduce the serum concentration and, in some cases, produced even better growth than the 10 percent control, demonstrating the tremendous potential of these protein hydrolysates to reduce serum and work synergistically with a serum to increase biomass production and sustain cell morphology. The concentrations of 1 and 10 mg/mL appear to have a negative effect on cell growth and survival and to be toxic to cell growth overall. Below this range, the toxicity of the protein hydrolysates was eliminated. Lugworm hydrolysates increased growth parameters while preserving cell morphology at a concentration of 0.1 mg/mL and 5% serum. At 5 percent serum, mussel hydrolysate restored lost serum activity at 0.01 mg/mL, while all other hydrolysates restored activity at 0.001 mg/mL. Thus, the 0.001-0.1 mg/mL range for protein hydrolysate appears to be the most effective range for reducing serum concentration by up to 50%. As the cell growth parameters decreased and morphological aberrations were observed, none of the protein hydrolysates could replace the serum completely. However, the cell numbers increased significantly relative to the basal media, indicating that the protein hydrolysates may have growth-promoting properties. At 0.001mg/mL most of the hydrolysates except of lugworm showed higher cell viability which is in corroboration with cell growth parameters as discussed above with % dye reduced in between approx. 51-64%. Overall, the cell viability determined using PrestoBlue was comparable to results obtained by microscope image analysis which presents a good cross reference test. The highest cell viability was produced with black soldier fly at 65% reduction.

Impact of various protein hydrolysates at 0.1- 0.001 mg/mL concentration at 2.5 and 1% serum levels

A serum concentration of 10 percent is the standard method for properly growing zebrafish embryonic stem cells. In our research, we reduced the serum concentration to 2.5, 1 and 0% to determine the effect on cell growth and morphology parameters. Figure illustrates the effect of serum at varying doses on numerous aspects of cell development.

The cells were grown in 10% serum containing mediums for all conditions to ensure proper adhesion and then changed to 2.5, 1 and 0% respectively. As expected, the cell growth lowered as the serum concentration was lowered. The cell numbers declined sharply at second day after the media change, for both 2.5, 1 and 0% media. At third day, the cell number dropped to 106, 8 x 10^5 and 4 x 10^5 cells for 2.5, 1 and 0% respectively from the gold standard of 1.5 x 106 cells at 10% serum concentration respectively. Overall, as compared to 10% control, the logarithmic reduction of 0.1, 0.2 and 1 were observed for 2.5, 1 and 0% serum concentrations respectively, all of which are statistically significant. The sharpest drop of cell growth was observed for 0% serum as expected. However, morphology at 2.5% serum was not affected as much but morphology of 1 and 0% serum containing media started showing various aberrations. This showed that although cell growth and proliferation dwindled at 2.5% serum concentration, however this amount of serum was enough to maintain the normal morphology of cells. At 1% serum the cells started showing some morphological aberrations and severe morphological anomalies were observed with complete serum starvation. Overall, as the serum reduced, the cell death increases as cells became rounded in formation, the cells lost their original spindle like fibroblast morphology and became more elongated, spiky, and starved in nature which was concluded earlier and is

very well documented (Pirkmajer & Chibalin, 2011). The cell viability results were in corroboration with the cell growth measurement obtained via imaging analysis.

