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BÁRBARA FLAIBAM

HIDRÓLISE ENZIMÁTICA DE RESÍDUOS AGROINDUSTRIAIS PARA OBTENÇÃO DE INSUMOS DE INTERESSE PARA CARNE CULTIVADA

ENZYMATIC HYDROLYSIS OF AGRO-INDUSTRIAL WASTES TO OBTAIN INPUTS OF INTEREST FOR CULTURED MEAT

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RESUMO

A demanda urgente por fontes alternativas de proteínas tem atraído a atenção para o desenvolvimento tecnológico da carne cultivada. A carne cultivada é a carne obtida a partir do crescimento de células em biorreatores, sem abate animal. No entanto, muitos obstáculos técnicos ainda precisam ser superados para a produção em larga escala, acessível aos consumidores. O entrave mais significativo do processo está relacionado ao meio de cultivo, que deve fornecer condições adequadas para a proliferação e diferenciação celular, apresentar baixo custo e ser livre de derivados animais, em especial o soro fetal bovino. Resíduos agroindustriais isentos de derivados animais podem ser explorados como potenciais fontes de obtenção de insumos para carne cultivada, tendo em vista que estão disponíveis em larga quantidade e apresentam características nutricionais favoráveis, além de outras funcionalidades que podem promover o crescimento celular animal *in vitro*. Dessa forma, este trabalho investigou resíduos agroindustriais com alto teor proteico e livres de derivados animais. Analisou a extração proteica assistida por enzima de farelo de soja e de amendoim e promoveu a maximização da hidrólise enzimática da matriz residual da extração, garantindo um melhor aproveitamento proteico do material de partida. Os insumos obtidos foram caracterizados para averiguação da composição nutricional e de possíveis outras funções convenientes aos cultivos celulares. Os resultados mostraram elevados rendimentos das extrações proteicas em relação ao método convencional, dada à assistência da enzima Viscozyme^{TM} L em condições de pH ajustadas. As hidrólises das matrizes residuais atingiram valores significativos de grau de hidrólise – acima de 30% – pela ação da enzima Alcalase^{TM} 2.4L e pela otimização dos parâmetros de reação: temperatura e concentração de enzima. Os extratos foram obtidos com teor proteico superior a 75% e análises de SDS-PAGE e cromatografia de exclusão por tamanho possibilitaram a identificação dos principais grupos proteicos dos materiais de partida. Os hidrolisados também apresentaram valores proteicos significativos, a composição de aminoácidos livres mostrou-se conveniente, com predominância de Leucina e Prolina, e a distribuição de pesos moleculares foi verificada na faixa entre 6,5 kDa e 0,137 kDa. Além disso, o sequenciamento proteômico dos insumos derivados do farelo de soja proporcionou a verificação de funcionalidades altamente relevantes ao crescimento celular animal. Por fim, a análise hipotética de custos em escala laboratorial validou a extração proteica e a hidrólise enzimática de resíduos agroindustriais não-animais como rotas promissoras e de baixo custo para obtenção de insumos de interesse para carne cultivada.

Palavras-chave: resíduos agroindustriais; extração de proteínas; hidrolisados de proteína; enzimas; carne cultivada.

ABSTRACT

The urgent demand for alternative protein sources has drawn attention to the technological development of cultured meat. Cultured meat is meat obtained from growing cells in bioreactors, without animal slaughter. However, many technical hurdles still need to be overcome for large-scale production that is accessible to consumers. The most significant obstacle in the process is related to the culture medium, which must provide adequate conditions for cell proliferation and differentiation, be inexpensive and be free of animal derivatives, especially fetal bovine serum. Agro-industrial wastes free of animal derivatives can be explored as potential sources of inputs for cultured meat, given that they are available in large quantities and have favorable nutritional characteristics, and functionalities that can promote animal cell growth *in vitro*. Thus, this work investigated agro-industrial wastes with high protein content and free of animal derivatives. Analyzed enzyme-assisted protein extraction of soy and peanut meal and promoted the maximization of enzymatic hydrolysis of the residual extraction matrix, ensuring better protein utilization of the starting material. The inputs obtained were characterized to verify their nutritional composition and possible other convenient functions for cell cultures. The results showed higher protein extractions yields than the conventional method, given the assistance of the Viscozyme^{TM} L enzyme under adjusted pH conditions. The hydrolysis of the residual matrices reached significant values of degree of hydrolysis – above 30% – by the action of the Alcalase^{TM} 2.4L enzyme and by the optimization of the reaction parameters: temperature and enzyme concentration. The extracts were obtained with a protein content greater than 75% and SDS-PAGE analysis, and size exclusion chromatography allowed the identification of the main protein groups of the starting materials. The hydrolysates also showed significant protein values, the composition of free amino acids was convenient, with a predominance of Leucine and Proline, and the distribution of molecular weights was verified in the range between 6.5 kDa and 0.137 kDa. In addition, the proteomic sequencing of inputs derived from soybean meal provided the verification of functionalities highly relevant to animal cell growth. Finally, the hypothetical laboratory-scale cost analysis validated protein extraction and enzymatic hydrolysis of non-animal agro-industrial waste as promising and low-cost routes to obtain inputs of interest for cultured meat.

Keywords: agro-industrial wastes; protein extraction; protein hydrolysates; enzymes; cultured meat.

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INTRODUÇÃO GERAL

A busca por fontes alternativas de proteínas é uma realidade que tem se intensificado devido às demandas nutricionais somadas ao crescimento populacional. Fontes proteicas com menores impactos ambientais, sem restrições éticas e com possibilidade de ampliação de escala são requeridas. Dentre as diversas vias exploradas, o cultivo de células animais destinado à obtenção de carne cultivada tem ganhado atenção, especialmente quando comparado às carnes convencionais obtidas por abate animal (SANTOS, 2019).

A carne cultivada é aquela obtida a partir do cultivo em biorreatores de células retiradas de animais vivos (HUBALEK *et al*., 2022), também designada por "carne de laboratório", "carne sintética", "carne limpa", "carne artificial", "carne baseada em células", ou ainda, "carne de célula animal" (LETTI *et al*., 2021). Embora atrelada aos conhecimentos de biotecnologia e da engenharia de tecidos, a produção em larga escala de células animais *in vitro* apresenta uma série de desafios (ALLAN *et al*., 2019), incluindo definições de linhagens celulares estáveis e hábeis para a diferenciação em tipos celulares relevantes; meios de cultivo de baixo custo e livres de componentes animais; biorreatores com volume, perfusão e condições adequadas e *scaffolds* com biocompatibilidade para o crescimento de células de alta qualidade (BOMKAMP *et al*., 2022; SPECHT *et al*., 2018).

Nesse sentido, o desenvolvimento de meios de cultivo isentos da aplicação de soro fetal bovino ou de outros componentes de origem animal é considerado o primeiro passo para a produção de carne cultivada em larga escala com custos acessíveis. O soro fetal bovino é considerado o suplemento universal de proteínas, aminoácidos, fatores de crescimento e de outros nutrientes para o cultivo de células animais (GUAN *et al*., 2021). Contudo, por estar associado ao abate animal e apresentar indefinição na composição, alto custo, variabilidade entre lotes e riscos de contaminação, o soro fetal bovino deve ser excluído do processo produtivo da carne cultivada (FANG *et al*., 2017; GUAN *et al*., 2021).

Diante de tais circunstâncias, resíduos agroindustriais isentos de derivados animais são matérias-primas promissoras para o fornecimento de insumos de interesse para a produção de carne cultivada. Esses resíduos são encontrados em massivas quantidades para aplicação em larga escala; possuem características nutricionais adequadas; podem apresentar componentes com propriedades bioativas; e o seu reaproveitamento minimiza o impacto ambiental gerado pelo descarte inadequado (CHABANON *et al*., 2008; HO *et al*., 2021; RUSS & MEYER-PITTROFF, 2004).

Proteínas e hidrolisados proteicos obtidos a partir de matrizes livres de derivados animais têm sido explorados como rotas potenciais para a obtenção de insumos para a suplementação dos meios de cultivo para carne cultivada (CHABANON *et al*., 2008; HARAGUCHI *et al*., 2022; YAO & ASAYAMA, 2017). Técnicas de extração proteica assistidas por enzimas e hidrólises enzimáticas são promissoras para a obtenção destes insumos, uma vez que a aplicação de enzimas pode maximizar o rendimento da extração e atingir graus de hidrólise adequados para a obtenção de aminoácidos e de peptídeos de baixo peso molecular, nutricionalmente favoráveis ao aumento da densidade celular nos cultivos (CHABANON *et al*., 2008; DEL MAR CONTRERAS *et al*., 2019; HO *et al*., 2021). A aplicação desses insumos visa a contribuir com o fornecimento de proteínas, vitaminas, aminoácidos, peptídeos de baixo peso molecular e até mesmo elementos-traço, como minerais para a cultura de células animais. Contudo, a falta de definição acerca das composições torna o uso dos extratos e dos hidrolisados limitado (YAO & ASAYAMA, 2017).

Dessa forma, este trabalho teve como objetivo a extração de proteínas a partir de resíduos agroindustriais livres de derivados animais e a otimização da hidrólise enzimática do material residual da extração, para a obtenção de outros insumos de interesse com vistas à produção de carne cultivada.

Inicialmente, foi realizado levantamento acerca de insumos alternativos e suas possíveis funcionalidades para a produção de carne cultivada.

Na sequência, resíduos agroindustriais livres de componentes animais e com diferentes composições químicas foram selecionados e testados quanto à extração de proteínas assistida por enzima e à hidrólise enzimática do material residual, maximizando a utilização proteica do material de partida. Foram estudados a atuação de enzimas sob diferentes condições e seus efeitos na extração e na hidrólise, além da otimização das condições de hidrólise para a obtenção de alta concentração de peptídeos e aminoácidos livres, análogos aos do soro fetal bovino, indicando uma rota eficiente para a obtenção de insumos para carne cultivada.

Por fim, as composições dos extratos e dos hidrolisados proteicos foram analisadas e comparadas ao soro fetal bovino quanto às funções favoráveis para o cultivo de células animais *in vitro*. Os custos de obtenção dos insumos também foram descritos e correlacionados com os valores comerciais do soro fetal bovino, possibilitando a avaliação inicial dos custos da rota estudada.

CAPÍTULO I: Non-animal protein hydrolysates from agro-industrial wastes: a prospect of alternative inputs for cultured meat

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Abstract

Due to the growing demand for alternative proteins, the production of laboratory-grown meat, obtained from cell culture without animal slaughter, has been highlighted as a potential solution to conventional meat. Cultured meat uses more sustainable methodologies, maximizing animals' well-being and minimizing the harmful effects on human health and the environment. However, the cell culture technique faces challenges such as the replacement of fetal bovine serum used in the supplementation of the culture medium, which burdens the process due to high cost, low availability, and ethical issues. This review prospects non-animal protein hydrolysates from agro-industrial wastes as possible substitutes for some critical fetal bovine serum components in cultured meat processing. Although the low definition of the composition of protein hydrolysates is one of the challenging aspects, the benefits of its application may outweigh the disadvantages, and future research may make cell production more accessible to consumers.

Keywords: enzymatic hydrolysis; agro-industrial waste; cultured meat; fetal bovine serum; alternative proteins

Highlights

- Non-animal protein hydrolysates may have potential use in the cultured meat process.

- Non-animal protein hydrolysates can replace fetal bovine serum components.
- The reuse of non-animal agro-industrial waste minimizes damage to the environment.
- Non-animal agro-industrial waste hydrolysates could make cultured meat cheaper.

1 Introduction

The projected increase in global demand for meat and meat products for the coming years has generated great concern since conventional large-scale meat production has been directly related to environmental problems, nutritional setbacks, public health complications, and associated animal suffering (Rubio et al., 2020). Although growth rates in the total amount of meat consumed vary across regions, this scenario has encouraged investors, researchers, and the industry to seek complementary alternatives to conventional meat production (Godfray et al., 2018; Letti et al., 2021). Among the emerging segments, cultured meat stands out for its *in vitro* cell culture without the traditional animal slaughter and for presenting the best possibilities of mimicking conventional meat in sensory and structural characteristics (Rubio et al., 2020). This multidisciplinary and biotechnological alternative unites tissue engineering knowledge with biomaterials, cultivating animal cells in large-scale bioreactors to obtain complex structures like conventional edible meat tissues. However, the production of cultured meat is still expensive, and achieving its large-scale production and commercialization in the coming years and consumer acceptance will need many technological efforts and the development of new tools (Letti et al., 2021; Singh et al., 2022).

Replacing fetal bovine serum, an essential ingredient for supplementing the cell culture medium responsible for the growth and proliferation of animal cells is the biggest obstacle encountered in the process so far. Fetal bovine serum interferes negatively with cultured meat production for several factors. Among them, the cost of fetal bovine serum stands out, since it can reach 95% of the total cost of the cell medium, in addition to its use being contradictory with the ethical aspects of the process (Letti et al., 2021; Thorrez & Vandenburgh, 2019).

Commercially available alternatives to bovine serum-free media are also expensive, mainly due to requiring proteins and growth factors normally obtained by genetic recombination (Thorrez & Vandenburgh, 2019). In this context, hydrolysates obtained from plant compounds and microorganism materials have attracted attention as potential complementary sources of proteins, peptides, amino acids, lipids, and vitamins for supplementing the culture medium to produce cultured meat in substitution of essential components of bovine fetal serum (Sung et al., 2004). The ideal culture medium should offer

the necessary nutrients and conditions to enable the maintenance and growth of animal cells, minimizing waste and associated cost (Andreassen et al., 2020). In addition, these hydrolysates also have essential functions that should not be neglected, such as antioxidant, antibacterial, antiapoptotic, immunomodulatory properties and other regulatory activities that can positively contribute to the cell production process, as well as add nutritional and sensory characteristics to cultured meat (Ho et al., 2021). Considering the recent applications, the importance, and the valorization of non-animal protein agro-industrial wastes, this article prospects the potential application of hydrolysates obtained from these wastes in the production of cultured meat, to replace the components of fetal bovine serum, minimize process costs, and reduce environmental damage. This application's advantages, disadvantages, and possibilities will be explored based on the current literature.

2 Culture meat and its prospects

Cultured meat, also known as “animal cell meat,” “artificial meat,” “*in vitro* meat,” “synthetic meat,” “clean meat,” and “laboratory meat,” among other denominations, refers to meat produced based on cultivating of animal cells in the laboratory without traditional animal slaughter and predicted less environmental damage. At the current level of technological development, the productive process of cultured meat can be divided into four distinct phases, namely: cell biopsy and initial growth; cell proliferation in bioreactors, in which the cells are cultivated under optimal conditions in biological reactors with nutrient-rich culture media and adequate energy sources; cell differentiation with specific differentiation techniques, such as the scaffold-based method or the self-organization; and the processing of the final product, to obtain custom meat similar to conventional meat in nutritional and sensory aspects, facilitating consumer acceptance (Guan et al., 2021; Zhang et al., 2020).

A preliminary comparative study between the production cycle of cultured meat and conventional meat showed reductions of 99% in land use, 96% in water use, and 78–96% in greenhouse gas emissions, although, without real values of the productive process of cultured meat, the data of this study can underestimate or overestimate the impacts of the production of cultured meat in the environment. Therefore, not definitive and little conclusive regarding this new productive modality compared with the conventional technique (Tuomisto & Teixeira de Mattos, 2011). Regarding energy consumption, the data are still controversial and generate debates about the amount of energy needed to maintain the bioreactors to meet the estimated demand, which can equal or even surpass the amount of energy consumed in conventional

models (Mattick et al., 2015). The use of renewable energies by the cultured meat industry may be a factor that will favor this new market (Tuomisto & Teixeira de Mattos, 2011).

Recently, indications of cleaner production and the possibility of minimizing animal suffering have also attracted the attention of investors. In 2020, the cultured meat production process had 14% of the capital (US$ 366 million) allocated to the alternative proteins sector, double the value attributed in 2019 and 72% greater than the amount invested from 2016 to 2020. The expansion of investments in recent years indicates growth and new opportunities in the sector (GFI, 2020).

Like any new technology, the cultured meat production process seeks improvements in the environment, in product quality and safety standards, and in maximizing yields (Ho et al., 2021). Even preceded by decades of knowledge accumulation in cell culture, stem cell biology, tissue engineering, fermentation, biomaterials, and bioprocesses, cultured meat production needs new strategies and tools to make meat production in bioreactors viable, competitive, and affordable for the final consumer since production is still very expensive (GFI, 2020; Guan et al., 2021).

The technological novelty of cultured meat requires a lot of effort to overcome some obstacles, including the determination of cell lineages that are genetically stable and favorable to the production of large cell masses for producing cultured meat on a large scale; developing low-cost culture media-free of fetal bovine serum or other components of animal origin; designing bioreactors with adequate perfusion and volume for producing large cell concentrations; and creating scaffolds suitable for cell culture and nutritionally favorable for human consumption (Datar & Betti, 2010; Specht, 2020). In this context, replacing fetal bovine serum or bovine serum albumin – an essential ingredient for supplementing the culture medium, responsible for the growth and proliferation of animal cells – is essential to meet the economic premise, since the bovine fetus whey overloads the production process (Letti et al., 2021; Thorrez & Vandenburgh, 2019).

3 The fetal bovine serum

In animal cell culture, large amounts of culture medium are required, and fetal bovine serum has been used as a standard supplement since it contains a mixture of stimulating and necessary factors for the growth and maintenance of different cell types (Brunner et al., 2010; Subbiahanadar Chelladurai et al., 2021). Fetal bovine serum is the supernatant of clotted blood collected mainly by cardiac puncture from a bovine fetus (Andreassen et al., 2020).

Worldwide production of 800,000 liters of fetal serum is estimated, which leads to the slaughter of more than 2 million bovine fetuses (van der Valk et al., 2017).

Its main functions are related to the hormonal factors that stimulate cell growth and promote differential functions; transport of proteins, minerals, trace elements (such as transferrin), lipids; stabilization of pH; and factors of maintenance or direct/indirect inhibition of proteases and other toxic molecules (Brunner et al., 2010). The fetal bovine serum also acts on the physicochemical properties of the culture medium, such as viscosity, osmolarity, and diffusivity, in addition to reducing the shear stresses from handling the cells and agitating the bioreactors (Yao & Asayama, 2017; Zheng et al., 2006).

However, using ingredients of animal origin becomes inappropriate due to ethical issues for the production of cultured meat. Other problems are also reported regarding the application of fetal bovine serum, such as the undefined composition, the inconsistent quality between batches, the possible presence of disease transmitters, the potential risk of microbial contamination with viruses, prions, and mycoplasma and the high costs involved, especially for scale-up since its supply is less than the demand (Andreassen et al., 2020; Cassiday, 2018; Ho et al., 2021; Ng & Kurisawa, 2021). Thus, the risks and disadvantages associated with using bovine serum in cell growth outweigh the benefits (Ho et al., 2021).

According to Specht (2020), 55–95% of the production costs of laboratory-grown meat are attributed to the culture medium used for cell growth. The fetal bovine serum accounts for approximately 95% of these costs (Cassiday, 2018). Aspects related to safety, production, and ethics of the meat culture process require the urgent replacement of fetal bovine serum or essential serum components in animal cell cultures, guaranteeing the strategic approach, the expected quality of the final product, and providing greater independence in commercial supply (Brunner et al., 2010; van der Valk et al., 2017).

3.1 Composition

The composition of fetal bovine serum is variable and not all ingredients are defined qualitatively and quantitatively. Its composition and biological complexity vary according to the seasons and geography, and the same producer can present batches with different compositions (van der Valk & Gstraunthaler, 2017).

More than 1,800 proteins and 4,000 metabolites are estimated in fetal bovine serum composition, but not all components are identified and quantified (Subbiahanadar Chelladurai et al., 2021). Albumin is the most abundant protein in fetal bovine serum and is a highly multifunctional component (Yao & Asayama, 2017). Other main components used in

supplementation to replace fetal bovine serum are insulin, FGF-2, TGF-β, recombinant growth factors, vitamins, minerals, and buffering supplements. Growth factors are proteins that bind to receptors on the surface of cells to activate cell proliferation and differentiation (Khandwala et al., 2000). They act in small amounts in the cells and some growth factors are quite versatile, stimulating cell division in different cell types, whereas others are specific to a particular cell type (Yao & Asayama, 2017; Zheng et al., 2006). In addition to growth factors and hormones, the fetal bovine serum has low levels of inhibitory factors and a wide range of nutrients, which makes supplementing bovine serum-free commercial culture media a complex task (O'Neill et al., 2021; Yao & Asayama, 2017; Zheng et al., 2006). Among the activities of fetal bovine serum regarding the contribution to the growth of animal cells and tissues, the high peptidase activity is another important parameter, since it breaks peptide chains, supplying the required amino acid content (Jones et al., 1975).

Fetal bovine serum-free production is an important step in the scientific development of cultured meat, which must be seen as a priority for managed production. The challenge in replacing fetal bovine serum in cultured meat production is basically to consider how it influences cell growth in bioreactors, to identify the necessary components for cell growth, to replace such components from animal-free inputs, and to determine the composition specific for each cell type since they have subtle metabolic differences, i.e., different types of cell have particular nutritional needs for their development, proliferation, and differentiation (Andreassen et al., 2020; O'Neill et al., 2021).

Commercially, fetal bovine serum-free culture media have quantitatively defined compositions, but are designed for a specific cell lineage, not meeting the needs of other lineages, which makes it difficult to regulate the proliferation and differentiation of "muscle tissue cells," which are composed of more than one type of cell, such as muscle satellite cells, myoblasts, myocytes (myotubes and myofibers), adipose tissue-derived stem cells, adipocytes, and fibroblasts, necessary in cultured meat products (O'Neill et al., 2021).

For reference, Table 1 shows the main known components of fetal bovine serum and describes its functionalities in animal cell growth, indicating how complex the task of formulating animal cell culture media-free from fetal bovine serum can be.

Table 1. Generic concentration of nutrients and functional components of fetal bovine serum

Constituents[a]	Function	Range of concentrations[a]
	Proteins	
	Polypeptides	
Serum albumin	Serum albumin supports cell growth, protects materials from oxidation, and is the most abundant protein in fetal bovine serum (Zheng et al., 2006).	20 - 50 g/L
Fetuin	Fetuin is responsible for increasing the cell's accessories (Freshney, 2005).	10 - 20 g/L
Fibronectin	Fibronectin promotes cell attachment (Freshney, 2005; Yamada & Geiger, 1997).	1 - 10 mg/L
Globulins	The globulin fraction includes hundreds of serum proteins, including carrier proteins, enzymes, complement, and immunoglobulins (Busher, 1990).	1 - 15 g/L
Protease inhibitors: α1-antitrypsin, α2-macroglobulin	α1-antitrypsin and α2-macroglobulin have trypsin inhibitory functions (Freshney, 2005).	0.5 – 2.5 g/L
Transferrin	Transferrin is responsible for binding to iron, making it less toxic and bioavailable (Freshney, 2005).	2 - 4 g/L
	Growth factors	
FGF	The fibroblast growth factor (FGF) is a potent mitogen for a variety of cell types *in vitro* since it may locally regulate cell growth and differentiation during angiogenesis (Ingber & Folkman, 1989; Zheng et al., 2006).	1 - 100 µg/L
GGF	The glial growth factor (GGF) was originally identified as a glycoprotein that interacts with the receptor tyrosine kinase (Zheng et al., 2006).	
IGF-I and IGF-II	The insulin-like growth factors possess growth-promoting activity, and *in vitro*, they are potent mitogens for cultured cells. However, IGF-II appears in greater amounts than IFG-I in fetal bovine serum (Honegger & Humbel, 1986; Zheng et al., 2006).	
PDGF	The platelet-derived growth factor (PDGF) corresponds to a family of polypeptides with essential mitogenic activity for cell growth since it stimulates growth in fibroblasts and glia (Freshney, 2005).	
TGF-β	The transforming growth factor β (TGF-β) is a multifunctional peptide that controls proliferation, differentiation, and other functions in a variety of cell types. TGF-β regulates the actions of many other peptide growth factors and determines a positive or negative direction for their effects (Zheng et al., 2006).	

(continued on next page)

Table 1 *(continued)*

Constituents[a]	Function	Range of concentrations[a]
	Hormones	
Hydrocortisone	Hydrocortisone can promote cell attachment and proliferation, but under special conditions, such as at high cell density, it can be cytostatic and induce cell differentiation (Freshney, 2005).	3.625 – 72.49 µg/L
Insulin	Insulin is responsible for the uptake of glucose and amino acids and may have mitogenic influence via the IGF-I receptor (Freshney, 2005).	5.778 – 577.8 mg/L
Triiodothyronine	Triiodothyronine is an important signaling molecule and exerts biological actions such as metabolism, thermogenesis, growth, and development (Yamauchi, 2021).	13.02 µg/L
Thyroxine	Thyroxine is a hormone that contains iodine. Converted to its active form, triiodothyronine, it increases the speed of chemical reactions in cells and helps control growth and development (National Cancer Institute, 2022).	77.69 µg/L
	Amino Acids	
Essential amino acids	The essential amino acids most required in animal cell culture are cystine and/or cysteine, arginine, glutamine, and tyrosine, although the individual amino acid requirements vary among different cell types (Freshney, 2005).	0.01 – 1.0 µM
	Lipids	
Cholesterol	Cholesterol is essential for cell development and the regulation of several enzymes (Martínez et al., 2001).	3.67 mg/L
Linoleic acid	Linoleic acid can be found in low concentrations in fetal bovine serum and is normally bound to proteins such as albumin (Freshney, 2005).	2.805 – 28.05 µg/L
Phospholipids	Phospholipids are important components of membranes and the cellular structural matrix (Carmo & Correia, 2009).	0.7 – 3.0 g/L
	Polyamines	
Putrescine, spermidine	Polyamines act in the production of proteins and nucleic acids. However, high concentrations of these components induce cellular apoptosis (Yao & Asayama, 2017).	8.815 – 88.15 µg/L
	Amides	
Urea	Urea acts as a source of non-protein nitrogen (Subbiahanadar Chelladurai et al., 2021).	130 - 170 mg/L

(continued on next page)

Table 1 *(continued)*

Constituents[a]	Function	Range of concentrations[a]
	Inorganics	
Calcium	Calcium ions are responsible for the release of hormones and neurotransmitters and their concentration can influence cell proliferation or differentiation (Bendich, 2001; Freshney, 2005).	160.3 – 280.6 mg/L
Chlorides	Sodium, chloride, and potassium ions are responsible for regulating the membrane potential (Freshney, 2005).	3.545 mg/L
Sodium		3.10 – 3.57 g/L
Potassium		195.5 – 586.5 mg/L
Iron	Iron and zinc are found connected to serum protein (Freshney, 2005).	0.559 – 2.793 mg/L
Zinc		6.538 – 65.38 µg/L
Phosphate	Phosphate can act as an alternative iron carrier in bovine serum-free culture media, in addition to its nutritional functions (Rasmussen & Toftlund, 1986).	189.9 – 474.9 mg/L
Selenium	Selenium helps detoxify free radicals as a cofactor of glutathione (GSH) synthetase (Freshney, 2005; McKEEHAN & Ham, 1976).	0.790 µg/L
	Carbohydrates	
Glucose	Glucose is metabolized to form pyruvate, which can be converted to lactate or acetoacetate (Freshney, 2005).	0.6 – 1.0 g/L
Hexosamine	Hexosamine is responsible for the production of precursors, such as N-acetylglucosamine (GlcNAc), necessary for protein glycosylation (Rao & McArthur, 2017).	0.6 – 1.0 g/L
Lactic acid	Lactic acid supplementation can reduce the production of lactate by cells and consequently reduce the production of ammonia (Freund & Croughan, 2018).	0.5 – 2.0 g/L
Pyruvic acid	Pyruvic acid is an essential component for animal cell culture since it stimulates cell maturation and modulation of substrate metabolism (Downs et al., 1997).	2 - 10 mg/L
	Vitamins	
Vitamin A	Vitamin A is responsible for regulating cell growth and division (Harvard School of Public Health, 2022a).	10 - 100 µg/L
Folate	Folate, also known as vitamin B9, contributes to the formation of DNA and RNA and is involved in protein metabolism. It plays a key role in the breakdown of homocysteine, an amino acid that can exert harmful effects if present in large amounts (Harvard School of Public Health, 2022b).	5 - 20 µg/L

[a] O'Neill et al. (2021 apud Freshney, 2005).

The individual nutritional characteristics of the cell lines must be taken into account when supplementing the culture medium without bovine serum, as the cells have individual nutritional needs, varying according to the metabolism and proliferation of the cells. As is the case of glutamine, which in addition to being an essential amino acid, also acts as a biosynthetic material for the composition of nucleic acids and proteins. For cells with high nutritional requirements, the addition of glutamine can be efficient, but in other cases, the addition can have a detrimental effect, as glutamine can break down and promote the formation of cytotoxic ammonia. Thus, the presence of glutamine in culture medium supplementation should be investigated and the application of degradation-resistant derivatives, such as L-alanyl-L-glutamine or glycyl-L-glutamine, should be considered (Yao & Asayama, 2017).

Usually, these serum-free media are deficient in some critical components for cell growth and multiplication such as albumin, amino acids (mainly glutamine and arginine), various low-concentration vitamins, inorganic salts, and cytokines, including growth factors such as FGF-2, TGF-β and hormones such as insulin, responsible for cell proliferation and differentiation of a wide range of cells (Guan et al., 2021; O'Neill et al., 2021; Siemensma et al., 2010). Furthermore, formulations of amino acids, proteins, and other micronutrients available for cell culture are not produced on a scale compatible with food production (Andreassen et al., 2020). And the costs of growth factors and recombinant proteins used in supplementation are extremely high (Humbird, 2021; O'Neill et al., 2021).

The most versatile animal derivative-free culture media for animal cell growth applications are Eagle's Minimal Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), Ham's F-12 (Ham, 1965), and Essential 8 (O'Neill et al., 2021; Specht, 2020). Currently, research for the development of serum-free culture media for cultured meat has described the B8 medium for pluripotent stem cells, which has been improved with the addition of recombinant albumin, giving rise to the so-called Beefy-9 (Stout et al., 2022).

There is still little evidence of long-term cell proliferation in these media and other aspects must also be carefully studied. Serum-free culture systems can promote a more efficient proliferation of non-intensional clones or cell subtypes, which does not occur in media with the application of fetal serum. Some impurities present in serum-free media can cause more significant damage to cell growth and proliferation than when supplemented with serum, as this has toxin-neutralizing activity. *In vitro* growth without fetal serum supplementation can make cells more susceptible to oxidative stress, impairing cell proliferation and tissue formation. In this case, the addition of antioxidants such as vitamins C and E, glutathione, selenite, β-mercaptoethanol, dithiothreitol, or lipoic acid is effective, as well as the complexation of free

iron and copper ions with appropriate transporters such as transferrin, albumin or chelating agents are essential to prevent the formation of reactive oxygen (Yao & Asayama, 2017).

4 The application of hydrolysates to replace the components of fetal bovine serum

Alternatives have been presented for replacing serum components to minimize the problems encountered with the application of fetal bovine serum (Okamoto et al., 2022). Developing inputs of interest include the combined use of recombinant proteins, hormones, lipids, and hydrolysates (Ho et al., 2021). Proteins such as albumin, transferrin, and insulin have been obtained via genetic recombination for application in cultured meat; however, even when the recombinant technology processes reach their lowest costs and highest yields, the inputs obtained with this method will still compromise the values of the production process of cultured meat, requiring an alternative route. Since they are significant factors in the cost of the culture medium, alternative routes for producing serum elements, as well as basic nutrients without impurities and variability, are necessary to minimize costs and make cultured meat accessible to consumers (Hubalek et al., 2022).

Currently, researchers are searching for alternative cell culture methods and synthetic or plant-based substitutes and yeast extracts for supplementing fetal bovine serum-free formulations to reduce costs (Cassiday, 2018). For several decades, applying hydrolysates to animal cell growth has been reported to have positive effects, since protein hydrolysates promote more efficient use of amino acids in the metabolism of mammalian cells compared with fetal bovine serum (Ho et al., 2021), indicating a performance far beyond the simple nutritional value. The low cost of this application combined with the use of agro-industrial wastes makes this technique extremely attractive for large-scale applications (Franěk et al., 2000). Vegetable matrices such as rice, soybeans, and wheat are studied as potential sources of amino acids, peptides, vitamins, and trace elements for cell culture (Okamoto et al., 2020; Sung et al., 2004). Hydrolysates from plant materials can act as albumin substitutes regarding the shear stresses of the cells, favoring their robustness (Siemensma et al., 2010). Replacing animal proteins by plant peptides with potential bioactivity and anti-apoptotic characteristics has shown a positive effect on the growth and biosecurity of mammalian cells (Burteau et al., 2003).

Peptides from soy hydrolysates have been investigated for stimulating cell growth in addition to having multiple bioactivities of interest to the food and pharmaceutical industries (Singh et al., 2014). Soy hydrolysates can also present compounds such as citrulline and ornithine, precursors of polyamines, necessary for cell multiplication (Richardson et al., 2015). The addition of hydrolysates obtained from plant proteins showed a performance in cell growth

compatible with media supplemented with fetal bovine serum (Burteau et al., 2003). Hydrolysates obtained from rice and wheat proteins may present some peptides that can interact with cell surface receptors, stimulating cell growth and protein biosynthesis (Ballez et al., 2004). Chickpea protein hydrolysate acted as a good substitute for fetal bovine serum in the growth of cells growing in suspension, but did not allow the growth of cells growing in monolayers (Giro´n-Calle et al., 2008).

The application of cyanobacterial hydrolysates has been studied as a source of energy and nutrients for cultivating muscle cells, lowering environmental impacts in the process (Tuomisto & Teixeira de Mattos, 2011). Yeast hydrolysates have also shown positive results in supplementing CHO cell culture media (Spearman et al., 2014). Currently, mammalian cells such as CHO are considered the most common cellular systems for the recombinant expression of monoclonal antibodies (Mosser et al., 2013).

Based on the literature and considering the context of sustainability and reuse of resources, by-products of food production have shown to be promising aspects, since they are available in high quantities (with material percentages around 5%–90% depending on the raw material processed), are cheap, and have adequate ingredients for replacing bovine serum components (Andreassen et al., 2020). Developing techniques that favor the use of agro-industrial wastes as raw materials for the production of a wide range of valuable products for cultured meat production is of great interest (Celus et al., 2007). However, for large-scale production and commercialization of cultured meat to be feasible in the coming years, the inputs used must meet factors such as process reproducibility, low cost, and, mainly, biosafety (Siemensma et al., 2010).

Thus, hydrolysis methods can be applied to obtain these essential nutrients from agro-industrial wastes free of animal components as shown in Figure 1, since it can assist the extraction of proteins, and promote the breakdown of polypeptide chains into bioactive peptides and other components with biological functions and nutritional characteristics suitable for animal cell growth (Contreras et al., 2019). A range of applications to be explored around protein hydrolysates can make cultured meat production cheaper and safer (Ho et al., 2021). Furthermore, in many cases, combining hydrolysates from different raw materials can significantly impact the yield and growth of animal cells (Siemensma et al., 2010).

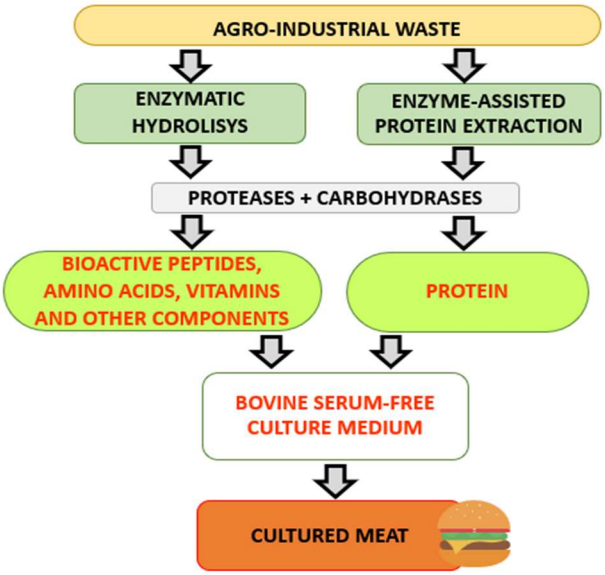

Figure 1. One of the possible schemes for obtaining inputs of interest for cultured meat from agro-industrial wastes.

Approximate protein profiles of different types of by-products that can be discussed as sources for protein extraction and obtaining other compounds with a focus on cultured meat production. Table 2 lists a series of agro-industrial wastes with different protein compositions that can act as raw materials to obtain inputs of interest.

Table 2. Protein-rich agro-industrial waste[a]

Agro-industrial waste	Crude Protein (%)
Peanut meal	48.2
Soybean meal	44.4
Brewer's yeast	41.8
Cottonseed meal	40.3
Canola meal	36.2
Sunflower bran	33.4
Cotton bran	28.1
Malt pomace	26.6
Coconut bran	22.2
Tomato pomace	22.1
Beet leaves	21.9
Radish leaves	19.4
Wheat bran	15.1
Rice bran	13.3
Lemon pomace	10.5
Corn bran	8.8
Banana peels	8.1

[a](Rostagno et al., 2011; Sharma et al., 2016).

However, few studies describe the application of non-animal protein hydrolysates from agro-industrial waste as substitutes for fetal bovine serum components for cultured meat production, as shown in Table 3.