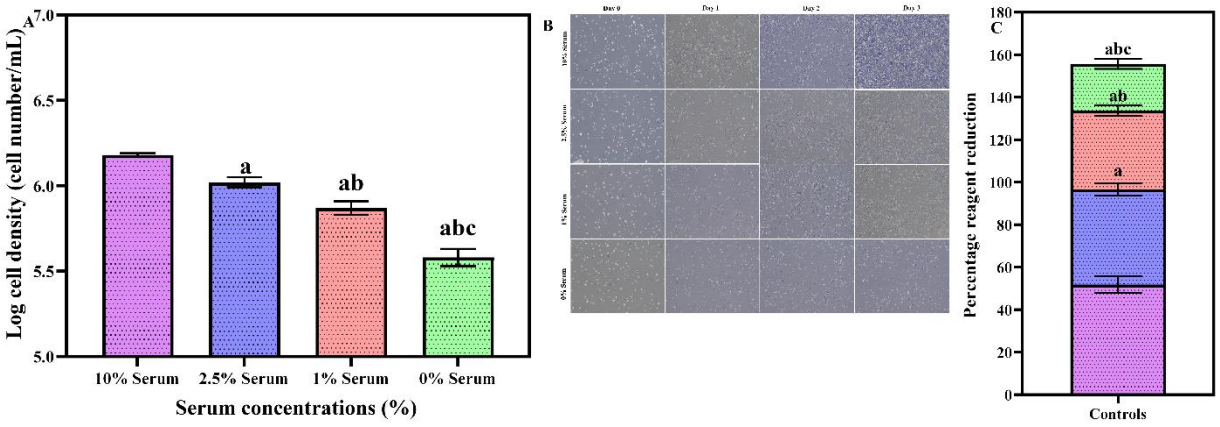


Figure 7: Cell growth parameters of ZEM2S cells at serum concentrations 10-0% obtained by imaging and PrestoBlue (A). Log transformed cell density at 3rd day. a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 2.5% control, c represents significantly different than 1% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage Dye reduced by cells at serum concentrations 10-0%.

The effect of 0.1 mg/mL concentration of all protein hydrolysate concentrations was positive as depicted by the figure

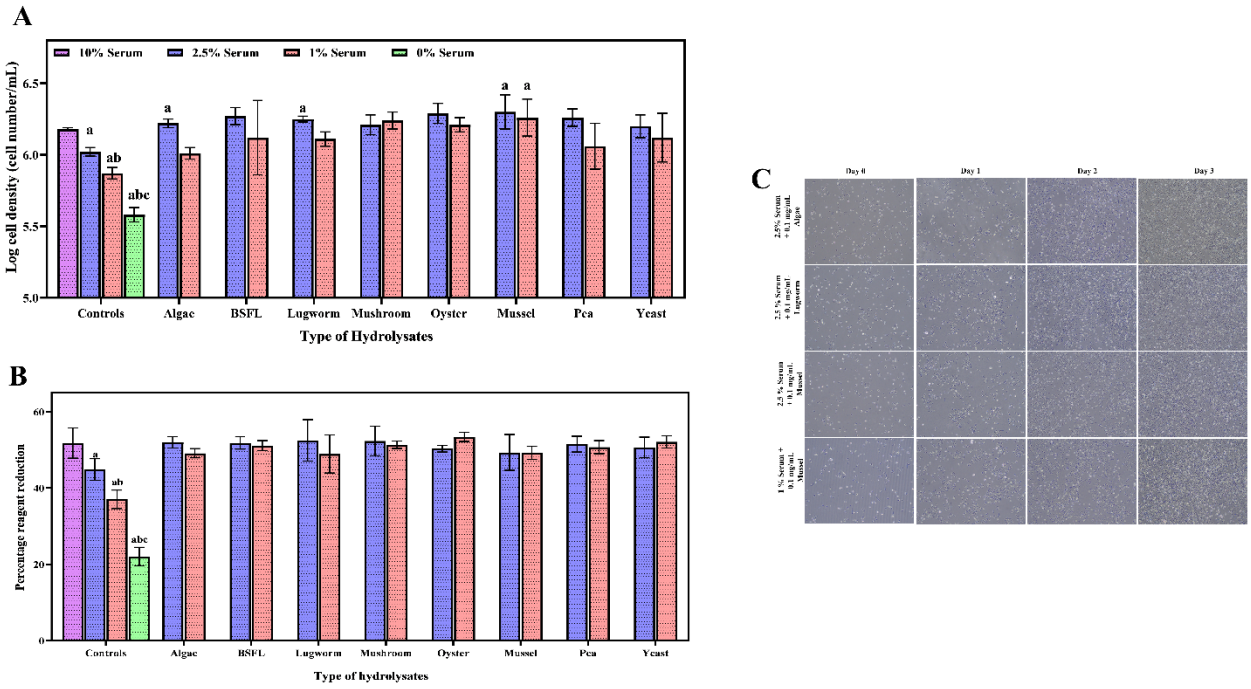
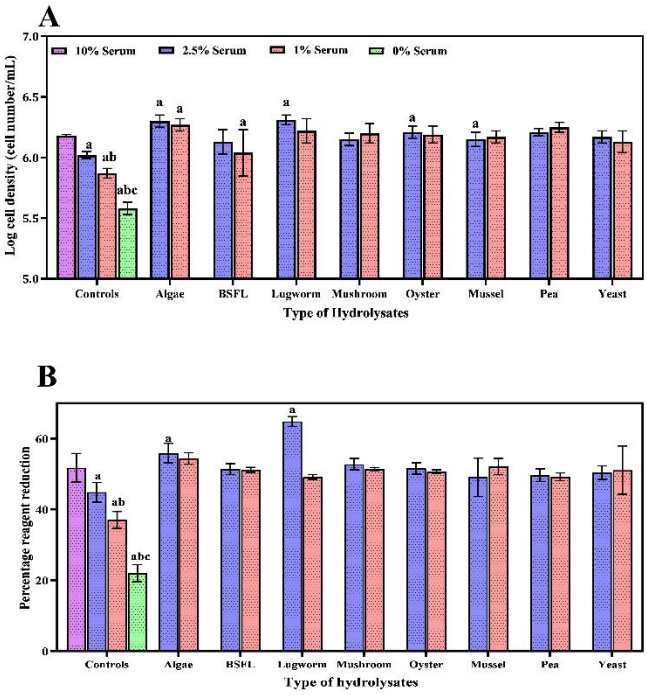