Table 3. Non-animal agro-industrial waste applied in the supplementation of animal cell culture

Non-animal agro-industrial waste	Compound obtained by	Replacement of FBS	Effects and Results	Reference
Soybean meal	Enzymatic hydrolysis of Alcalase™	Tests carried out with 0, 2.5, and 5% FBS + 5, 2.5 and 5% of the hydrolysate in the growth media, respectively. Positive control: 10% FBS in the media.	Hydrolysates were able to replace up to 50% of FBS maintaining the ability to proliferate and differentiate in pig muscle stem cell culture.	Kim et al., 2023
Soybean meal	Enzymatic hydrolysis of Bromelain and Phyzyme™	The final concentration of hydrolysates tested in the medium was 0.4% (w/v). The suspension medium was used as negative control and suspension medium with 10% (v/v) FBS was used as positive control.	Apparent effects of supplementation with different concentrations of hydrolysates were observed in CHO-2 cell culture	Gupta, 2015.
Rapeseed meal	Enzymatic hydrolysis of Alcalase™, Esperase™, Neutrase™, Orientase™, and Pronase™	Hydrolysates were added at different concentrations in serum-free medium containing transferrin, albumin and insulin for the growth of CHO cells.	Hydrolysates with a high degree of hydrolysis allowed an increase in maximum cell density.	Chabanon et al., 2008
Proteins isolated from flaxseed oil cake	Enzymatic hydrolysis of Alcalase™, Neutrase™, and Protamex™	Tests carried out with: 5% FBS + 5% or 1% of the hydrolysate in the growth media. Positive control: 10% FBS in the media.	No positive response to the addition of hydrolysate in terms of growth rate and productivity was verified in the CHO culture in this study.	Logarušić et al., 2021
Cottonseed meal	Commercial peptones	Chemically defined media were supplemented with 2 g/L of cottonseed meal hydrolysate compared to medium without supplementation.	Significant increases in cell density and relative production of recombinant protein were seen in the CHO-320 culture.	Barbau et al., 2010
Rapeseed meal	Alkali extraction of protein meals	Supplementation of Beefy-9 medium with 0.4 mg/ml protein isolate.	Rapeseed meal protein isolates improved bovine satellite cell growth when compared to Beefy-9 medium, maintaining cell phenotype and myogenicity.	Stout et al., 2023

Since many components of protein hydrolysates already compose commercial culture media, free of bovine serum, it is essential to better understand how protein hydrolysates can play a role in cellular metabolism. It is necessary to explore how the chemical composition, the origin of the starting material, and the bioactive principles of the hydrolysates can influence cell growth, proliferation, and differentiation, to ensure greater cell densities in the process (Lobo-Alfonso et al., 2010; Siemensma et al., 2010). Therefore, a technical-economic analysis and a scale-up study must be carried out to ensure optimized and viable conditions for the process (Preece et al., 2017).

4.1 Obtaining the hydrolysates

The hydrolysis of protein from agro-industrial wastes can occur by alkaline, acidic, or enzymatic processes and is characterized as a reaction that involves breaking proteins down into smaller peptides and several other components necessary for the survival and growth of animal cells, such as peptides, amino acids, minerals, vitamins, carbohydrates, and lipids similar to the starting waste (Andreassen et al., 2020; Ho et al., 2021).

The use of enzymes in hydrolysis has been widely explored due to the milder, more sustainable conditions, with higher extraction yields and less degradation of other bioactive compounds (X. Guo et al., 2013; Sari et al., 2015). Furthermore, enzymatic hydrolysis also provides greater control of the process and the final product, improves the functional and nutritional characteristics of the starting material, and is the most suitable method for obtaining bioactive compounds, promoting adequate reuse of low-value-added waste, and increasing the yield and quality of the product obtained (Zamora-sillero et al., 2018; Fiorese et al., 2018).

The use and nature of the enzymes employed determine a possible increase in the availability of proteins and/or which peptides will be produced. Hydrolysates formed by combining various enzymes can have different functionalities depending on the degree of hydrolysis achieved (Celus et al., 2007; Zamora-sillero et al., 2018). Using enzymes simplifies the hydrolysis process, and, depending on the enzyme used, protein hydrolysates can have different side chains such as carboxyl, amino, imidazole, and sulfhydryl, among others that can play specific roles in the proliferation and growth of animal cells (Cruz-Casas et al., 2021; Pasupuleti & Braun, 2010).

Thus, for enzymatic hydrolysis to be successful, the enzyme should be adequate to the characteristics of the substrate and the product of interest (Pasupuleti & Braun, 2010). Furthermore, some independent variables must be controlled according to the optimal ranges of enzyme activity, including temperature, pH, reaction time, enzyme:substrate ratio, and the

initial protein concentration in the material (Adler-Nissen, 1984). The role of these independent variables in enzymatic hydrolysis is determined by the degree of hydrolysis, which relates the number of broken peptide bonds with the number of peptide bonds initially present in the protein (Zamora-sillero et al., 2018).

Enzyme-assisted protein extraction and protein hydrolysis from plant wastes are obtained by applying simple or complex enzymes such as proteases and carbohydrases, used sequentially or mixed (Piovesana et al., 2018; Sari et al., 2015). Enzyme preparations, such as Viscozyme™ L – containing a cocktail of different carbohydrases (β-glucanase, cellulase, xylanase, hemicellulase) – have successfully increased protein recovery in the extraction from cereals and other plant wastes (Contreras et al., 2019). However, simple proteases, such as trypsin, pepsin, and papain generate limited degrees of hydrolysis, which can be improved from the combination of endo and exoproteases (Chiang et al., 1999). Currently, many studies have applied a mixture of endo- and exoproteases, commercially known as Flavourzyme™, to improve the sensory characteristics of protein hydrolysates since applying proteases can generate undesirable bitter flavors by forming polypeptides of certain chain lengths or by generating peptides with a hydrophobic portion at the end of the chain, this negative effect on flavor can be easily solved with this mix of enzymes, favoring a greater use of hydrolysates in food industry applications (Hamada, 2000).

However, studies reported in the literature indicate Alcalase™ as a reference protease for research aimed at the hydrolysis of agro-industrial wastes from vegetables and microorganisms. It is an alkaline bacterial protease capable of generating protein hydrolysates with a high degree of hydrolysis and wide specificity with hydrophobic side chains and peptide bonds (Graycar et al., 2013).

Yust et al. (2003) showed higher yields in the extraction of proteins from sunflower flour based on the application of the enzyme Alcalase™, which also promoted greater solubility in hydrolysates in acidic solutions, favoring their applications in the food industry. Peptides with chelating bioactivity were obtained from the enzymatic hydrolysis of chickpeas with Alcalase™ and Flavourzyme™ (Megías et al., 2007). González-García et al. (2014) evaluated the protein extraction from plum by-products by different enzymes, indicating that the hydrolysates obtained from Alcalase™ activity showed higher isolation of antioxidant peptides and antihypertensive potentials. Montone et al. (2019) extracted bioactive compounds from Alcalase™ asparagus by-products, expanding the added value of this waste from the biological activity of the peptides obtained, suitable for application as functional ingredients in foods. Mazloomi et al. (2020) conducted a study to obtain peptides with bioactive properties from the

hydrolysis of orange seeds with Alcalase™. Ozón et al. (2022) presented the first report of antithrombotic peptides from by-products of Chia seed (*Salvia hispanica*) by enzymatic hydrolysis by Alcalase™ with Flavourzyme™.

However, complementary steps for the removal of undesirable materials and adequate storage of the hydrolysates are extremely necessary to guarantee the quality and effectiveness in the application, as well as the use of advanced techniques such as metabolomics and proteomics to identify and investigate of the components (Ho et al., 2021).

4.2 Composition of hydrolysates

Proteins and hydrolysates obtained from animal-free wastes have great potential as bioactive compounds. They may have a complex composition of nutrients such as peptides, amino acids, minerals, carbohydrates, lipids, and proteins similar to the starting material, with beneficial effects for the proliferation and growth of animal cells. Protein hydrolysates can act as a direct source of amino acids, as a stimulator of cell growth, and in the protection of cells against deformation (Siemensma et al., 2010), characteristics that favor the increase of cell density and promote higher yields regarding cell production.

The main and most abundant component of these protein hydrolysates are peptides, formed by a combination of 2 to 20 amino acids, which may have beneficial bioactive properties such as anti-apoptosis, antioxidant, immunomodulatory effect, and antibacterial properties, in addition to their nutritional value (Andreassen et al., 2020; Ho et al., 2021). Low molecular weight peptides effectively contribute to increase the nutritional value when applied to supplement culture media for animal cells (Heidemann et al., 2000) but they can also mimic growth factors or interact with other ligands resulting in specific mechanical and biological responses in cell development (Rubert Pérez et al., 2015).

For protein hydrolysates to act as an amino acids source in place of the amino acids from fetal bovine serum, they must have the highest possible concentration of free amino acids or short-chain peptides, such as dipeptides or tripeptides (Siemensma et al., 2010). Studies indicate that hydrolysates with low degrees of hydrolysis do not significantly affect cell growth, whereas hydrolysates with a high degree of hydrolysis (DH>20%) contribute to increase cell density. However, a greater number of cleaved peptide bonds does not increase bioavailability (Chabanon et al., 2008; Ho et al., 2021).

In this context, a greater understanding of the composition and influence of protein residue hydrolysates on cell growth is necessary for them to act as successful alternatives in replacing fetal bovine serum for laboratory meat production (Ho et al., 2021), since they can

nutritionally complement the medium, increase glutamine stability and biomass (Ho et al., 2021; Hubalek et al., 2022).

4.3 Bioactive peptides

Bioactive peptides are products of protein hydrolysis, which in their original protein conformation have an inactive biological activity. According to proteomic analyses, more than 6,000 protein molecules may contain bioactive peptides and plant matrices have been widely explored as a source of biopeptides. In this context, exploiting agro-industrial wastes to obtain these peptides is a promising pathway due to the low cost and the reuse of these materials (Maestri et al., 2016).

The bioactivity of peptides obtained from protein hydrolysis is largely dependent on the hydrolysis conditions and the starting material proteins, varying according to composition, amino acid sequence, hydrophobicity, and molecular weight (Meshginfar et al., 2018).

The size of the peptides formed in hydrolysis is one of the preponderant factors that directly influence their biological functions, techno-functional properties, and subsequent applications (Pasupuleti & Braun, 2010). Studies indicate that low molecular weight peptides, from 1 to 3 kDa, obtained from plant hydrolysates, generally have more effective antioxidant activity than peptides with molecular weights greater than 3 kDa (Hwang et al., 2016; Zhou et al., 2013). However, peptides with higher molecular weights can also be cited as substitutes for cell growth or survival factors, since they can reduce apoptosis effects and regulate cellular parameters via external molecular signals (Franěk et al., 2000). Several studies cited that oligopeptides obtained from yeast hydrolysates promote growth and productivity in cell cultures (Spearman et al., 2014). For applications such as growth promoters, cell protection, and metabolic stimulation, the three-dimensional sequence of amino acids and the physicochemical character of the peptide chain are extremely relevant (Siemensma et al., 2010).

The presence of specific amino acids within certain peptide sequences plays a key role in the biological activity of peptides (Maestri et al., 2016; Norris & FitzGerald, 2013). Other studies have shown a direct relationship between these biological activities and the occurrence of hydrophobic residues in the peptide chain, mainly regarding antioxidant activity (Chen et al., 1998; Li et al., 2007). The antioxidant activity of the peptides is related to the scavenging of radicals, the inhibition of lipid peroxidation, and the chelating of metal ions, widely detected in hydrolyzed soybean peptides (Singh et al., 2014). Chickpea, sesame, and

sunflower hydrolysates also present peptides effective as chelators due to the presence of cysteine, histidine, aspartic acid, and glutamic acid (L. Guo et al., 2014).

The presence of hydrophilic and basic amino acids in the chain, such as histidine and lysine, also favors the antioxidant capacity of peptides (Chen et al., 1998; Hattori et al., 1998). Amino acids such as alanine, proline, and phenylalanine have free radical scavenging activity, whereas amino acids such as leucine and valine can play an important antioxidant role since their aliphatic groups interact with sensitive fatty acids (Chen et al., 1998; Park et al., 2001). Tryptophan and tyrosine can act as free radical scavengers (Pihlanto, 2006).

The immunomodulatory activity of cells can also be activated by low molecular weight, positively charged peptides, as seen in hydrolyzed soy protein peptides (Singh et al., 2014). Although studies on the immunomodulatory activity of peptides obtained from hydrolysates are still limited, the presence of specific amino acids such as glycine, valine, leucine, proline, glutamine, and tyrosine possibly indicate this ability (Chalamaiah et al., 2018). Regarding antimicrobial activity, peptides obtained from hydrolysates such as cottonseed and chia seeds, have this reported property which varies according to the enzyme applied for hydrolysis. As for anti-apoptotic activities, the specific sequence of peptides from non-animal waste hydrolysates is still unknown (Ho et al., 2021).

To evaluate the bioactivity of peptides obtained from protein hydrolysates, separating the starting material is necessary since non-protein materials from the hydrolysates can also present bioactivities that interfere with the detection of the activity of the peptide. Isoflavones, saponins, and phytic acid are some examples of components present in plant matrices that have antioxidant activity and can be confused with the activity of peptides (Piovesana et al., 2018; Xiong, 2010). However, identifying the sequences of the peptides responsible for the bioactivities of these molecules is not always an easy task, which ends up being neglected, especially for matrices from non-animal agro-industrial wastes. Therefore, databases of bioactive peptides have been used to help in this task, since they describe sequences with known bioactivity, as well as algorithms capable of predicting the bioactivity of a peptide (Piovesana et al., 2018). The BIOPEP-UWM is one of the most cited platforms in research to validate the activity of bioactive peptides from the identified amino acid sequence (Minkiewicz et al., 2019). However, an in-depth study regarding the delivery and availability of peptides for animal cell culture *in vitro* is necessary to elucidate their performance and efficacy (Maestri et al., 2016).

4.4 Contaminants and by-products of hydrolysates

Despite the contributions described regarding of the bioactivity of the peptides present in the hydrolysates, contaminants from the starting material and by-products generated in enzymatic hydrolysis can negatively affect animal cell growth, impairing the yield, quality, and safety of cultured meat (Ho et al., 2021). The presence of non-peptides or the formation of peptides conjugates with other organic substances during processing can compromise the biological activities of the hydrolysates (Franěk et al., 2000); however, since, bioactive peptides are highly selective with their targets, the formation of toxic metabolites from them is very unlikely (Maestri et al., 2016).

In general, bioactive peptides produced from hydrolysis with food-grade enzymes are identified as GRAS (Generally Recognized as Safe) (Ulug et al., 2021). However, the toxicity of the peptides must be evaluated regarding cell proliferation and differentiation, since some amino acids (Cys, His, Asn, and Pro) and amino acid fragments (Phe-Lys-Lys, Leu-Lys-Leu, Lys-Lys-Leu-Leu, Lys-Trp-Lys, and Cys-Tyr-Cys-Arg) are identified in peptides considered toxic for human consumption (Chaudhary et al., 2016). Hydrolysates obtained with high-ash and low-protein content can also negatively affect animal cell growth (Pasupuleti & Braun, 2010).

Also note the risk of toxicity due to exposure of the starting material to high concentrations of fertilizers and agrochemicals, especially when working with agro-industrial plant wastes (Okamoto et al., 2020). Microorganisms from these wastes, such as viruses, bacteria, mycoplasma, and endotoxins, can also compromise the cultured meat processing (Yao & Asayama, 2017). Thus, a process for eliminating these toxins must be studied before protein extraction and enzymatic hydrolysis (White et al., 2014).

5 Challenges, research needs, and future directions

Promising approaches are seen regarding the application of protein hydrolysates to replace some essential components of fetal bovine serum for cell growth in cultured meat production. As with all new technology, real efforts must be made to strengthen this field of innovation, and cell growth performance tests must be diligently performed to verify the advantages and limitations of applying protein hydrolysates in place of fetal bovine serum (Lobo-Alfonso et al., 2010). The potential of hydrolysates still requires extensive investigation in the future, as large-scale application involves greater physical and metabolic stress on cells that may compromise cultured meat production. In addition to the challenge of replacing essential components of fetal bovine serum, the application of non-animal protein hydrolysates

can also be investigated to help overcome other obstacles related to sensory, nutritional and consumer acceptance issues (O'Neill et al., 2021).

The constant maintenance of the biochemical profile of the hydrolysate in each batch produced can be one of the most significant effects to be studied. This variability in composition between batches can be minimized with ultrafiltration processes, which limit particle size from molecular weight based on membrane cutoff size (Spearman et al., 2014).

Continuous investigations of cell metabolism and mechanisms are also needed to effectively understand the performance of fetal bovine serum components and replace them with cheaper and ethically appropriate components (O'Neill et al., 2021). Special attention must be given to possible interactions between the components of the basal medium chosen for cultivation and the supplements to be used, as the components do not act alone and these interactions must be investigated in order not to negatively compromise cell growth and differentiation (Yao & Asayama, 2017).

Other factors that can significantly compromise the culture meat production are the cost of the enzyme used to obtain the hydrolysates, its commercial availability, and its ability to stay active. These parameters must be evaluated for the process to be economically viable and capable of scaling up, and enzymatic immobilization techniques can be suggested as alternative ways of reducing process costs (Contreras et al., 2019; Cruz-Casas et al., 2021). New methodologies associated with enzymatic hydrolysis should also be investigated to maximize reaction rates and reduce the amount of enzyme used. Microwaves, ultrasound, high-pressure assisted hydrolysis, hydrolysis using ultrafiltration membranes and high-voltage pulsed electric field are some of these techniques (Rizzello et al., 2016). Sensory aspects of the hydrolysates must also be evaluated, preventing undesirable bitter flavors from remaining in the cultured cells and interfering with the quality of the final product (Hamada, 2000), as well as the label characteristic of protein hydrolysates, since high temperatures or physical trauma can compromise the quality of the hydrolysates used for supplementation of the culture media (Lobo-Alfonso et al., 2010).

6 Conclusion

This review summarized technological approaches and new perspectives for the cultured meat process regarding the supplementation of the culture medium based on the substitution of essential and basic components of fetal bovine serum, minimizing environmental and health issues. There will likely be multiple approaches to replacing critical components and various combinations of them, but the use of non-animal protein agro-industrial waste

hydrolysates is a path to be explored and studied for the development of nutritionally favorable and protective culture media for the formation of cells at the high densities required in the processing of cultured meat. Although protein hydrolysates are a promising category for replacing critical compounds, many challenges still need to be overcome and worked on in future research.

Alongside the identification of critical fetal bovine serum molecules in animal cell growth, special attention must be paid to differences between species and cell types. Studying these gaps in an interdisciplinary way can make cultured meat cheaper and more accessible to the population in the future, reaching the potential of the technology. Furthermore, it proposes a new route for using and valuing non-animal agro-industrial waste for food production.

Declaration of competing interest

None.