Figure 8: Cell growth parameters of ZEM2S cells at various serum concentrations obtained by imaging and PrestoBlue analysis at protein hydrolysate concentration 0.1mg/mL. (A). Log transformed cell density at 3rd day a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 2.5% control, c represents significantly different than 1% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage Dye reduced by cells at various conditions

Most protein hydrolysates exhibited great cell growth profiles at a serum concentration of 2.5%, as evidenced by their growth rates and population doubling times. The cell viability profiles of all protein hydrolysates did not differ significantly from the 10% standard. Compared to the 10% serum standard, algae, lugworm, and mussel hydrolysate displayed significantly higher growth. This suggests that protein hydrolysates may synergistically affect cell growth with serum. All other protein hydrolysates did not differ significantly from the 10% control, indicating that 7.5% of serum required by the cells was successfully replaced. Overall, 0.1 mg/mL concentration efficiently reduced the serum levels by up to 75% without affecting cell viability or growth profile.

At 1% serum, only mussel hydrolysate exhibited significantly more significant growth than the control at 10% serum, indicating a synergistic effect with serum and improving its function. Compared to the 10% control, algae, pea, and lugworm hydrolysates exhibited decreased cell proliferation, showing antagonistic interactions between serum and these protein hydrolysates. The remaining protein hydrolysates exhibited no significant differences in cell growth compared to a control group of 10%, demonstrating the effective restoration of up to 90% of serum. In addition, none of the protein hydrolysates demonstrated a significant change in cell viability compared to a 10% control.