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CAPÍTULO II: Effects of enzymes on protein extraction and post-extraction hydrolysis of non-animal agro-industrial wastes to obtain inputs for cultured meat

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Abstract

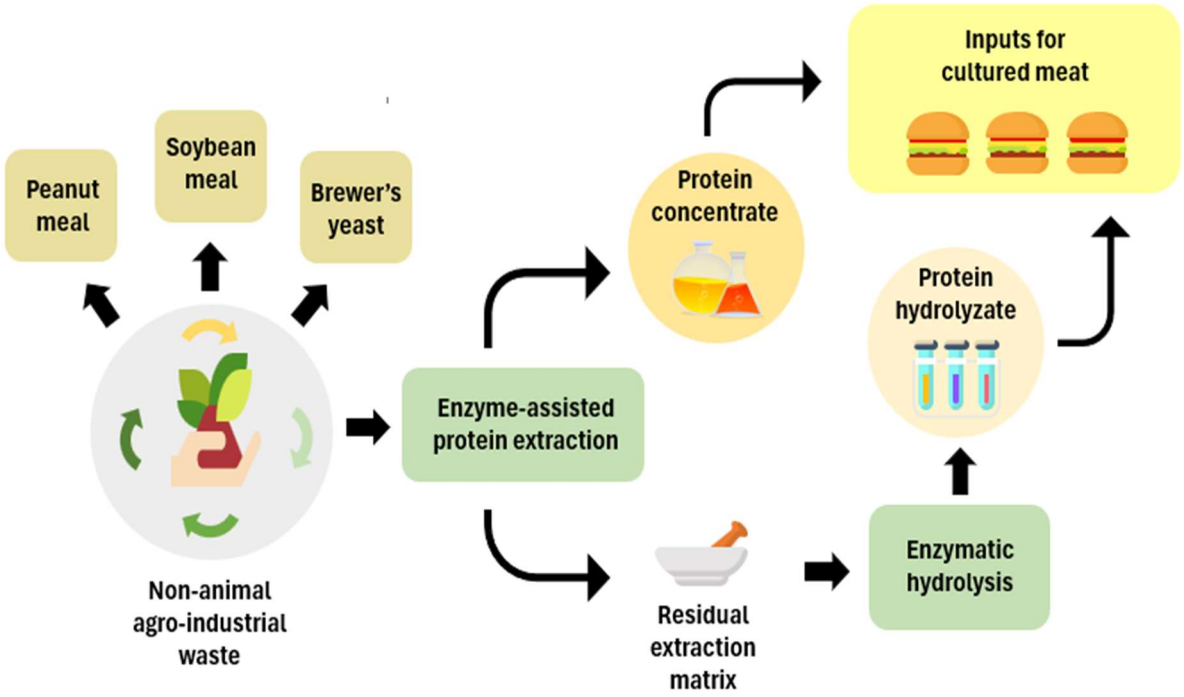
Non-animal agro-industrial wastes have a diverse and valuable nutritional composition for the alternative protein market. In this study, two routes for obtaining inputs of interest for cultured meat were evaluated, enzyme-assisted protein extraction from animal-free agro-industrial wastes and enzymatic hydrolysis of the extraction residue. Soybean meal, peanut meal, and brewer's yeast were the wastes evaluated and characterized, with soybean meal and peanut meal chosen based on their nutritional composition for the following studies. Viscozyme™ L assisted protein extraction was analyzed and compared with conventional methods. The protein extraction yield from these wastes increased considerably with the application of the enzyme and adjustments in the pH levels of the reaction medium. The residual protein extraction matrices were then subjected to enzymatic hydrolysis using different non-animal commercial enzymes (Alcalase™ 2.4L and Neutrase™ 0.8L). The application of Alcalase™ 2.4L proved to be the most indicated to maximize the degree of hydrolysis and optimize the reaction conditions, in order to obtain free amino acids and low molecular weight peptides required as inputs of interest for the supplementation of nutrients in cell cultures for processing cultured meat. The highest degree of hydrolysis obtained was 31.76 ± 0.43% for the soybean meal protein extraction residue and 30.59 ± 1.68% for the peanut meal protein extraction residue; at 50 °C, pH 8.0 and enzyme:substrate ratio of 3.5% and 5.0%, respectively. The results showed that the application of enzymes can maximize the use of protein from non-animal agro-industrial wastes and gives rise to a low-cost route, with less environmental impact and with nutritional potential for the generation of inputs required for cultured meat.

Keywords: wastes; enzymes; protein extraction; enzymatic hydrolysates; cultured meat

Highlights

- Proteins from non-animal agro-industrial wastes can be exploited to generate inputs for cultured meat.
- Non-animal agro-industrial wastes used to generate inputs for cultured meat is a cheaper process.
- Proteins, peptides, and amino acids are inputs required in high amounts for cultured meat.
- Application of carbohydrases in protein extraction can increase extraction yields.
- Proteases can maximize the degree of hydrolysis to obtain peptides and even free amino acids.

Graphical Abstract



1 Introduction

Population growth and the ethical and environmental limitations related to animal slaughter has encouraged the search for alternative protein sources (González-Pérez and Arellano, 2009; Sarkar, 2021). Accordingly, the world demand for protein obtained from non-animal derivatives, and their hydrolysates, should intensify in the coming years. Mainly with regards to the supply of nutrients required for animal cell cultivation in cultured meat

production (Rubio et al., 2020). The prospect of replacing fetal bovine serum (FBS) *in vitro* animal cell growth using low-cost components and free of animal derivatives, that could potentially reach industrial scale, is a great opportunity for this emerging technology in the food industry (Specht, 2020).

In this context, non-animal agro-industrial wastes have promising food quality components that can be found in large quantities (Contreras et al., 2019). Non-animal agro-industrial wastes with adequate nutritional properties and protein content can act as inputs to supplement animal cell culture medium, increasing cell density (Andreassen et al., 2020). In addition, the bioactivity of the obtained peptides, and the available free amino acids after hydrolysis of the wastes, can exert nutritional and physiological functions that favor the performance and regulation of cell cultures in the production of cultured meat (Andreassen et al., 2020; Contreras et al., 2019). The advantages of utilizing these materials, along with other inputs, for cultured meat goes beyond the nutritional benefits, as it can reduce the process costs and minimize the environmental impacts generated both by conventional livestock and by the incorrect disposal of these wastes (Pastore et al., 2013; Russ and Pittroff, 2004).

Among the non-animal agro-industrial wastes, oilseed cake or meal, which is obtained after oil extraction, stand out as a protein-rich biomass. These by-products contain an average of 35 – 45% protein. However, meals from sunflower and peanut reach an average composition of 40 – 50% and 50 – 60% protein, respectively. The abundance of production also favors the application of these biomasses, with soybeans responsible for 69% of world production (González-Pérez and Arellano, 2009).

The extraction of proteins from different sources of non-animal biomass can be performed using different methodologies with or without the addition of enzymes. Among the different conditions that can be used for extraction, pH is a significant parameter, as it interferes with the solubility of proteins, thus favoring extraction (Sari et al., 2013). The addition of enzymes such as proteases or carbohydrases, the type of biomass, and the extraction temperature are other factors that can contribute positively to protein extraction (Sari et al., 2013, 2015). Carbohydrases, such as Cellulases, Pectinases, and Viscozyme^{TM} L, have all been reported in the literature as enzymes that increase the protein extraction yield, as they hydrolyze the polysaccharides of the plant cell wall matrices and release proteins (Kim and Lim, 2016). However, the study of protein extraction from agro-industrial wastes using Viscozyme^{TM} L has currently been limited.

As for agro-industrial waste protein hydrolysis, they can be carried out using chemical or enzymatic pathways. Under enzymatic hydrolysis, the cleavage of peptide bonds

occurs from the application of proteases, such as Alcalase™ 2.4L and Neutrase™ 0.8 L. The product of the hydrolytic reaction is a mixture of peptides with different molecular weights and even free amino acids depending on the degree of hydrolysis achieved (Adler-Nissen, 1986; Pereira et al., 2019). However, for hydrolysates destined to be applied as inputs, and for them to have significant effects on animal cell growth in cultured meat, the maximum degree of hydrolysis is desired. Hydrolysis degree values greater than 20% contribute to the increase in cell density (Chabanon et al., 2008; Ho et al., 2021). Choosing the right enzyme and determining the reaction conditions (temperature, pH, and enzyme concentration) are essential to optimize the process (Adler-Nissen, 1986; Pereira et al., 2019).

Several studies of enzyme-assisted protein extraction and optimization of enzymatic hydrolysis of proteins have been developed. However, there are limitations in research regarding the method of obtaining low-cost inputs with less environmental impact for cultured meat. In this article, non-animal agro-industrial wastes with different chemical compositions were selected and tested for protein extractability, as well as for enzymatic hydrolysis of the residual extraction matrix. The enzymatic effects on protein extraction from non-animal agro-industrial wastes was one of the evaluated aspects. Furthermore, the optimization of the protein hydrolysates preparation methodology using the residual matrix from protein extraction, and investigation of the effect of hydrolysis using different enzymes were also evaluated. This study sought maximum protein utilization of the starting matrix, to demonstrate a cheap and efficient route to obtain inputs for cultured meat.

2 Material and methods

2.1 Agro-industrial waste

Different agro-industrial wastes (peanut meal, soybean meal, and brewer's yeast) with high protein content, obtained from local commerce, were evaluated. The meals were evaluated in their natural condition and defatted with hexane in the ratio of 1:4 (w/v), under constant agitation for 3 hours at room temperature. The hexane was removed by vacuum filtration and the meals were dried at room temperature (Feyzi et al., 2015). All wastes were submitted to a granulometric analysis and the size of the particles was standardized to values less than or equal to 0.4 mm for the subsequent steps.

2.2 Enzymes and reagents

A multi-enzyme complex of carbohydrases, Viscozyme™ L (100 FBG/g), was used for protein extraction. Alcalase™ 2.4L (2.4 AU-A/g) and Neutrase™ 0.8L (0.8 AU-N/g)

obtained from *Bacillus licheniformis* and *Bacillus amyloliquefaciens*, respectively, were used for enzymatic hydrolysis. The enzymes and analytical-grade chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3 Approximate composition of agro-industrial waste

Moisture, protein, lipid, crude fiber, and minerals (ashes) content were determined according to the AOAC (1998). For moisture determination, samples were dried in an oven at 105 °C until constant weight. Minerals were quantified by calcining the samples in a muffle at 550 °C for 12 hours. Total lipids were determined by direct extraction in a Soxhlet, using petroleum ether as a solvent, and crude dietary fibers were determined through the acid and basic digestion method, with subsequent muffle burning at 550 °C. Proteins were quantified by total nitrogen using the classical method of Kjeldahl. The protein content was calculated using a conversion factor of 6.25 for soybean meal, 5.46 for peanut meal, and 5.75 for brewer's yeast (Singh & Singh, 1991; Sgarbieri, 1996). The total carbohydrate content was obtained by difference.

2.4 Enzyme activity assay

The activity of the Viscozyme^{TM} L multienzyme complex was quantified through the determination of reducing sugars released during laminarin hydrolysis. The release of reducing sugars occurred from a reaction system containing 250 µL of laminarin solution (1%) in sodium acetate buffer (50 mM), pH = 5.0, and 125 µL of enzymatic solution. The reaction medium was maintained for 30 min at 37 °C and ended with the addition of 1.5 mL of 3,5-dinitrosalicylic acid. The released reducing sugars was determined by the spectrophotometric method at 550 nm (Bauermeister et al., 2015). The unit of fungal beta-glucanase (FBG) corresponds to the amount of enzyme preparation required for the hydrolysis of β-glucan to reduce carbohydrates corresponding to 1 µmol of glucose per min (Tu et al., 2015).

The proteolytic activity of Alcalase^{TM} 2.4L and Neutrase^{TM} 0.8L enzymes were measured using the protocol proposed by Charney and Tomarelli (1947) and adapted by De Castro and Sato (2014), using azocasein as substrate. Aliquots of 0.5 mL of azocasein (0.5% m/v) in 0.1 mol/L Tris-HCl buffer (pH 8.0) and 0.5 mL of enzyme preparation were incubated in a water bath at 60 °C for 40 min. The reaction was terminated with the addition of 0.5 mL of trichloroacetic acid (TCA) (10% m/v). Samples were centrifuged at 17,000 × g at 25 °C for 15 min and 1 mL of the supernatant was neutralized with 1 mL of KOH (5 mol/L), and absorbance

was measured at a wavelength of 428 nm. A proteolytic unit (U) is defined as the amount of enzyme required to change the absorbance by 0.01 unit per reaction minute.

2.5 Protein extraction from agro-industrial waste

Conventional, alkaline, and enzyme-assisted Viscozyme^{TM} L extraction methods were investigated for protein extraction from the studied agro-industrial waste, as described by Tu et al. (2015), with modifications. All processes were performed in triplicate. Conventional extraction was carried out in a single step, mixing the agro-industrial wastes with deionized water at a mass ratio of 1:10, in a thermostatic orbital shaker, under conditions described in Table 1. The alkaline extraction was carried out under the same conditions as the conventional extraction, but with a pH adjusted to 10.0. For the enzyme-assisted Viscozyme^{TM} L method, a greater detailing of the studies required studies for protein extraction and solubilization. For this, enzyme-assisted extractions were performed with simple or sequential steps under different pH conditions, in order to favor enzymatic activity in the optimal pH range and promote maximum protein solubilization. For this, 250 ml Erlenmeyer flasks containing 10 g of agro-industrial waste and 100 ml of deionized water were incubated in a thermostatic orbital shaker. The amount of Viscozyme^{TM} L enzyme added was 5 FBG per gram of dry mass, and the pH was adjusted following the conditions and steps also described in Table 1.

Table 1. Experimental conditions of protein extraction

Extraction methods	pH		Temperature (°C)		Extraction time (min)		Mixing rate (rpm)	
	1st step	2nd step	1st step	2nd step	1st step	2nd step	1st step	2nd step
Conventional	7	-	30	-	120	-	200	-
Alkaline	10	-	30	-	120	-	200	-
	5	-	50	-	120	-	200	-
	7	-	50	-	120	-	200	-
Enzyme-assisted	5	10	50	30	120	120	200	200
	6.5	10	50	30	120	120	200	200
	7	10	50	30	120	120	200	200

After incubation, the samples were centrifuged at 3,500 rpm at 20 °C for 20 min to remove solids. The supernatant was adjusted to a pH 4.5 using 0.1 M HCl for protein coagulation at the isoelectric point. The coagulate was separated by centrifugation at 5,000 rpm at 20 °C for 20 min and redissolved in distilled water. The protein coagulation procedure was

repeated twice more times to increase the protein concentration rate. The protein concentrate obtained was frozen and lyophilized, later its content was characterized by the Kjeldahl digestion method as described in item 2.3, and its molecular weight distribution was analyzed using the SDS-polyacrylamide gel electrophoresis method (SDS-PAGE), as described in item 2.5.1. The solids were dried in an oven at 30 °C for 24 h and followed by enzymatic hydrolysis of residual proteins, in order to obtain other inputs of interest.

2.5.1 SDS-PAGE electrophoresis

The protein concentrates obtained were investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970). 2.5 mg of protein concentrates were mixed with 1,5 mL sample buffer (deionized water, 0.5 M Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, glycerol, and β-mercaptoethanol). Protein markers and samples (10 µL) were loaded onto 12,5% of the separation gel and 4% of the stacking gel. The electrophoresis system was set and run under a constant current of 150 V. The sample-loaded gel were stained overnight in a solution of Coomassie Brilliant Blue and de-stained in acetic acid/methanol solution under gentle shaking. The molecular weight of proteins was estimated by comparing their electrophoretic mobility with that of known protein standards. Protein standards used in the electrophoretic analysis were purchased from Thermo Fischer Scientific (Waltham, MA, USA), with molecular weight range between 10 and 180 kDa.

2.6 Study of residue hydrolysis conditions

The residual material from protein extraction was enzymatically hydrolyzed for maximum use of waste. For this, a kinetic study was carried out by utilizing the enzymes and conditions described by Schlegel et al. (2020) (Table 2); either individually or in combination, and simultaneously or sequentially.

Table 2. Experimental hydrolysis conditions for different systems

Code	Enzymatic systems	Temperature (°C)	pH
A	Alcalase^{TM} 2.4L	50.0	8.0
N	Neutrase^{TM} 0.8L	50.0	7.0
A+N	Alcalase^{TM} 2.4L + Neutrase^{TM}	50.0	8.0
A->N	Alcalase^{TM} 2.4L -> Neutrase^{TM}	50.0	8.0 -> 7.0

(1) The enzymatic combination represented by Alcalase^{TM} 2.4L + Neutrase^{TM} 0.8L indicates simultaneous hydrolysis with the two enzymes.

(2) The system described by Alcalase^{TM} 2.4L -> Neutrase^{TM} 0.8L represents sequential hydrolysis employing the two enzymes.

A residue suspension (residue:water ratio of 1:10 w/v) was heated and pH adjusted using a 2 M NaOH solution, according to the conditions given in Table 2, for each enzymatic system. The enzyme or enzyme combination (50:50 ratio) was added at an enzyme:substrate ratio initially set at 1:100, to minimize influence on the process cost (Zhao et al., 2012). For the sequentially conducted enzyme system, the second enzyme was added to the reaction medium 1 hour after the addition of the first enzyme (Schlegel et al., 2020). The pH was controlled during the reaction and adjusted every time it differed by 0.1 unit from the initial pH, done through the addition of 2 M NaOH using a burette.

The response variable in the enzymatic kinetics study was the degree of hydrolysis (DH, %) determined from the pH-stat method, based on the titration of the α-amino groups released at constant pH and temperature during enzymatic hydrolysis (Adler-Nissen, 1986). To calculate the degree of hydrolysis, the volume of the base consumed was recorded at 5 min intervals in the first hour, every 10 min in the second hour, every 15 min for the next two hours, and then every 30 min. The reaction time was evaluated until variations of base consumption in the degree of hydrolysis analysis were insignificant (Nchienzia et al., 2010).

Proteases were inactivated through heating in a water bath at 85 °C for 10 min and the hydrolysates were then centrifuged at 20 °C for 15 min at 5,000 rpm to separate impurities. The supernatants were vacuum filtered and kept at -20 °C or lyophilized (Zhao et al., 2012). After knowing the hydrolysis kinetics of the different systems and ensuring their correlation with the degree of hydrolysis, the hydrolysis conditions were determined in an optimization study.

2.7 Optimization of hydrolysis conditions

The system with the best correlation between the reaction time and the degree of hydrolysis highlighted in the enzymatic kinetics study, was selected for the optimization of hydrolysis conditions. The independent variables studied were temperature and enzyme:substrate ratio and the response variable analyzed was the degree of hydrolysis. The temperature range was evaluated according to the conditions listed by the manufacturer of the enzymes, and enzyme:substrate ratio was determined from a defined variation between 0.5% and 5% (v/v) (Santos et al., 2020). The pH was not analyzed as an independent variable, and was kept fixed at the optimal value for the enzyme chosen in the study, which follows the results described in the literature (Santos et al., 2020). For this, a Composite Rotational Central Design - DCCR (2^2) with 11 trials, contemplating 4 axial points and 3 repetitions in the central point

was conducted. The reaction was also monitored through the pH-stat method, and statistical analysis of the factorial design was performed using the Protimiza Experimental Design software at 5% significance. For model validation, the degree of hydrolysis generated from the enzymatic hydrolysis of the residues under optimal conditions was tested and compared with the estimated values.

The general scheme referring to the stages of this methodology for the maximum use of proteins from non-animal agro-industrial wastes is presented in Figure 1.