The effect of 0.01 mg/mL concentration of all protein hydrolysate concentrations was positive as depicted by the figure



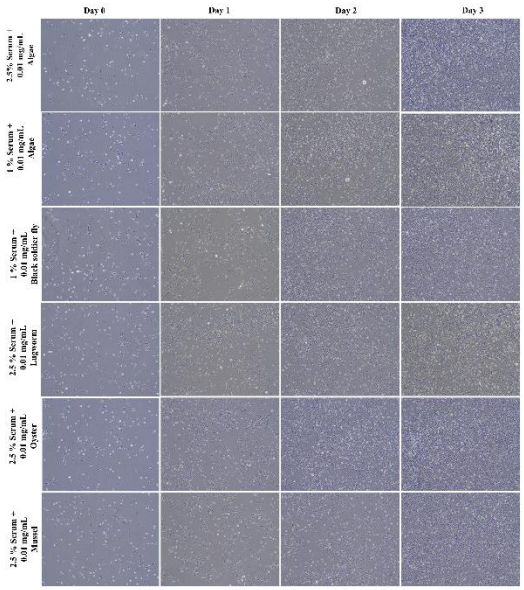


Figure 9: Cell growth parameters of ZEM2S cells at various serum concentrations obtained by imaging and PrestoBlue analysis at protein hydrolysate concentration 0.01mg/mL. (A). Log transformed cell density at 3rd day a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 2.5% control, c represents significantly different than 1% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200μm) magnification. (C) Percentage Dye reduced by cells at various conditions

Algae, lugworm, oyster, and mussel hydrolysate exhibited significantly greater cell proliferation at 2.5 percent serum than 10 percent serum control, thus enhancing the serum's function. The remaining protein hydrolysates showed no significant variation from the 10% control, showing that up to 75% of the serum was successfully replaced with affecting cell growth parameters. At 1% serum, only algae and BSFL exhibited considerably higher cell proliferation than the 10% control, whereas all other hydrolysates did not differ significantly. In addition, the cell viability of only algae and lugworm was significantly greater than 10 % control; the cell viability of the remaining hydrolysates did not change significantly. This demonstrated that all protein hydrolysates could reduce serum levels by up to 90 percent at a concentration of 0.01 mg/mL.

The effect of 0.01 mg/mL concentration of all protein hydrolysate concentrations was positive as depicted by the figure