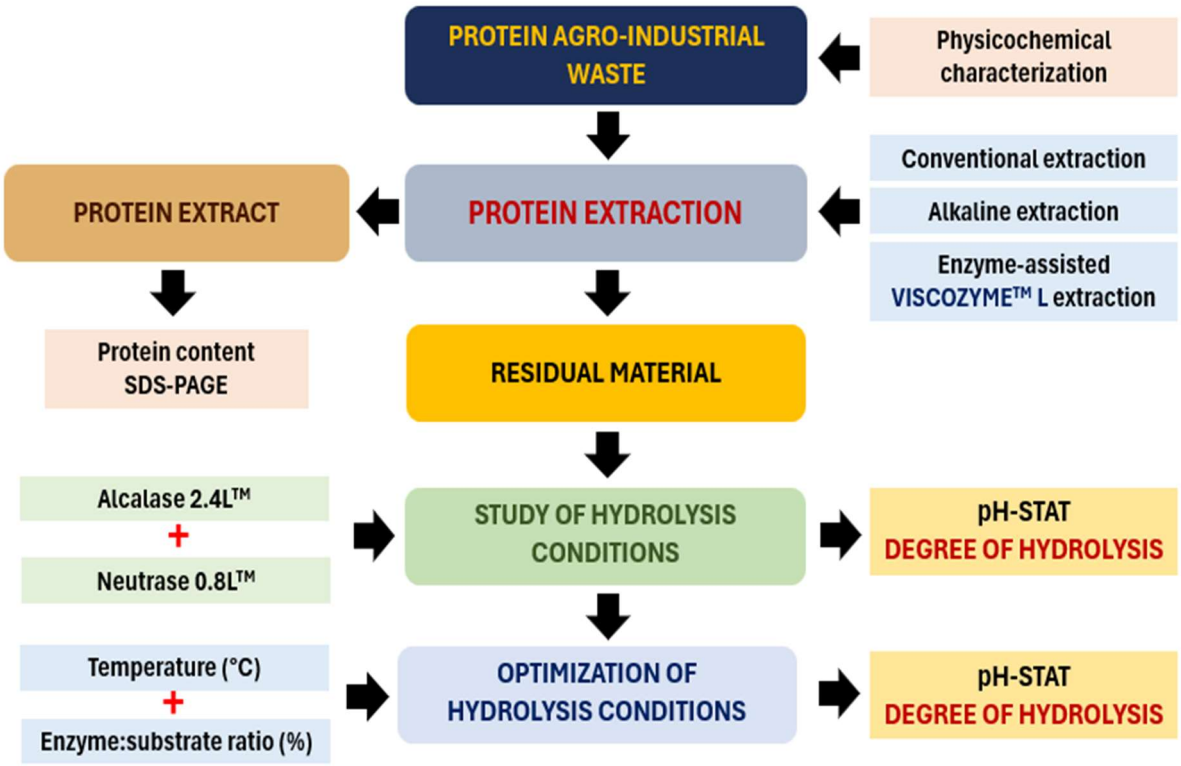


Figure 1. Flowchart of the methodology with the steps applied for the maximum use of proteins from agro-industrial wastes.

3 Results and discussion

3.1 Approximate composition of agro-industrial waste

The characterization of agro-industrial wastes was carried out to define the best matrices for obtaining inputs of interest for cultured meat based on protein content, in relation to protein availability compared to lipid content and ash content that can negatively compromise the animal cell growth (Pasupuleti & Braun, 2010). Data on the approximate composition of the analyzed agro-industrial wastes are presented in Table 3.

Table 3. Approximate composition of the agro-industrial wastes analyzed

Composition (%, w/w)	Soybean meal	Defatted soybean meal	Peanut meal	Defatted peanut meal	Brewer's yeast
Moisture	10.57 ± 0.02	9.73 ± 0.05	7.33 ± 0.52	7.23 ± 0.22	5.07 ± 0.06
Crude protein	47.57 ± 0.10	48.29 ± 0.03	43.15 ± 0.03	43.64 ± 0.42	29.00 ± 0.03
Crude oil	1.46 ± 0.12	0.68 ± 0.14	2.05 ± 0.15	1.05 ± 0.08	1.11 ± 0.38
Crude fiber	6.89 ± 0.33	6.38 ± 0.90	11.18 ± 0.42	11.53 ± 0.89	0.60 ± 0.25
Ash	6.26 ± 0.04	6.30 ± 0.02	6.63 ± 0.14	6.47 ± 0.12	24.49 ± 0.20
Total sugar	27.25 ± 0.61	28.62 ± 1.14	29.66 ± 1.26	30.08 ± 1.74	39.73 ± 0.92

From the results presented in Table 3, a higher protein content was observed in soybean meal and peanut. Regarding the protein and lipid differences between the defatted meal and the not-defatted meal, the results did not show greater protein availability with the defatting method, since the not-defatted meal already had a low lipid concentration, not providing an effective increase in the protein content. Thus, the degreasing method proved to be unnecessary for the matrices under study. In addition, the elimination of hexane, a highly toxic solvent, from the process is extremely favorable. Thus, soybean and peanut meal were the two matrices selected for the following steps of the methodology, as they have a protein content of around 47.57% and 43.15%, respectively. The values obtained for soybean meal and peanut meal are close to the protein contents reported in the literature, these being 44.4% and 48.2% respectively (Rostagno et al., 2011). Based on the other components, special attention should be given to the higher crude fiber content of peanut meal compared to soybean meal, which can make protein extraction difficult and even compromise the quality of the extracted protein.

It is worth noting that the evaluated brewer's yeast had a lower protein content and a high ash composition when compared to the values indicated in the literature (41.8% and 3.64% respectively) (Rostagno et al., 2011). According to information provided by the supplier, the analyzed brewer's yeast contained mineral salt supplementation, for use as animal feed, which may have compromised the values; and therefore, distancing them from the values cited in the literature.

3.2 Protein extraction from agro-industrial waste

Concerning protein extraction from soybean and peanut meal, the yields obtained are shown in Table 4.

Table 4. Yields for protein extraction methods of soybean and peanut meal

Waste	Extraction methods	pH		Extraction yield (%)	Protein content (%)	Extracted protein yield (%)	Increase to conventional method (%)
		1st step	2nd step				
Soybean meal	Conventional	7	-	3.24 ± 0.34	78.09 ± 4.34	2.66 ± 0.29	-
	Alkaline	10	-	17.18 ± 0.98	78.63 ± 2.36	13.51 ± 0.77	407.89
	Enzyme-assisted	5	-	1.45 ± 0.002	75.95 ± 1.52	1.16 ± 0.09	-56.39
	Enzyme-assisted	7	-	7.56 ± 0.47	77.83 ± 0.58	5.89 ± 0.25	121.43
	Enzyme-assisted	5	10	21.13 ± 0.39	75.32 ± 1.61	15.52 ± 0.71	483.46
	Enzyme-assisted	6.5	10	27.95 ± 1.60	80.46 ± 0.15	22.49 ± 1.29	745.49
	Enzyme-assisted	7	10	31.69 ± 0.90	86.45 ± 1.19	27.40 ± 0.96	930.08
Peanut meal	Conventional	7	-	4.53 ± 0.41	72,13 ± 0.50	3.27 ± 0.30	-
	Alkaline	10	-	28.52 ± 0.89	77.96 ± 1.79	21.29 ± 0.66	551.07
	Enzyme-assisted	5	-	0.77 ± 0.11	70.38 ± 0.80	0.54 ± 0.08	-83.49
	Enzyme-assisted	7	-	7.50 ± 0.21	69.70 ± 1.65	5.66 ± 0.76	73.09
	Enzyme-assisted	5	10	23.29 ± 0.90	70.61 ± 0.91	16.44 ± 0.63	402.75
	Enzyme-assisted	6.5	10	27.51 ± 0.26	70.16 ± 0.76	18.49 ± 0.41	465.44
	Enzyme-assisted	7	10	33.98 ± 1.23	76.80 ± 1.20	26.10 ± 0.95	698.17

From the data presented in Table 4, it is verified that the application of Viscozyme™ L (95 FBG/g) considerably increases the protein extraction yields from the evaluated agro-industrial wastes, depending on the different extraction parameters. Except for the single step analysis at pH 5.0, the extraction yields were substantially higher than those of conventional extraction. The highest protein extraction yield obtained occurred from Viscozyme™ L enzyme-assisted extraction in 2 steps, with a neutral initial pH and protein solubilization at pH 10.0. Under these conditions, the yield obtained from soybean meal was 27.40 ± 0.96% (930.08% higher than conventional extraction); and for peanut meal it was 26.10 ± 0.95% (698.17% higher than conventional extraction). The higher extraction yield for soybean meal compared to peanut meal may be related to the greater protein availability in soybean meal, but also to the lower fiber content present.

The lowest yields were observed from extractions with pH values close to the isoelectric point (pH 4.5), since the lowest solubility of peanut and soybean meal protein occurs in this region, as described by Feyzi et al. (2015) and Wang et al. (2009), respectively. At the isoelectric point (pI), proteins carry no net charge and with the reduction of electrostatic repulsion, protein aggregation and precipitation occur. Electrostatic repulsion in more acidic and alkaline media increases protein solubilization (Feyzi et al., 2015), which explains the higher extraction yields for solubilization at an alkaline pH. However, the use of Viscozyme™ L under the described optimal conditions, with pH being the main characteristic evaluated, also

promoted a significant increase in the proteins extracted; when compared to the pure alkaline method.

Enzyme-assisted aqueous extraction and alkaline solubilization favored the extraction of the fraction of “albumin” from the waste that is soluble in water; “glutelin” which is soluble in dilute basic solutions; and “globulins”, which are soluble in dilute saline solutions due to the presence of minerals in the plant matrix (Osborne, 1924; Villareal and Juliano, 1981). The high presence of these protein fractions in the used wastes results in the increase in the increased extraction values obtained when applying the enzymatic pre-treatment with alkaline solubilization (Makeri et al., 2017; Silva et al., 2012). It is worth noting that peanut proteins have good solubility in water, since the polar groups are positioned on their surfaces (Yu et al., 2007a); and the high polar amino acid content present in the wastes studied, favors the extraction of proteins using these methodologies, with about 47% of the amino acids present in soybean meal and 50.5% in peanut meal being polar (Feyzi et al., 2015).

Consequently, pretreatment with Viscozyme^{TM} L for cell wall polysaccharides hydrolysis had a more significant effect on protein extraction at pH values farther from the isoelectric point; indicating that pH directly affects protein extraction yields, due to protein solubility. However, the effectiveness of Viscozyme^{TM} L in protein extraction cannot be generalized to other plant matrices, as the performance of this enzyme can change according to the plant matrix used (Görçüc et al., 2019).

Thus, protein extraction for soybean and peanuts meal was conducted using the two-step enzyme-assisted method with Viscozyme^{TM} L, at an initial pH of 7.0, with solubilization at pH 10.0; to guarantee maximum yield. The pH values greater than 10 were not evaluated, as the literature cites undesirable changes and even protein denaturation under these conditions (Yu et al., 2007b). Furthermore, a detailed study of the effects of Viscozyme^{TM} L concentration and temperature on the extraction of these agro-industrial wastes may be a point of future study to improve yields since Rosset et al., (2012) achieved values around 56.2% for the extraction of defatted soy flour with higher process temperatures (60 °C); greater than the optimal range indicated by the manufacturer (40 to 50 °C) (Novozymes, 2023).

Considering that cell proliferation and differentiation medium used in the cultivation of animal cells requires the addition of purified proteins (Yamanaka et al., 2023), the exploration of this methodological route for protein extraction becomes a promising aspect for obtaining inputs used in the cultured meat industry. However, to obtain purified protein for application as an input in cultured meat, additional steps must be added to the methodology; these include dialysis, precipitation, and centrifugation (Hamada, 1997).

3.2.1 SDS-PAGE electrophoresis

Proteins extracted using the Viscozyme^{TM} L assisted method under maximum yield conditions were separated according to their molecular weight (MW) distribution by SDS-PAGE, as shown in Figure 2.

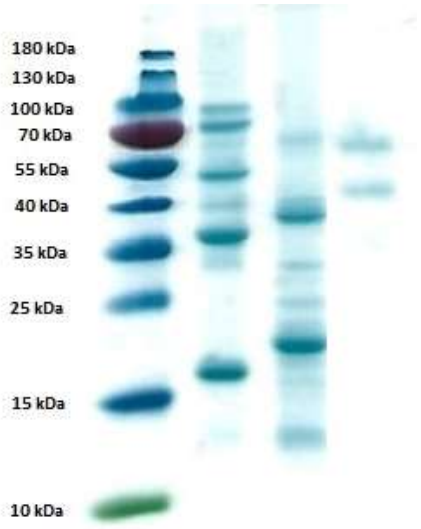


Figure 2. SDS-PAGE profile of the protein concentrates obtained through enzymatic extraction of soybean meal (a), peanut meal (b), and the protein profile of the Viscozyme^{TM} L enzyme used in the extraction (c).

The bands present in the Viscozyme^{TM} L enzyme profile (Figure 2 (c)) were verified in the protein extracts profiles (Figure 2 (a) and (b)), and were not considered in the analysis of proteins present in the extracts.

The main protein bands obtained from soybean meal were around 35 – 40 kDa and 15 – 25 kDa. Bands with lower intensity are identified in the regions of 100 kDa, 70 kDa, 55 kDa, and between 25 – 35 kDa. In the low molecular weight region, a subtle band can be seen between 10 – 15 kDa. González-Pérez and Arellano, (2009) point out that glycinin (11S globulin) and β-conglycinin (7S globulin) are the most important proteins in soybeans. Glycinin is composed of a basic polypeptide (20 kDa) linked to an acidic polypeptide (38 kDa) via a disulfide bond. While, β-conglycinin is characterized as a trimeric glycoprotein, with three subunits in different combinations around 57 – 72 kDa, 57 – 68 kDa, and 45 – 52 kDa. The albumins present in soybeans, on the other hand, belong to a broad family of proteins, are compact and globular with high solubility in water, and have lower molecular weights, ranging

from around 10 to 18 kDa. However, Gorinstein et al. (2001) studied soy albumin and found a higher concentration in the range of 35 to 80 kDa. In general, the albumins present in soy are complex and have a greater number of bands with larger variation in the molecular weight of their protein fraction (Ciabotti et al., 2016).

The compatibility of the bands found in the SDS-PAGE analysis of soy meal protein extract with the molecular weights described in the literature demonstrates the presence of globulins and albumins in the protein extract. The protein content of the soybean meal extraction can be explained by the methodology employed since albumin extraction via an aqueous solution is normally used in plant matrices (Amagliani et al., 2017). However, contamination by globulins is frequent in the extracts due to the minerals present in plant matrices, which dissolve in water and promote greater solubility of globulins, especially in saline solutions (Villareal and Juliano, 1981). Since albumin is one of the main types of protein required as an input for the growth of animal cells, and globulins also a part of the composition of fetal bovine serum used to supplement the culture medium in mammalian cell cultures (O'Neill et al., 2021); the protein extract obtained from soybean meal can be evaluated as a possible source of protein input for cultured meat.

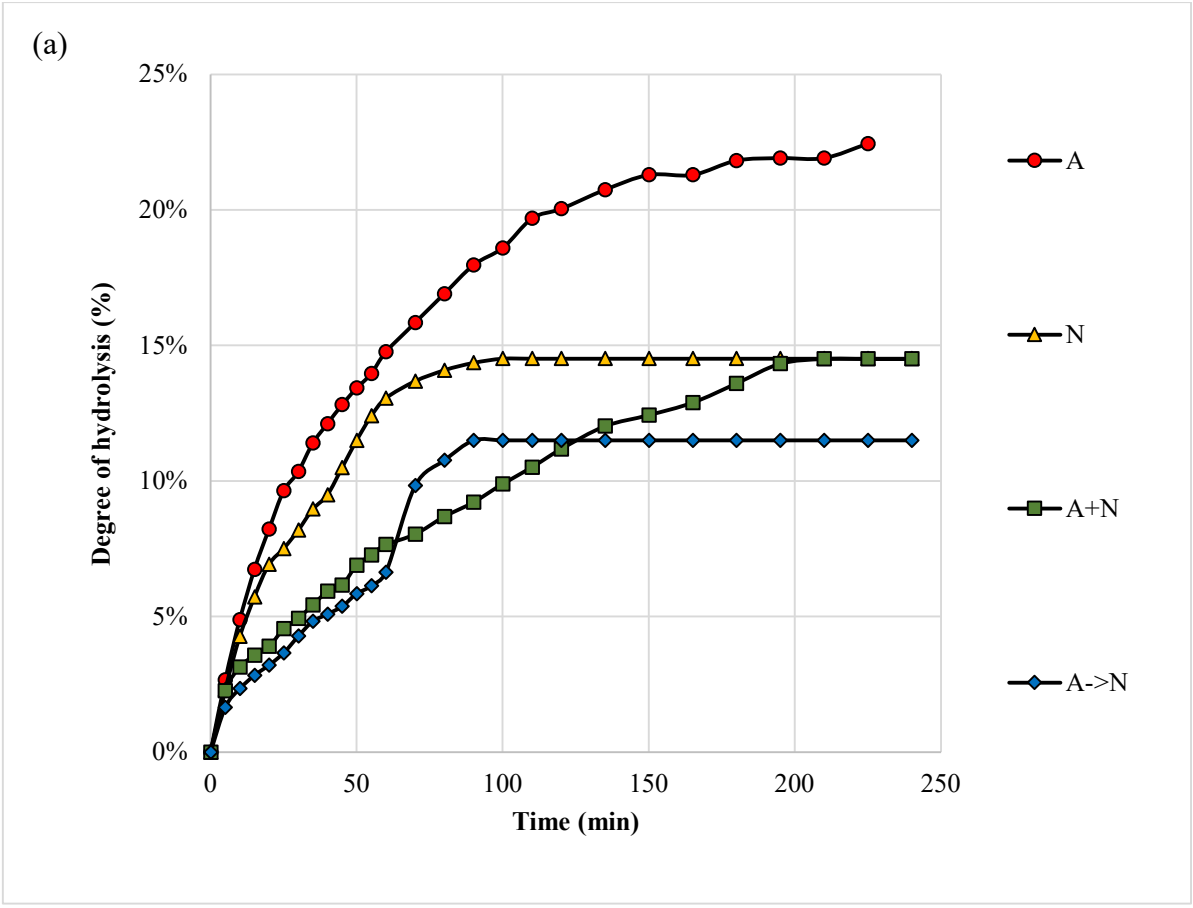
In regards to the protein extract obtained from peanut meal, bands with low intensity were found at 10 – 15 kDa and 25 – 35 kDa. Bands with higher intensities were identified in the 15 – 25 kDa and 35 – 40 kDa regions. The literature indicates that the main proteins present in peanuts, as well as in soybeans, are albumins and globulins. Among the globulins, arachin, and conarachin are the two main ones, comprising more than 85% of peanut protein. The conarachin content is equivalent to only 25% of the globulins and the remainder is composed of the arachin subunits (Seifert, 2010). Accordingly, five main protein subunits present in peanuts can be highlighted: the conarachin region (MW > 50 kDa), the arachin acid region (MW 38 – 49.9 kDa), the intermediate MW region (23 – 37.9 kDa), the basic region of arachine (MW 18 – 22.9 kDa) and the low MW region of the protein (14 – 17.9 kDa) (Bianchi-Hall et al., 1993). However, from the protein profile identified through SDS-PAGE for the peanut meal protein extract, only bands with a molecular weight below 50 kDa were clearly identified.

The likely presence of albumin in the samples can be demonstrated through a comparison with a study conducted by Bueno-Díaz et al. (2021), the study reported that albumin isolated from different oilseeds, such as pistachios, almonds, walnuts, pine nuts, sesame, and others, had a molecular weight in the range of 12 to 15 kDa; when separated using SDS-PAGE. Furthermore, Apostolovic et al. (2021) also reported that peanut allergens caused by the 2S albumin protein family were identified as bands around 15 to 20 kDa. These bands are also

observed from proteins extracted from peanut meal in current study, indicating the potential presence of albumins and globulins in the obtained protein extract. Together with soybean meal, protein extraction from peanut meal can be seen as a possible route for the development of cultured meat inputs.

3.3 Study of residue hydrolysis conditions

After obtaining the protein extract, the residual material from the extraction was subjected to enzymatic kinetic analysis for 240 min under conditions defined by Schlegel et al. (2020); to verify the influence and of the specificity of different enzymes (Alcalase^{TM} 2.4L – 18,000 U mL^{-1} and Neutrase^{TM} 0.8L – 200,000 U mL^{-1}), and enzymatic combinations have on the degree of hydrolysis on these residual matrices. The system with the most significant result, regarding the degree of hydrolysis was used for the subsequent studies. The kinetic curves obtained are shown in Figure 3 (a) and (b), for soybean and peanut meal residues from protein extraction, respectively.