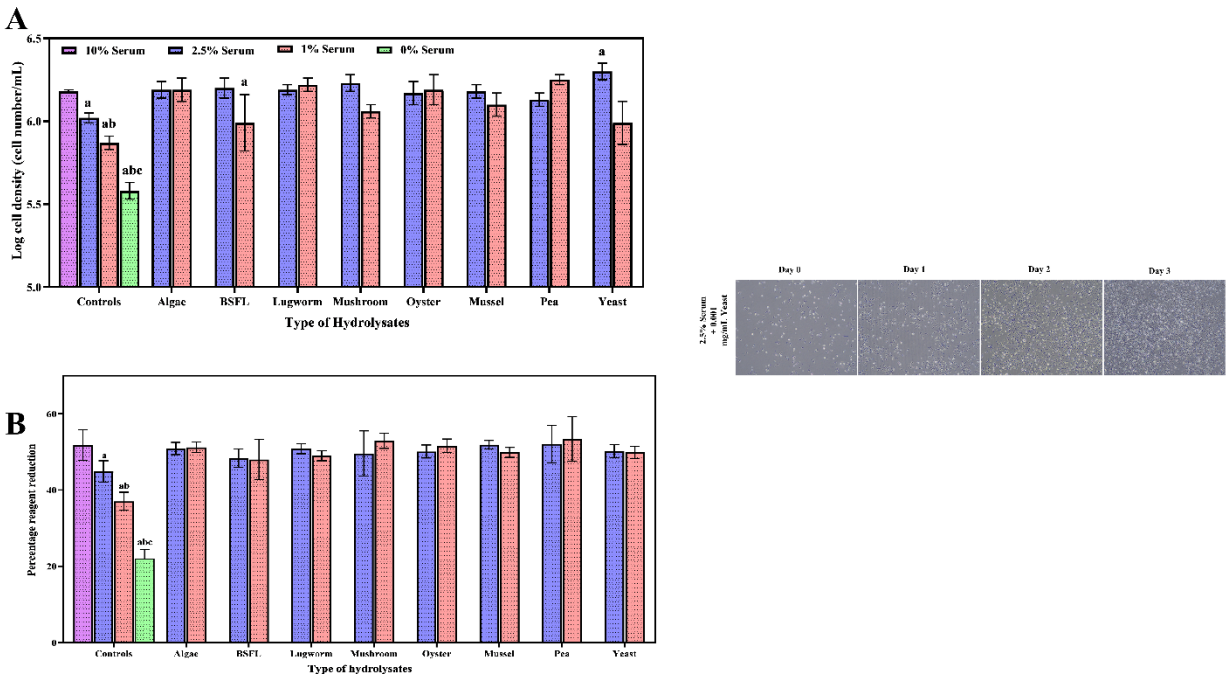


Figure 10: Cell growth parameters of ZEM2S cells at various serum concentrations obtained by imaging and PrestoBlue analysis at protein hydrolysate concentration 0.001mg/mL. (A). Log transformed cell density at 3rd day. a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 2.5% control, c represents significantly different than 1% control. (B) Micrographs of ZEM2S cells obtained on all four days at 10X (200µm) magnification. (C) Percentage Dye reduced by cells at various conditions

At a serum concentration of 2.5%, nearly all protein hydrolysates demonstrated cell growth characteristics and viability that did not statistically differ from the control at 10%. At a 1 percent serum concentration, only the black soldier fly displayed significantly better cell development than the 1 percent control, indicating an improvement in the serum's efficacy. The other six did not differ significantly from the 10% control group except for the mushroom hydrolysate. In addition, there was no significant difference in cell viability between the protein hydrolysates and the 10 % control group. This suggests that seven hydrolysates could reduce serum concentrations by up to 90%.

Almost all protein hydrolysate concentrations between 0.01 and 0.001 demonstrated statistically better or similar growth and viability compared to the 10 % control, showing that the serum concentration can be decreased by 75 to 90 percent. Morphologically, there was no discernible difference between the protein hydrolysates at any of the serum concentrations and 10% control. This demonstrates that protein hydrolysates between 0.1-0.001 mg/mL can restore cell growth, viability parameters, and morphological integrity. All protein hydrolysates, except mussel, were chosen for additional morphological and cell health testing at 0.01 mg/mL concentrations because they performed better than or similar to the 10 percent gold standard. The ideal concentration of mussel hydrolysate was established to be 0.1 mg/mL, as the cell growth parameters were significantly higher than at 10% control.

Fluorescent staining

Hoechst dye is an effective and reliable fluorophore with a long history of visualizing DNA content and nuclear structure using a fluorescence microscope. It is a fluorescent dye that can stain both living and fixed cells and emits fluorescence in the blue light spectrum. Serum deprivation induces apoptosis or necrosis, which can be recognized by karyopyknosis and visually identified by staining cells with hoescht dye (Bucevičius et al., 2018; Huang et al., 2018). Actin is another ubiquitous protein in eukaryotic animal cells, particularly in the filamentous (F) form. These proteins maintain cellular shape and structure, signaling, and cell division (Dominguez & Holmes, 2011). It is known that serum deprivation impairs actin protein, which can potentially negatively impact essential cell-based features such as cell shape, cell-cell matrix connections, and proliferative capacity (Wallenstein et al., 2010). Unfortunately, the process of serum deprivation is

poorly understood, as it differs from cell to cell. For this study, cells grown at all serum conditions (0-10%) and all protein hydrolysates selected from the previous section were stained with Hoescht and actin green. All these conditions are depicted in the figure.

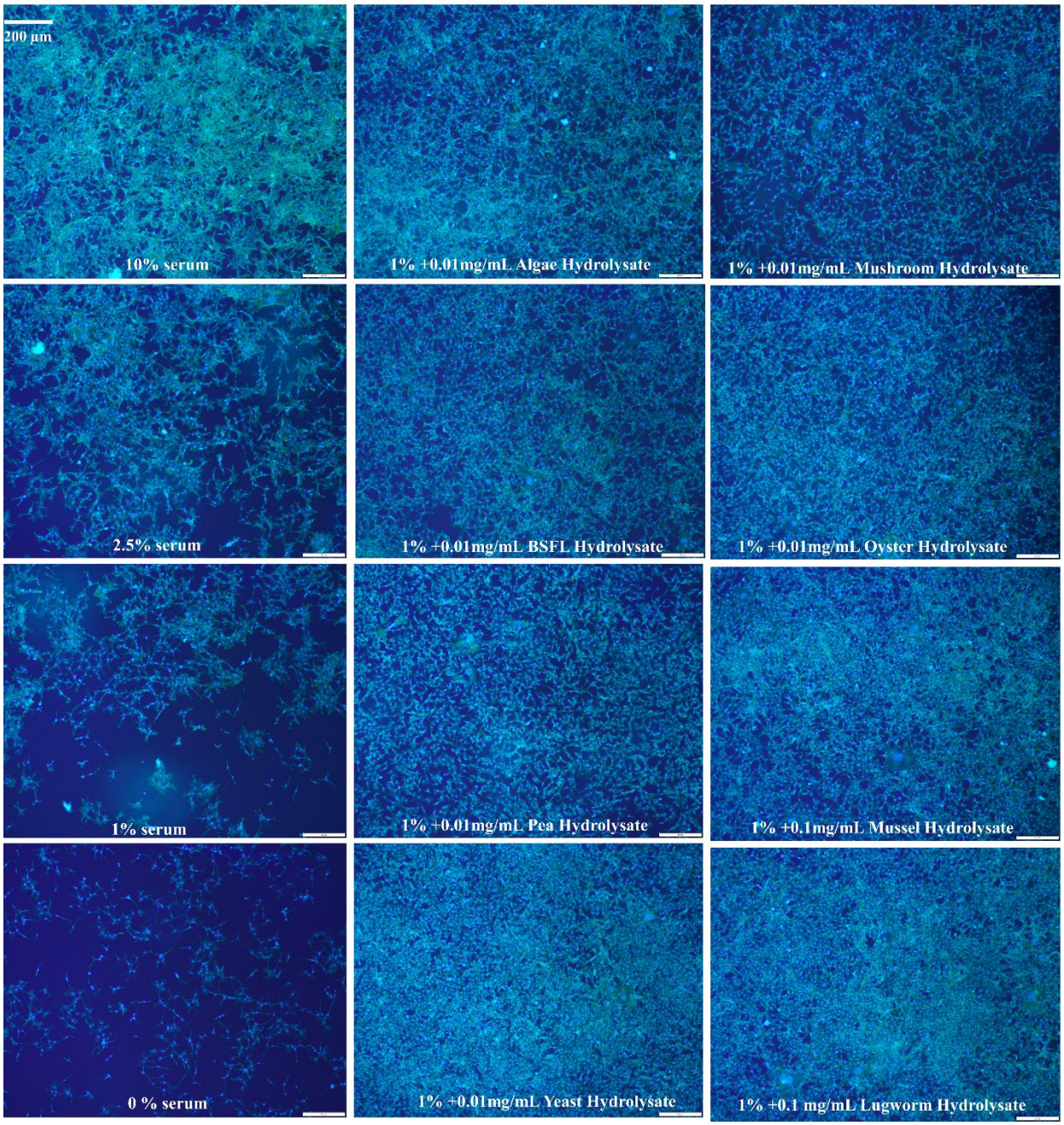


Figure 11: Hoechst and actin green, fluorescent staining of cells at all serum and protein hydrolysate conditions