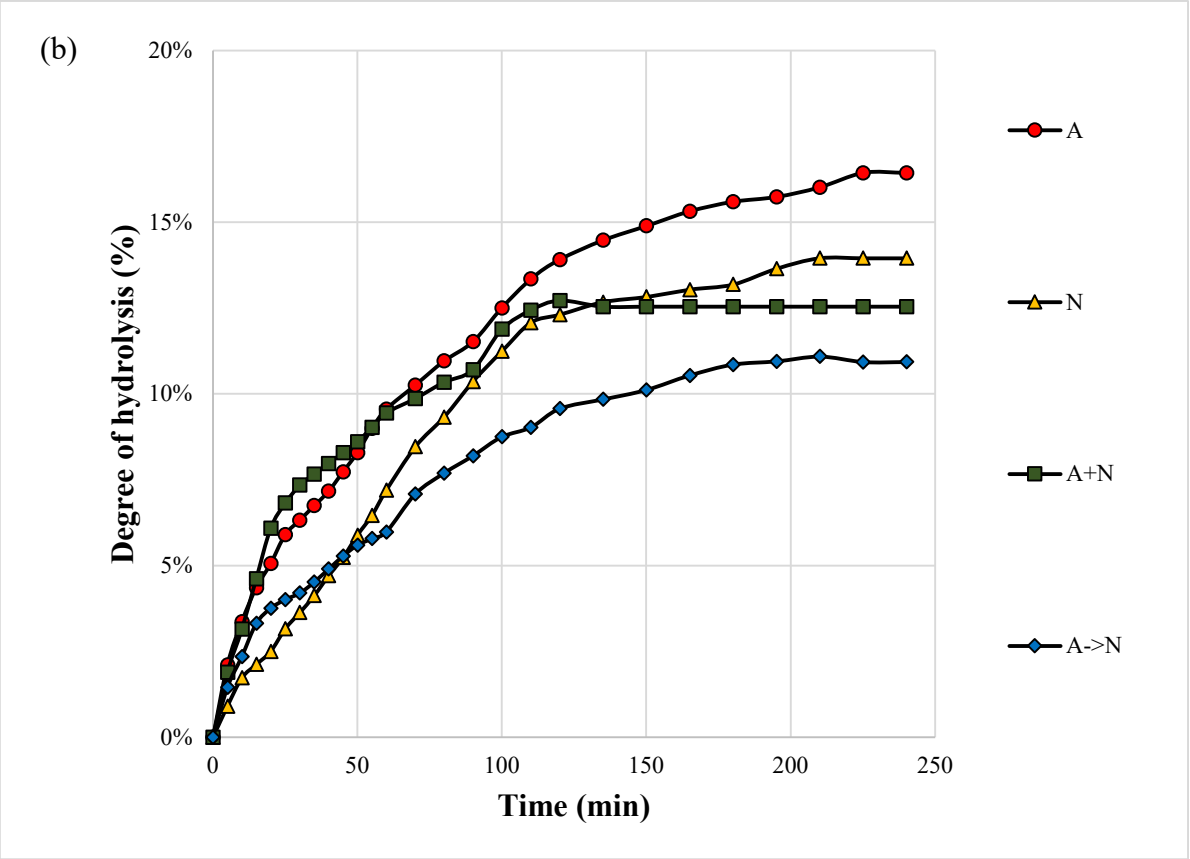


Figure 3. Kinetics of the hydrolysis from soybean meal (a) and peanut meal (b) protein extraction residues.

Based on the results for the reaction systems evaluated, a high reaction rate is observed in the initial minutes with an increase in the degree of hydrolysis. This is due to the extensive breakdown of peptide bonds. Subsequently, there is a decrease in the reaction rate, with a slight increase in the value of the degree of hydrolysis, followed by a constant maximum value. The reaction rate reductions observed over time for the evaluated systems have been described in the literature, and may be related to factors such as: the reduction in the number of available peptide bonds, due to enzymatic hydrolysis; and a possible inhibition of enzymes caused by hydrolysis products or by enzyme inactivation (Kılıç Apar and Özbek, 2007).

Concerning the analyzed matrices, the enzymatic kinetics related to hydrolysis from the sequential combination of enzymes Alcalase™ 2.4L and Neutrase™ 0.8L, showed an increase in the degree of hydrolysis after 60 min of reaction with the addition of the second enzyme into the system; which justifies the sigmoidal shape of the curve. This increase in degree of hydrolysis was more pronounced for the soybean meal extraction residue than for the peanut meal extraction residue. However, this combination was responsible for the lowest verified degree of hydrolysis, achieving a maximum of 11.50% for the soybean meal residue

and 10.93% for peanut meal residue. Although the combined use of enzymes did not give significant results for the analyzed hydrolysis, the literature indicates that pre-treatment with microfluidization of matrices exposed to hydrolysis can increase the breakdown of different proteins with the application of different enzymes; as core groups hidden in the protein structure become more exposed, although further explorations in this areas are still needed (Chen et al., 2016; Zhang et al., 2021). Furthermore, this work focused only on the degree of hydrolysis, the combination of proteases in enzymatic hydrolysis can favor the obtaining of peptides with different bioactivities, depending on the required needs (Ceylan et al., 2022).

The kinetic curves resulting from the other studied enzymatic hydrolysis systems had a similar behavior and showed standard asymptotic formats. For both residues, the highest degree of hydrolysis was obtained with the exclusive application of Alcalase^{TM} 2.4L, achieving a maximum of 22.44% for the soybean meal protein extraction residue and 16.44% for the peanut meal protein extraction residue.

Alcalase^{TM} 2.4L, is a non-specific protease and contains a complex of endopeptidases and displays differences in activity and selectivity (Andreassen et al., 2020); which may explain the degree of hydrolysis results when compared to the results of the other enzymatic systems evaluated, under the same reaction conditions used. Similar actions of Alcalase^{TM} 2.4L have also been reported during hydrolysis of chia expeller (Ozón et al., 2022), chia seeds (Urbizo-Reyes et al., 2019), corn protein (Jin et al., 2016) and canola proteins (Cumby et al., 2008). This behavior is related to the fact that Alcalase^{TM} 2.4L has a wide specificity for peptide bonds, promoting degrees of hydrolysis around 20 – 25%, in short time periods and moderate reaction conditions (Graycar et al., 2013; Ozón et al., 2022).

Since the objective of this study was to identify the enzymatic system with the highest values of degree of hydrolysis, to obtain free amino acids and low molecular weight bioactive peptides for the supplementation of culture media for animal cell growth, the Alcalase^{TM} 2.4L was found to be the best protease for the optimization step of the enzymatic hydrolysis process of residues from the protein extraction of soybean meal and peanuts.

3.4 Optimization of hydrolysis conditions

To improve amino acid and bioactive peptide availability in culture medium used for animal cell growth, it is essential to replace the components of fetal bovine serum for cultured meat production. Therefore, the enzymatic hydrolysis of agro-industrial protein residues is necessary for the maximum degree of hydrolysis (Chabanon et al., 2008; Ho et al., 2021). For this, variables such as temperature and the enzyme:substrate ratio must be carefully

evaluated to maximize their influence on the degree of hydrolysis. Therefore, experimental planning for the development of an optimized and economical process is essential (Adler-Nissen, 1986).

The time period assessed for enzymatic hydrolysis was set at 2 hours, based on the preliminary enzymatic kinetic study; after this initial time, the reaction rates become less significant. Thus, a suspension (residue:water) in the proportion 1:10 (w/w) was heated, had the pH adjusted to 8.0 with the application of 1 M NaOH and 1 M HCl solution, and received Alcalase^{TM} 2.4L, according to the conditions indicated in the design matrix. The design matrix and the results for the obtained for the soybean and peanut meal protein extraction residue tests are presented in Table 5.

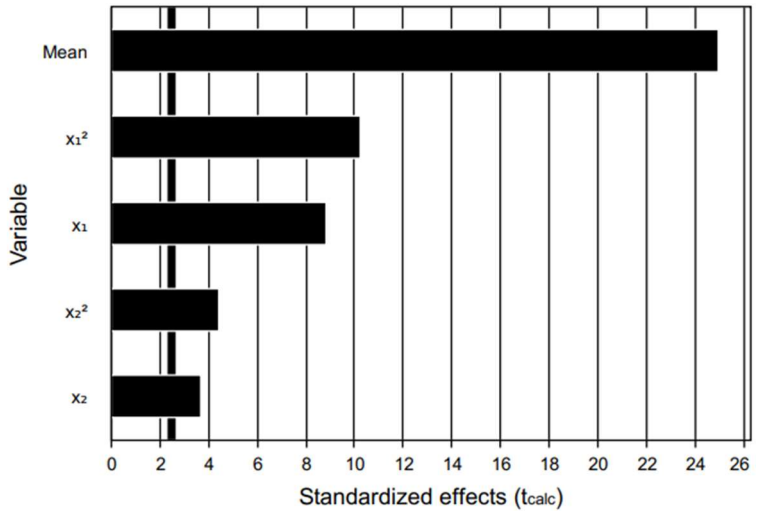
Table 5. Tests and respective degrees of hydrolysis obtained from soybean and peanut meal protein extraction residues.

Assay	X_1*	X_2*	Temperature (ºC)	Enzyme:substrate ratio (%)	Degree of hydrolysis (%) of Soybean meal residue	Degree of hydrolysis (%) of Peanut meal residue
1	-1	-1	30.8	1.15	5.02	8.55
2	1	-1	59.2	1.15	15.78	14.15
3	-1	1	30.8	4.34	7.90	11.64
4	1	1	59.2	4.34	21.04	21.81
5	-1.41	0	25.0	2.75	4.47	7.91
6	1.41	0	65.0	2.75	16.53	16.19
7	0	-1.41	45.0	0.50	15.33	13.54
8	0	1.41	45.0	5.00	21.68	25.81
9	0	0	45.0	2.75	23.57	21.32
10	0	0	45.0	2.75	23.32	21.26
11	0	0	45.0	2.75	23.78	20.68

*X_1 = temperature (ºC); X_2 = enzyme:substrate ratio (%).

From the results presented in Table 5, the repetitions at the central points for soybean meal residue enzymatic hydrolysis achieved highest values of degree of hydrolysis, around 23.57%. While for the peanut meal residue, assay 8 achieved the highest degree of hydrolysis with 25.81%. In terms of the influence of the variables studied, the Pareto charts shown in Figures 4 (a) and (b), indicate the effects of temperature and enzyme:substrate ratio have on the degree of hydrolysis; with a significance level of 5% ($p<0.05$).

(a)



(b)

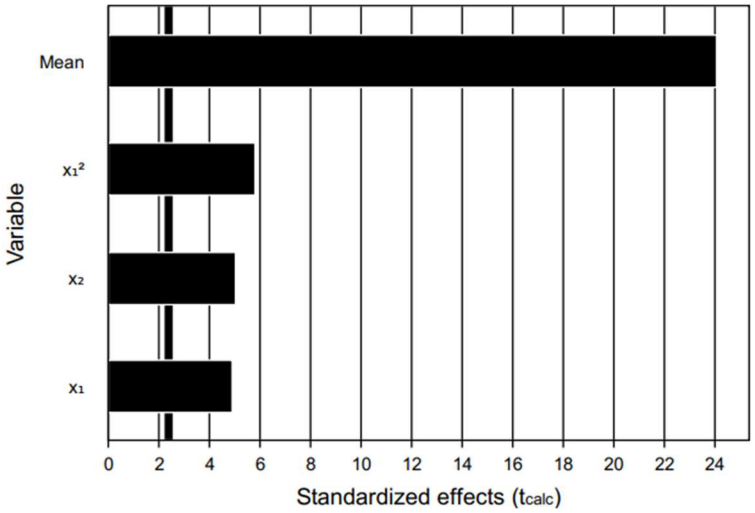


Figure 4. Pareto chart for the enzymatic hydrolysis of soybean meal (a) and peanut meal (b) residues ($p<0.05$).

From the Pareto chart in Figure 4 (a), it can be seen that the linear and quadratic terms of temperature and the enzyme:substrate ratio were significant for the enzymatic hydrolysis of the soybean meal protein extraction residue with Alcalase^{TM} 2.4L, for the 5% significance level analyzed ($p<0.05$). However, interactions between variables were not significant in this study. The Pareto chart in Figure 4 (b), it was possible to analyze that the linear and quadratic terms of temperature were significant for the enzymatic hydrolysis of the peanut meal protein extraction residue with Alcalase^{TM} 2.4L, as well as the linear term of enzyme concentration for the 5% significance level analyzed ($p<0.05$). Interactions between variables were not significant and neither was the quadratic term of enzyme concentration in this study. It was also possible to perform an analysis of variance (ANOVA) using the Pareto

analysis. Analysis of variance (ANOVA) indicated a percentage of explained variation (R^2) of 97.08% for the soybean meal protein extraction residue and 92.28% for the peanut meal residue, in addition to a F_{calc} value higher than F_{tab} for both situations. The R^2 value obtained for the two situations under study suggests that the model is satisfactory for evaluating the degree of hydrolysis of the residue from the meal protein extraction residue.

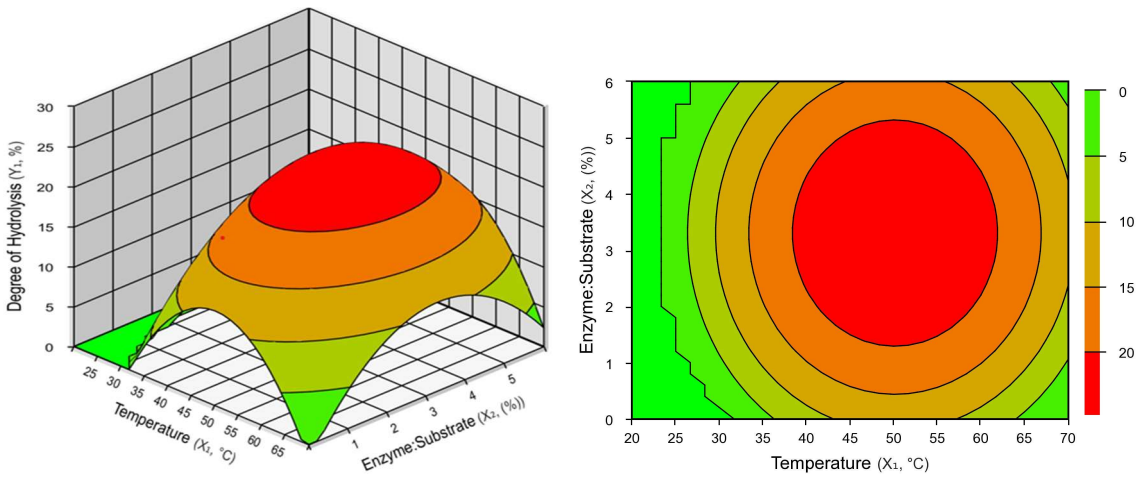
From the ANOVA, a model that describes the degree of hydrolysis as a function of coded variables that were statistically significant was built. The predicted models for the soybean meal protein extraction residue and the peanut meal protein extraction residue are shown in the respective equations below:

$$DH\ (\%) = 23.56 + 5.12\ X_1 - 7.05\ X_1^2 + 2.14\ X_2 - 3.04\ X_2^2$$

$$DH\ (\%) = 19.99 + 3.43\ X_1 - 4.63\ X_1^2 + 3.51\ X_2$$

where: DH = degree of hydrolysis; X_1 = temperature (ºC); X_2 = enzyme:substrate ratio (%).

Using ANOVA, the response surfaces and contour curves were also constructed, and can be seen in Figures 5 (a) and (b), respectively, for the soybean meal protein extraction residue.



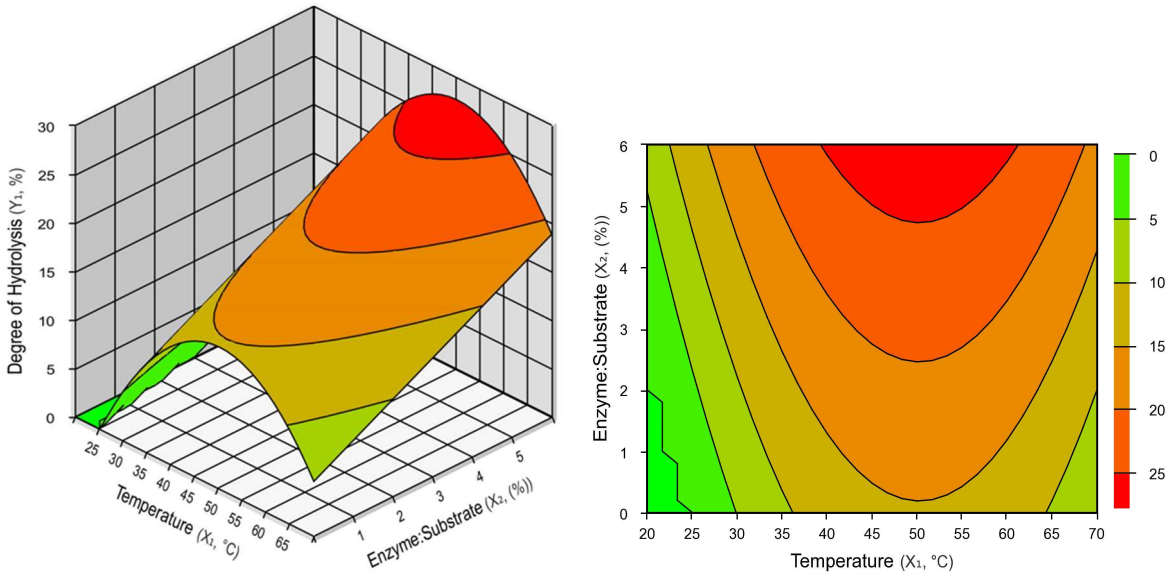
(a) (b)

Figure 5. Response surface (a) and contour curves (b) as a function of temperature and enzyme:substrate ratio for the degree of hydrolysis for soybean meal residue with Alcalase™ 2.4L, at a fixed pH equal to 8.0.

Observing the results presented in Figure 5, which correlates the degree of hydrolysis as a function of temperature and amount of enzyme, an optimal region can be delimited in the temperature range of 50 °C and enzyme concentration around 3.5% for the pH condition fixed at 8.0. Therefore, maintaining this binomial (temperature and enzyme:substrate

ratio) is an important factor for obtaining the maximum degree of hydrolysis for soybean meal residue in the current study; as well as for obtaining an economical and optimized process. Therefore, for the pre-established range of studies with pH fixed at 8.0, it can be said that the optimal condition for the enzymatic hydrolysis of the soybean meal protein extraction residue with Alcalase^{TM} 2.4L was 50 °C and at an enzyme:substrate ratio of 3.5%.

The response surface and contour curves for the peanut meal protein extraction residue can be seen in Figures 6 (a) and (b), respectively.



(a) (b)

Figure 6. Response surface (a) and contour curves (b) as a function of temperature and enzyme:substrate ratio for the degree of hydrolysis for peanut meal residue with Alcalase^{TM} 2.4L, at a fixed pH equal to 8.0.

From Figure 6, an optimal region can be delimited in the temperature range of 50 °C and enzyme concentration around 5% for pH fixed at 8.0. Therefore, maintaining this binomial (temperature and enzyme:substrate ratio) is also an important factor for obtaining the maximum degree of hydrolysis for the peanut meal residue in this current study. Thus, for the pre-established range of studies with the pH fixed at 8.0, the optimal condition established for the enzymatic hydrolysis of peanut meal protein extraction residue with Alcalase^{TM} 2.4L was 50 °C and at an enzyme:substrate ratio of 5.0%.

To validate the experimental design, a new a new enzymatic kinetics curve was undertaken using the optimized conditions for each residue, shown in Figures 7 (a) and (b).

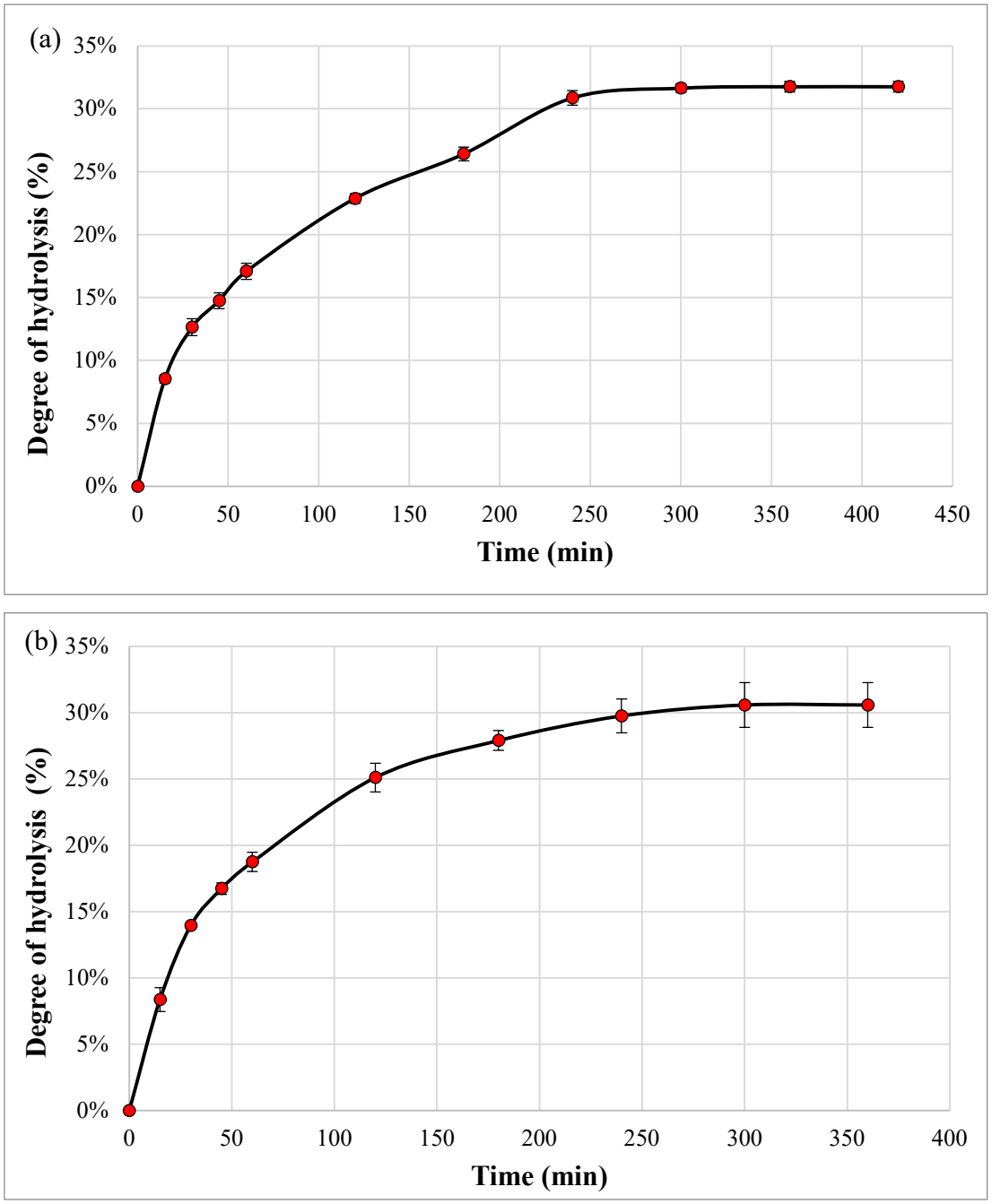


Figure 7. Enzymatic kinetics under optimized conditions for experimental model validation of enzymatic hydrolysis from soybean meal (a) and peanut meal (b) protein extraction residues.

Under these conditions, the model predicts a theoretical degree of hydrolysis of 24.82% for soybean meal protein extraction residue in 2 hours of reaction; and the degree of hydrolysis obtained experimentally for the same hydrolysis time was equal to 22.88%. Although the relative error is 7.82%, the model can be validated, despite the pH-stat method being performed from manual titration, and subject to analyst errors.

For the peanut meal protein extraction residue, the model was also satisfactorily validated since the model predicts a theoretical degree of hydrolysis of 25.57% in 2 hours of

reaction; and the degree of hydrolysis obtained experimentally for the same hydrolysis time was equal to 25.12%. With the obtained value being consistent with the predicted value, with a relative error of only 1.76%.

From the kinetics obtained, an asymptotic trend of the curves with the occurrence of the highest rates of enzymatic hydrolysis in the first hour of reaction and reduction of values, until stabilization (in a plateau) around 300 min for both matrices, was observed. Enzymatic hydrolysis systems for the evaluated residues that seek to maximize the degree of hydrolysis values should, therefore, extend the reaction time until the reaction is stabilized; provided that the relevance for this maximization is greater than the cost of this time increment. Thus, in the plateau observed at 300 min of reaction, the highest values for the degree of hydrolysis were obtained, with an average of 31.76 ± 0.43% for soybean meal protein extraction residue and 30.59 ± 1.68% for peanut meal protein extraction residue.

The maximum values obtained for the degree of in the current study allows for the procurement of low molecular weight peptides and free amino acids suitable for the metabolism and growth of animal cells. Mainly due to these values being above the 20% indicated in the literature, which is the reference percentage required for the significant breakdown of proteins and to obtain nutrients favorable for the increase in cell density for the *in vitro* culture of animal cells (Chabanon et al., 2008; Ho et al., 2021). From the results obtained, the enzymatic hydrolysis with the application of Alcalase^{TM} 2.4L in agro-industrial wastes is highlighted as a favorable route for obtaining inputs for cultured meat.

4 Conclusion

The application of enzymes during protein extraction and protein hydrolysis can be favorable in maximizing the yields of these processes; since the demand for alternative proteins has grown with the rise of the plant-based market and the new challenges for supplementation of culture media for cultured meat production. The application of the enzyme Viscozyme^{TM} L for the extraction of proteins from non-animal agro-industrial wastes significantly increased the yield of the process, combined with the optimal conditions for enzyme performance and the solubility of the proteins present in the matrices. However, an additional study to design the optimal enzyme-assisted extraction conditions must be performed to guarantee the maximization of this process with a good cost-benefit, ensuring the effectiveness of the method. Aiming for the full use of non-animal agro-industrial wastes, sequential, simultaneous, or individual protein hydrolysis was carried out using Alcalase^{TM} 2.4L and Neutrase^{TM} 0.8L in the residual extraction matrices, to obtain the highest possible degree of hydrolysis. Based on the

results, a single application of Alcalase^{TM} 2.4L enzyme was satisfactory to maximize the degree of hydrolysis. Optimum reaction conditions were determined to be at 50°C, pH = 8.0, and enzyme:substrate ratio of 3.5% for soybean meal residual matrix and 5.0% for peanut meal. Proteins and hydrolysates obtained through enzyme-assisted processes are good candidates for the composition of food-grade protein alternatives. Proteins and hydrolysates with a high degree of hydrolysis can mainly act as inputs free of animal derivatives, and are low cost options for the enrichment of the cell culture medium in the cultured meat process; replacing the commonly used fetal bovine serum. However, detailing the characteristics of extracted proteins and hydrolysates is needed to elucidate the type and availability of nutrients required for animal cellular metabolism. Thus, the results suggest that the optimization of the enzymatic hydrolysis achieved is satisfactory to proceed with characterization and cost studies of the hydrolysates; to verify the technical and economic viability of these inputs in the supplementation of culture medium for cultured meat production. These details will be the subject of future studies by this group, opening doors to the potential development of cheaper and safer routes for the production of cultured meat inputs.

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Supporting Information

Table S1. Kinetics of the hydrolysis from soybean meal protein extraction residue

Alcalase™ 2.4L			Neutrase™ 0.8L			Alcalase™ 2.4L + Neutrase™ 0.8L			Alcalase™ 2.4L -> Neutrase™ 0.8L		
Time (min)	**DH* (%)**	**SD** (%)**	**Time (min)**	**DH* (%)**	**SD** (%)**	**Time (min)**	**DH* (%)**	**SD** (%)**	**Time (min)**	**DH* (%)**	**SD** (%)**
0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
5	2.66	0.57	5	2.31	1.30	5	2.26	0.60	5	1.65	0.19
10	4.89	0.73	10	4.27	2.30	10	3.14	0.58	10	2.34	0.95
15	6.74	0.48	15	5.72	2.52	15	3.58	0.62	15	2.83	0.96
20	8.23	0.64	20	6.93	2.47	20	3.90	0.57	20	3.21	1.16
25	9.64	0.66	25	7.51	2.61	25	4.55	0.56	25	3.66	0.81
30	10.35	1.41	30	8.19	2.67	30	4.93	0.54	30	4.28	0.65
35	11.40	1.42	35	8.96	2.48	35	5.43	0.40	35	4.83	0.87
40	12.11	1.70	40	9.49	2.20	40	5.93	0.30	40	5.09	1.33
45	12.82	1.32	45	10.50	2.03	45	6.16	0.48	45	5.38	1.57
50	13.43	1.28	50	11.50	1.59	50	6.89	0.78	50	5.84	1.43
55	13.96	1.60	55	12.40	1.61	55	7.27	0.73	55	6.13	1.71
60	14.77	1.55	60	13.06	1.22	60	7.66	0.68	60	6.63	2.08
70	15.84	1.38	70	13.68	0.80	70	8.04	0.63	70	9.82	3.46
80	16.90	1.25	80	14.09	1.04	80	8.69	0.35	80	10.76	1.97
90	17.97	1.26	90	14.36	1.23	90	9.22	0.20	90	11.50	1.74
100	18.59	1.72	100	14.51	1.37	100	9.89	0.62	100	11.50	1.74
110	19.69	0.81	110	14.51	1.37	110	10.51	0.84	110	11.50	1.74
120	20.04	1.17	120	14.51	1.37	120	11.18	1.15	120	11.50	1.74
135	20.74	1.22	135	14.51	1.37	135	12.02	0.87	135	11.50	1.74
150	21.29	2.16	150	14.51	1.37	150	12.43	0.60	150	11.50	1.74
165	21.29	2.16	165	14.51	1.37	165	12.89	0.43	165	11.50	1.74
180	21.82	2.18	180	14.51	1.37	180	13.60	0.39	180	11.50	1.74
195	21.91	2.33	195	14.51	1.37	195	14.33	0.72	195	11.50	1.74
210	21.91	2.33	210	14.51	1.37	210	14.50	0.59	210	11.50	1.74
225	22.44	3.03	225	14.51	1.37	225	14.50	0.59	225	11.50	1.74
240	-	-	240	14.51	1.37	240	14.50	0.59	240	11.50	1.74

*DH = Degree of Hydrolysis
**SD = Standard Deviation

Table S2. Kinetics of the hydrolysis from peanut meal protein extraction residue

Alcalase™ 2.4L			Neutrase™ 0.8L			Alcalase™ 2.4L + Neutrase™ 0.8L			Alcalase™ 2.4L -> Neutrase™ 0.8L		
Time (min)	**DH* (%)**	**SD** (%)**	**Time (min)**	**DH* (%)**	**SD** (%)**	**Time (min)**	**DH* (%)**	**SD** (%)**	**Time (min)**	**DH* (%)**	**SD** (%)**
0	0.00	0.00	0	0.00	0.00	0	0.00	0.00	0	0.00	0.00
5	2.11	0.01	5	0.91	0.21	5	1.89	0.25	5	1.46	0.46
10	3.37	0.73	10	1.73	0.45	10	3.15	0.23	10	2.35	0.45
15	4.36	0.25	15	2.12	0.24	15	4.62	0.46	15	3.32	0.51
20	5.06	0.01	20	2.50	0.24	20	6.09	1.69	20	3.76	0.35
25	5.90	0.42	25	3.16	0.32	25	6.82	1.43	25	4.01	0.26
30	6.33	0.42	30	3.63	0.10	30	7.35	1.39	30	4.20	0.26
35	6.75	0.42	35	4.13	0.45	35	7.66	1.55	35	4.52	0.36
40	7.17	0.42	40	4.71	0.15	40	7.98	1.56	40	4.90	0.49
45	7.73	0.24	45	5.24	0.39	45	8.29	1.59	45	5.28	0.75
50	8.29	0.22	50	5.89	0.40	50	8.61	1.64	50	5.60	1.13
55	9.00	0.47	55	6.46	0.63	55	9.03	1.74	55	5.79	1.32
60	9.56	0.95	60	7.19	0.96	60	9.45	1.88	60	5.98	1.51
70	10.26	1.19	70	8.46	0.60	70	9.87	1.88	70	7.09	0.79
80	10.96	1.44	80	9.33	0.23	80	10.34	2.02	80	7.70	0.99
90	11.52	1.92	90	10.36	0.71	90	10.71	1.88	90	8.20	0.91
100	12.51	2.44	100	11.24	1.13	100	11.89	1.30	100	8.75	1.28
110	13.35	1.87	110	12.08	1.47	110	12.44	0.73	110	9.03	1.33
120	13.91	1.90	120	12.31	1.53	120	12.72	0.31	120	9.57	0.47
135	14.48	1.87	135	12.67	1.68	135	12.54	0.11	135	9.84	0.34
150	14.90	1.67	150	12.83	1.62	150	12.54	0.11	150	10.12	0.68
165	15.32	1.56	165	13.04	1.40	165	12.54	0.11	165	10.54	1.54
180	15.60	1.80	180	13.19	1.14	180	12.54	0.11	180	10.86	1.09
195	15.74	1.91	195	13.65	0.71	195	12.54	0.11	195	10.95	0.96
210	16.02	2.19	210	13.95	0.19	210	12.54	0.11	210	11.09	0.71
225	16.44	2.53	225	13.95	0.19	225	12.54	0.11	225	10.93	0.71
240	16.44	2.53	240	13.95	0.19	240	12.54	0.11	240	10.93	0.78

*DH = Degree of Hydrolysis
**SD = Standard Deviation

Table S3. Analysis of variance for the degree of hydrolysis of evaluated residues

Variation Source		Sum of Squares	Degrees of Freedom	Mean Square	F_{calc}	p-valor
Soybean meal residue*	Regression	532.4	4	133.1	49.9	0.00010
	Residual	16.0	6	2.7		
	Total	548.4	10			
Peanut meal residue**	Regression	325.8	3	108.6	27.9	0.00029
	Residual	27.3	7	3.9		
	Total	353.1	10			

*% Explained variation (R^2) = 97.08%; Ftab 4/6; 0.05 = 4.53
**% Explained variation (R^2) = 92.28%; Ftab 3/7; 0.05 = 4.35

CAPÍTULO III: Low-cost protein extracts and hydrolysates from non-animal agro-industrial waste: properties and analyzes for application as inputs of interest for cultured meat

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Abstract

The first challenge for large-scale cultured meat production is the replacement of fetal bovine serum (FBS). Currently, fetal bovine serum has been used as a universal supplement for animal cell culture medium. Protein extracts and hydrolysates obtained from non-animal agro-industrial wastes are low-cost, and nutritionally favorable inputs with bioactive potential, which can favor animal cell growth *in vitro*. In this work, soybean and peanut meal were used as agro-industrial sources free of animal compounds to obtain protein extracts from assisted extraction by the enzyme Viscozyme™ L. Residual matrices from protein extraction were reused and hydrolyzed with Alcalase™ 2.4L to obtain free amino acids and low molecular weight peptides required for animal cell growth. The protein extracts were characterized for protein molecular weight distribution, and the hydrolysates were characterized for free amino acids and peptide sizes. Proteomic sequencing was performed for the protein extract and hydrolysate obtained from soybean meal. Based on the presence of essential factors for cell survival and proliferation, the results show favorable perspectives for the application of these inputs in animal cell culture, not only in nutritional terms. The extracts obtained with a high protein content showed a predominance of globulins and albumins, based on a comparative analysis of molecular weight with the main groups of starting matrices. For the hydrolysates, the measured protein content was 69.43% for the soybean meal residual matrix and 54.23% for the peanut meal residual

matrix, values close to the protein content in the dry basis of fetal bovine serum, 69.80%. The free amino acid content was computed as 2.96% for the hydrolysate from soybean meal and 5.05% for the hydrolysate from peanut meal, with Leucine (Leu) and Proline (Pro) being the most available amino acids in both samples. And the molecular weight distribution of the peptides in the hydrolysates indicated the highest concentration in the range between 6.5 kDa and 0.137 kDa, achieved by the high degree of hydrolysis. Finally, a laboratory-scale cost analysis was carried out, which demonstrated that obtaining protein inputs from non-animal agro-industrial waste can constitute a low-cost and promising alternative route to favor cell growth in cultured meat processing.

Keywords: soybean meal; peanut meal; protein extracts; hydrolysates; cultured meat

1 Introduction

The rise of the alternative protein market has intensified the search for protein sources free of animal derivatives, with nutritional and sensory quality comparable to conventionally raised meat (Sarkar, 2021). In this context, cultured meat has attracted the attention of researchers, investors, and the industry as an innovative technology and a possible alternative to complement the meat supply and minimize the environmental impacts of traditional production (Andreassen et al., 2020; Letti et al., 2021). Cultured meat is the meat obtained from the culture of cells in bioreactors. Despite the various challenges that still compromise the development of this technology on a large scale, the use of fetal bovine serum as a supplement to the culture medium is considered the greatest obstacle, since it significantly burdens the sustainability and economic aspects of the process (Humbird, 2021; Kolkmann et al., 2020).

The fetal bovine serum has been extensively used as the main supplement for animal cell culture medium, serving as a source of amino acids, proteins (albumin and globulins), lipids, hormones, growth factors, and trace elements (Yao & Asayama, 2017). However, the disadvantages of its application in the cultured meat process significantly outweigh its effective action on animal cell growth (Chabanon et al., 2008).

The search for culture media for animal cells free of fetal bovine serum or for inputs free of animal derivatives to supplement basal media has been one of the prominent areas in the technological development of cultured meat (Okamoto et al., 2022). Since the maintenance of animal cell growth and differentiation without the application of fetal bovine serum is desired, the development of routes to obtain components such as proteins, amino acids, enzymes,

hormones, and, growth factors free of animal derivatives is necessary (Andreassen et al., 2020; Ho et al., 2021).

Studies indicate the use of hydrolysates obtained from plant matrices as a form of supplementation of the serum-free culture medium for the growth of even more cells (Chabanon et al., 2008). The use of hydrolysates as inputs for the culture medium can also complement the demands of non-essential amino acids presented by the cells in the culture. Normally, non-essential amino acids are biosynthesized by cells, but not all cell types can produce sufficient amounts of these amino acids. Supplementation of non-essential amino acids can generate positive results in animal cell growth, in addition to minimizing the biosynthetic load of cells (Yao & Asayama, 2017).