From these fluorescent staining, it is evident that serum deprivation had direct effects on the cytoskeleton, although there was no discernible effect on the nucleus other than a reduction in numbers. At 10 % control, a high number of nuclei and abundant actin protein stains were observed. At a serum concentration of 2.5%, there was no visible effect on the nucleus other than a reduction in the number. However, the actin filaments were stained less, indicating a decrease in the presence of actin proteins, and the cell area was increased. As the serum content declined from 1 to 0%, the actin protein exhibited minor actin staining, and the cells were more dispersed and obtained a greater surface area. Overall, the actin protein presumably decreased along with the serum concentration, which altered cell shape. This result is supported by a second investigation demonstrating a decrease in actin protein staining under serum-starved circumstances (Wallenstein et al., 2010). The selected protein hydrolysates from the first section exhibited the same number of nuclei and cytoskeleton as the 10 % serum control, demonstrating that the protein hydrolysates were able to restore actin protein in these cells and, consequently, the original cytoskeleton.

LDH staining

The figure displays the LDH activity of the selected protein hydrolysate concentration at 2.5 and 1% serum concentration and 10 and 0% control.

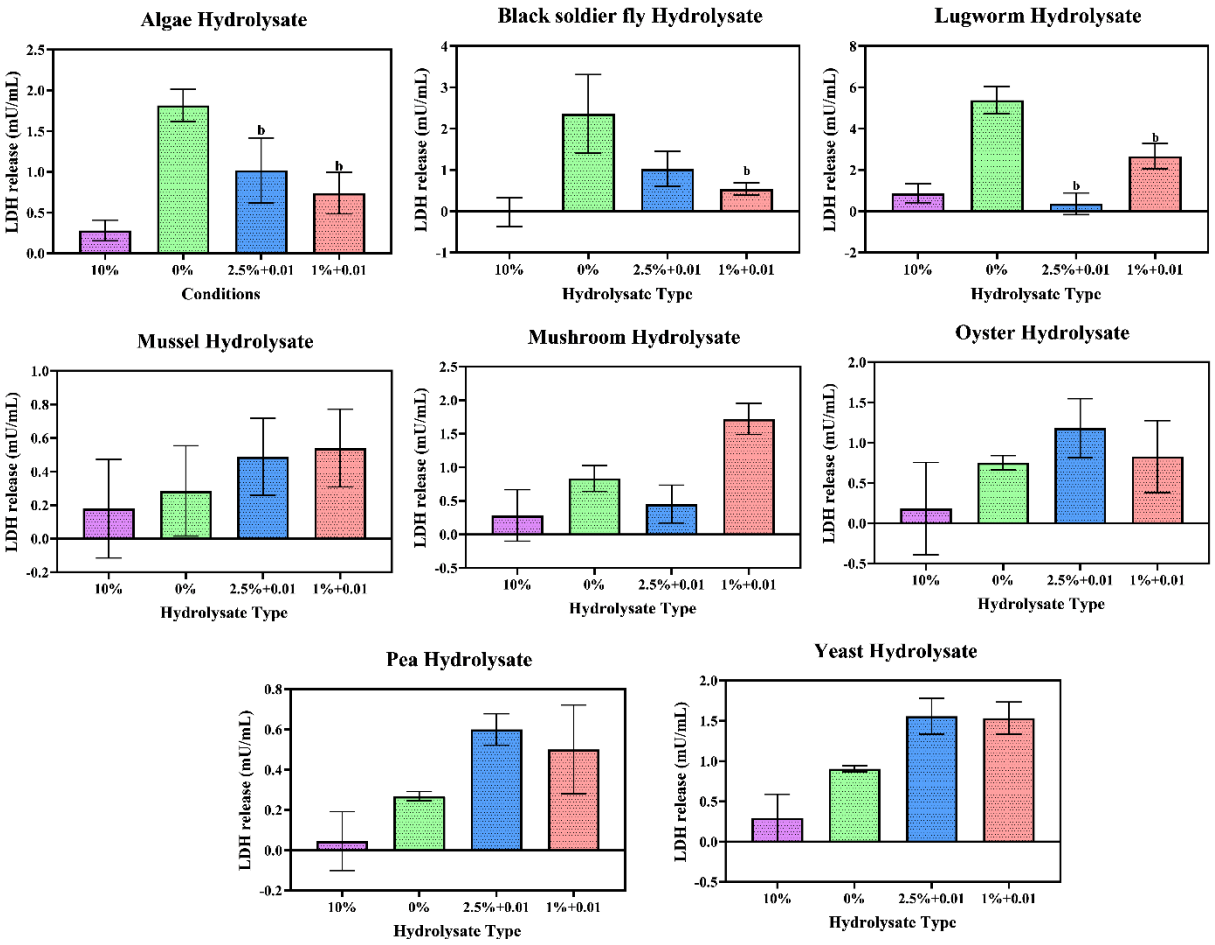


Figure 12: Lactate dehydrogenase assay conducted for protein hydrolysate conditions at 2.5 and 1% serum concentrations. a represent significantly different ($p<0.05$) from 10% control, b represents significantly different ($p<0.05$) from 0% control

In general, a serum concentration of 0 % showed a more significant release of LDH activity, indicating damage to the plasma membrane. In contrast, a serum concentration of 10 % had the lowest LDH activity, in some cases reaching negative levels. These results suggest that the integrity of the cell membrane was compromised, resulting in the release of LDH into the medium, which can indicate apoptosis and necrosis. Overall, the results were highly variable due to the serum's inherent LDH activity, which can interfere with the kit; however, blanks were collected for every condition. In addition, less

accuracy and sensitivity of the absorbance-based colorimetric tests utilized in this study contributes to the higher variability.

All other groups had significantly greater LDH release than the 10% control group. Only Algae, black soldier fly, and lugworm hydrolysates significantly reduced LDH release relative to the 0% control group. All other hydrolysates exhibited a greater LDH release than the 0% serum control, indicating that these protein hydrolysates can cause a greater degree of cell membrane damage than in serum-free conditions (Chan et al., 2013). Additionally, a higher LDH activity level may be associated with the presence of tumorigenic cells (Mishra & Banerjee, 2019). Adding protein hydrolysates to the culture media may have caused the cells to become malignant because of the Warburg effect-induced rise of LDH in the culture media. However, no other approach was made to confirm this assertion; therefore, it remains only a hypothesis.

Conclusion

In this study, eight protein hydrolysates were produced, and one already-prepared protein hydrolysate was characterized and evaluated for its ability to reduce or replace serum in Zebrafish embryonic stem cells. Black Soldier Fly protein hydrolysates proved ideal in terms of characterization and functional properties. It had a high yield, productivity, protein content, and quality, as well as a satisfactory level of hydrolysis. In addition, it possesses exceptional functional properties that allow its practical application in bioreactor conditions and enhance lipid-based components' bioavailability. All hydrolysates demonstrated the ability to replace at least fifty percent of serum in cell culture conditions, reducing the overall cost of producing cell-based meat. However, none of the hydrolysates could completely replace serum as cell growth lowered

drastically, accompanied by morphological aberrations. Based on the results of this study, the black Soldier Fly hydrolysate is the best protein hydrolysate for use in cell culture media due to its superior functional and nutritional properties. A subset of hydrolysates examined in a prior study was employed in this investigation. At 2.5 and 1 % serum concentrations, nearly all protein hydrolysates demonstrated excellent cell growth characteristics and viability at 0.1-0.001mg/mL concentrations. Additionally, fluorescence imaging has shown that protein hydrolysates can improve cytoskeleton density to a similar extent to 10% control. However, only Lugworm and black soldier fly hydrolysate exhibited the lowest LDH activity, indicating the least damage to the cell membrane, making it the optimum and most effective protein hydrolysate for a 90 percent serum decrease without compromising cell health indicators. Potentially with antifoaming chemicals, cellular agriculture can also utilize pea and algal hydrolysate to make media devoid of animal components.

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