In this context, researchers have been investigating and exploring the use of proteins and protein hydrolysates from food waste to promote animal cell growth, increase production scale and reduce process costs (Andreassen et al., 2020; Ho et al., 2021). Additionally, the use of non-animal agro-industrial waste to obtain nutrients for cultured meat contributes to the ethical requirements of production and favors the circular economy since it reduces the amount of waste discarded in the environment (Andreassen et al., 2020).

Soybean meal and peanut meal are the main by-products of oil extraction from their respective starting matrices and are promising non-animal agro-industrial wastes for the cultured meat industry. Soybean meal and peanut meal are good sources of protein, generally contains a good distribution of amino acids required for animal cell growth, and are available in high amounts. It is possible from these wastes to obtain inputs capable of supplying the protein portion derived from fetal bovine serum in animal cell growth *in vitro*, convenient for the production of cultured meat. Currently, the reuse of these wastes is limited only to use as animal feed or fertilizers (González-Pérez & Arellano, 2009; Zhang et al., 2022).

The use of enzymes has been highlighted in protein extraction and hydrolysis pathways, mainly to obtain safe and suitable nutrients for food production. The application of enzymes promotes milder, more sustainable conditions with less degradation of the bioactive compounds resulting from the process (Guo et al., 2013). The product resulting from enzymatic extraction and hydrolysis normally resembles the components of the starting matrix of the process. In the extraction, depending on the conditions employed, the extracts show a predominance of the most abundant proteins in the starting material, despite the presence of other charged molecules in the extraction process (Sari et al., 2013). In hydrolysates, a complex mixture of peptides, amino acids, residual proteins, minerals, carbohydrates, and lipids is observed (Ho et al., 2021).

However, the application of protein extracts and hydrolysates from non-animal agro-industrial waste as a supplement to the animal cell culture medium still has limitations, mainly about their undefined composition. In this study, based on a detailed analysis of the composition, molecular characteristics and estimated costs at laboratory-scale, we provide evidence that proteins obtained by enzyme-assisted extraction and hydrolysates obtained from the residual matrix of protein extraction can compose a new independent production route of nutrients and inputs derived from non-animal agro-industrial wastes for processing low-cost cultured meat with higher sustainability criteria.

2 Material and methods

2.1 Agro-industrial wastes, enzymes, and reagents

Soybean meal and peanut meal with high protein content, obtained from local commerce, were used as agro-industrial matrices for the development of this study. The matrices were standardized with granulometry ≤ 0.4 mm. For protein extraction, a multi-enzyme complex of carbohydrases, Viscozyme™ L (100 FBG/g) was used. Alcalase™ 2.4L (2.4 AU-A/g), a serine protease from *Bacillus licheniformis*, was used for enzymatic hydrolysis. The enzymes and analytical-grade chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sterile, preservative-free fetal bovine serum from Nutricell (Campinas, SP, Brazil) (Brazilian Ministry of Agriculture registration: nº 9244/2006) was used in the comparative studies.

2.2 Enzyme activity assay

The β-glucanase activity of the Viscozyme™ L enzyme was evaluated using the method for quantifying reducing sugars from laminarin as substrate, as described by Bauermeister et al. (2015). The unit of fungal beta-glucanase (FBG) represents the amount of enzyme preparation required to promote the breakdown of β-glucan equivalent to 1 μmol of glucose per minute (Tu et al., 2015).

The total proteolytic activity of the enzymatic preparation Alcalase™ 2.4L was evaluated using azocasein as substrate, as described by Charney and Tomarelli (1947) and adapted by De Castro and Sato (2013). The activity was determined from the absorbance reading at a wavelength of 428 nm, where a proteolytic unit (U) is the amount of enzyme necessary to change the absorbance by 0.01 unit per reaction minute.

2.3 Total amino acids analysis

The determination of total amino acids of the raw material was performed according to the method described by White et al. (1986), and Hagen et al. (1989) reverse phase column (C18 from Phenomenex) chromatography in a high-performance liquid chromatograph (HPLC, SHIMADZU®). The release of individual amino acids occurred in acid hydrolysis with 6 M hydrochloric acid solution and phenol. After hydrolysis, α-aminobutyric acid was added as an internal standard. Derivatization of the hydrolysate with solution of methanol, ultrapure water, triethylamine and phenylisothiocyanate (PITC) was performed in the sequence. The amino acids were the dissolved in diluent and introduced into the column. The tryptophan content was not determined, since it is destroyed in the acid hydrolysis.

2.4 Preparation of protein extract

The preparation of the protein extract was carried out using the enzyme-assisted method divided into two steps at pH 7.0 for protein extraction with Viscozyme^{TM} L and pH 10.0 for protein solubility. For this, 250 ml Erlenmeyer flasks containing 10 g of agro-industrial waste and 100 ml of deionized water were incubated in a thermostatic orbital shaker at 50 °C and 200 rpm for 2 hours. The amount of Viscozyme^{TM} L enzyme added was 5 FBG per gram of dry mass.

2.5 Enzymatic hydrolysis

Hydrolysis experiments were performed in a 100 mL jacketed batch reactor using the pH-stat method with Alcalase^{TM} 2.4 L. A residue suspension (residue:water ratio of 1:10 w/v) was heated at 50°C and pH 8.0 for 5 hours. The Alcalase^{TM} 2.4L enzyme:substrate ratio was 3.5% for hydrolysis of soybean meal residual matrix and 5.0% for peanut meal residual matrix. The degree of hydrolysis was determined using the pH-stat method, which determines the percentage ratio between the number of cleaved peptide bonds and the total number of peptide bonds available for hydrolysis, according to Adler-Nissen (1986). The general scheme referring to the maximum use of proteins from the evaluated non-animal agro-industrial wastes is shown in Figure 1.

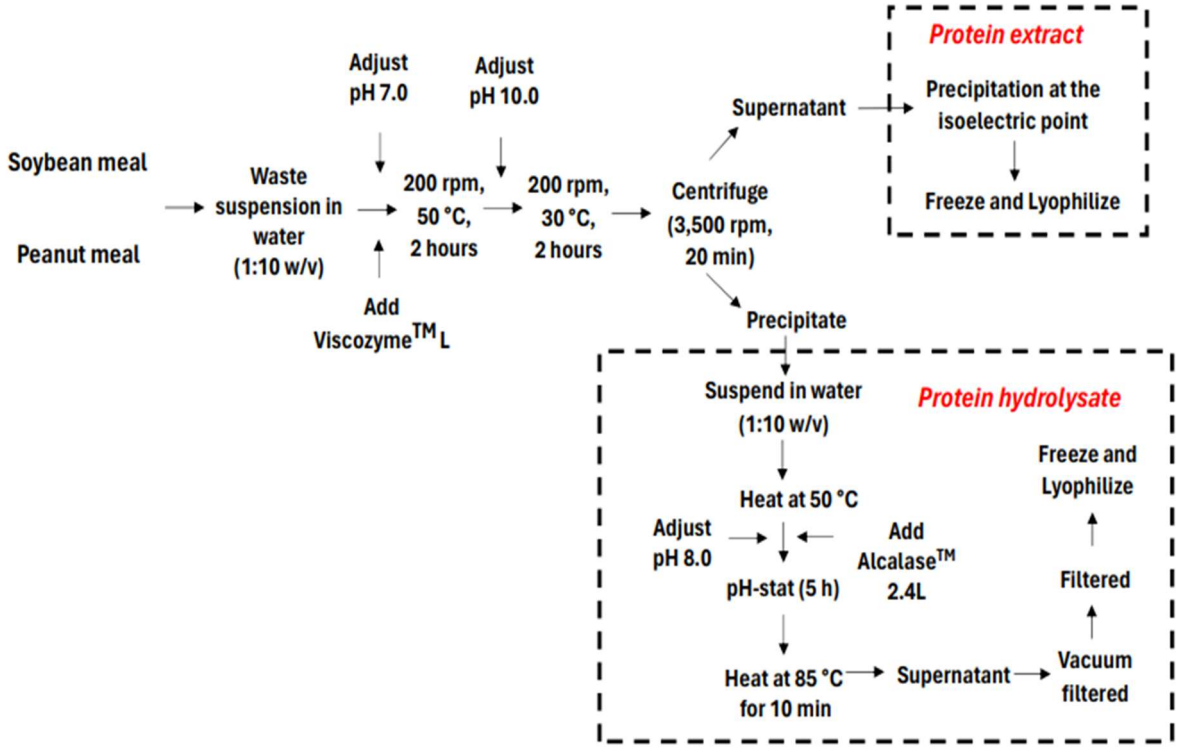


Figure 1. Simplified flowchart for maximum utilization of proteins from evaluated wastes.

2.6 Characterization of protein extracts

Protein extracts were characterized by molecular weight distribution by size exclusion chromatography (SEC). And proteomic sequencing of the protein extract was carried out for the extract with the highest protein content, in order to identify the main protein groups present and relate them to the biological activities described in the literature.

SEC analyses were applied aiming to compare the molecular weight distribution of proteins and peptides contained in the different samples. Fetal bovine serum was also evaluated. For analysis, sample solutions were prepared in water (0.1% TFA) at a concentration of 1 mg/mL The solutions were stirred for 10 min, centrifuged for 15 min, 10,000 × g at 25 °C, and filtered through a 0.45 µm PVDF filter. The separation was performed on a BioSep SEC-S3000 column (5 µm, 600 x 7.8 mm, Phenomenex) in an isocratic mode in the proportion of 70% phase A (water containing 0.1% (v/v) trifluoroacetic acid) and 30% phase B (acetonitrile 0.1% trifluoroacetic acid), flow 0.4 mL/min, in a Shimadzu HPLC (model Prominence UFLC, Kyoto, Japan) equipped with binary pump (LC-20AD), degassing unit (DGU-20A3R), automatic injector (SIL-20AHT) and column furnace (CTO-20A), coupled to a diode array detector (DAD) (SPD-M20A). Absorbance was monitored at 214 nm. The chromatogram was divided into seven molecular weight ranges: bigger than 670 kDa, between 670 and 150 kDa, between 150 kDa and 66 kDa, between 66 kDa and 13.7 kDa, between 13.7 kDa and 6.5 kDa, between

6.5 kDa and 137 Da and less than 137 Da. For comparison purposes, the following proteins standards were used: Bovine Thyroglobulin (670 kDa) = 27.7 min; Globulins from Bovine Blood (150 kDa) = 29.6 min; Bovine Serum Albumin (66 kDa) (A8531) = 30.8 min; Ribonuclease A (13.7 kDa) = 39.3 min; Aprotinin (6.5 kDa) = 42.1 min (A3886); pABA (137 Da) = 63 min. The percentage of molecular weight distribution was expressed as the area of the fraction of the total area of the chromatogram.

Proteomic sequencing was performed from an untargeted proteomic analysis with injection into capillary liquid chromatography coupled to mass spectrometry (LC-MS/MS) and data processing of samples from the filtered hydrolysate. The samples were treated with Zip Tip C18 and the hydrolysates were obtained from digestion with Promega Trypsin in a 1:100 (w/w) enzyme:protein ratio. Identification of the proteins present in the protein extract samples was performed with a Proteome ID from UniProt (Swiss-Prot/TrEMBL) specific database (*Glycine max* (Soybean) (*Glycine hispida*) (cv. Williams 82) reviewed. All protein hits were identified with the confidence of > 95%.

2.7 Characterization of hydrolysates

The protein hydrolysates were characterized in terms of protein content, free amino acids and size of the peptides obtained for a comparative analysis with the components normally found in fetal bovine serum. Likewise, proteomic sequencing was performed for the hydrolysate with the highest protein content and highest degree of hydrolysis achieved, in order to identify the peptide sequences present in the hydrolysate and compare them with sequences with bioactivity listed in an online database.

Proteins were quantified by total nitrogen by the classic Kjeldahl method (AOAC, 1998). The protein content was calculated using a conversion factor of 6.25 for soybean meal, 5.46 for peanut meal (Singh & Singh, 1991), and 6.25 for fetal bovine serum.

Free amino acids were extracted with an acidic solution (0.1 M HCl) and stirred for 30 min. An aliquot of the filtrate was derivatized following the same method of total amino acids described in item 2.3 (except acid hydrolysis) (Hagen et al., 1989; White et al., 1986).

The molecular size distribution of peptides for each hydrolysate was analyzed, following the same procedure described for protein extracts in item 2.6. The percentage of molecular weight distribution was also expressed as the area of the fraction of the total area of the chromatogram.

Proteomic sequencing was performed from an untargeted proteomic analysis with injection into capillary liquid chromatography coupled to mass spectrometry (LC-MS/MS) and

data processing of samples from the filtered hydrolysate. The samples were treated with Zip Tip C18. Identification of the proteins present in the protein extract samples was performed with a Proteome ID from UniProt (Swiss-Prot/TrEMBL) specific database (*Glycine max* (Soybean) (*Glycine hispida*) (cv. Williams 82) reviewed.

2.8 Extraction and hydrolysis cost analysis

A laboratory-scale scenario was analyzed to obtain inputs of interest for the protein supplementation of the culture medium in terms of raw material cost (C_{RM}), cost of equipment (C_{Eq}), and cost of operation (C_{Op}). The calculations were performed based on the cost analysis methodology described by Osma et al. (2011). For the C_{RM} determination, the values were composed of the market prices of the enzymes and reagents used obtained from Sigma-Aldrich (St. Louis, MO, USA). In the case of agro-industrial wastes used, the costs were determined from market values in tons. To determine equipment and operating costs (C_{Eq} and C_{Op}), only essential equipment for protein extraction and enzymatic hydrolysis was listed. The equipment cost (C_{Eq}) was measured according to the lifetime and the real price of the equipment involved in the process, calculated according to the manufacturer's specifications. For the protein extract, the C_{Eq} was related to the costs of the thermostatic orbital shake, the centrifuge, and the lyophilizer, for a total yield of 3 kg of protein extract before freeze-drying. As for the residual matrix hydrolysate from protein extraction, the C_{Eq} was related to the costs of the water bath with reciprocal shaking, the centrifuge, and the lyophilizer. The cost of each piece of equipment was divided by the number of operating cycles, its lifetime, and its capacity. For the production of protein extracts, the capacity of the lyophilizer (3 kg) was decisive in the process, as it takes 4.3 days to produce the maximum amount of protein extract to be loaded into the lyophilizer, meeting the capacity indicated by the manufacturer. Thus, 58 production cycles were computed for the production of protein extract, operating 252 days a year and 24 hours a day. As for the protein hydrolysate, the maximum amount of protein hydrolysate is produced every 48 h, due to the limitation of the lyophilizer (3 kg), which needs to perform two cycles of 24 h for the total load to be completely lyophilized. Thus, the number of cycles for the production of hydrolysates was 126 per year, considering 252 days of operation per year and 24 hours per day. As the operational capacity is limited by the lyophilizer, this will be used as the total capacity of the equipment involved. The cost of operation (C_{Op}) took into account the electrical energy spent per hour with the use of the equipment and the operating conditions. And the cost of labor was not considered in the calculations, as it is a value dependent on automation and

knowledge of the process. The total cost (C_{Prod}) of the inputs obtained was calculated from the sum of the three costs involved.

3 Results and discussion

3.1 Total amino acids analysis

Soybean meal and peanut meal are agro-industrial wastes, free of animal derivatives, with high protein content and availability for use as inputs for the most diverse areas of bioprocessing (González-Pérez & Arellano, 2009; Rostagno et al., 2011). The balanced amino acid composition of these wastes has attracted attention for the production of inputs for cultured meat, since obtaining protein inputs from these wastes can potentially contribute to replacing the protein portion of fetal bovine serum in the supplementation of animal cell culture media (Ho et al., 2021). The profile of amino acids in soybean meal and peanut meal under study are shown in Table 1.

Table 1. Total amino acid composition of soybean meal and peanut meal (g/100 g)

Amino Acids		Soybean meal	Peanut meal
Essential Amino Acids (EAAs)	Isoleucine (Ile)	2.03 ± 0.01	1.52 ± 0.05
	Leucine (Leu)	3.59 ± 0.01	3.13 ± 0.11
	Phenylalanine (Phe)	2.53 ± 0.03	2.43 ± 0.03
	Lysine (Lys)	2.89 ± 0.003	1.52 ± 0.005
	Histidine (His)	1.22 ± 0.12	1.18 ± 0.02
	Threonine (Thr)	2.03 ± 0.01	1.17 ± 0.001
	Valine (Val)	2.22 ± 0.02	1.93 ± 0.003
	Methionine (Met)	0.62 ± 0.01	0.55 ± 0.003
Non-Essential Amino Acids (NEAAs)	Aspartic acid/Asparagine (Asp, Asn)	5.47 ± 0.01	5.55 ± 0.04
	Glutamic acid/Glutamine (Glu, Gln)	8.65 ± 0.03	9.04 ± 0.05
	Serine (Ser)	2.61 ± 0.004	2.43 ± 0.01
	Glycine (Gly)	2.00 ± 0.005	2.88 ± 0.02
	Arginine (Arg)	3.55 ± 0.01	5.52 ± 0.02
	Alanine (Ala)	2.02 ± 0.01	1.84 ± 0.02
	Proline (Pro)	2.61 ± 0.02	2.03 ± 0.01
	Tyrosine (Tyr)	1.95 ± 0.0004	2.07 ± 0.02
	Cysteine (Cys)	0.32 ± 0.03	0.30 ± 0.03
Total Amino Acids		**46.30 ± 0.22**	**45.11 ± 0.06**

Glu/Gln, Asp/Asn, Arg, and Leu were the amino acids observed in greater amounts in the wastes under study. The results showed that soybean meal contains a notable amount of acidic amino acids (Glu/Gln and Asp/Asn), which corresponds to approximately 30.5% of the

total amino acids present in the waste. The same acidic amino acids present in soybean meal represent 32.3% of the total amino acids in peanut meal. However, these wastes are deficient in sulfur-containing amino acids, Met and Cys. The amino acid distribution found corroborates the amino acid profiles already described in the literature for the wastes under study (Parsons et al., 1992; Batal et al., 2005).

In terms of essential amino acids, the values found for soybean meal were higher than for peanut meal, 17.13 g/100 g and 13.43 g/100 g of waste, respectively. However, many animal species (cattle, chickens, fish, and pigs) include Cys and Tyr in the list of essential amino acids (Hou & Wu, 2018). For general animal cell growth, the amino acids required in the culture medium are Cys, Arg, Glu/Gln, and Tyr, although individual amino acids requirements vary between different cell types (Freshney, 2011). Mainly, the Glu/Gln requirement in animal cell growth is relative since can generate beneficial effects for the growth of cells with high nutritional requirements, but can have undesirable effects for other cells with fewer requirements, promoting the formation of cytotoxic ammonia (Yao & Asayama, 2017).

Special attention should also be given to the Cys content which is deficient in both wastes and may need additional supplementation for the development of cultured meat inputs.

In general, few differences were verified in the total amino acid profile of the residues. Thus, studies were maintained for both matrices to detect functional differences in the extracted proteins and hydrolysates obtained that could be significant in performance as cultured meat inputs.

3.2 Characterization of the protein extracts

Firstly, the protein extracts obtained were analyzed for molecular weight distribution using size exclusion chromatography (SEC). The separation of the protein fractions obtained from the SEC analysis for the extracts can be seen in Figure 2, as well as the comparison between the protein fractions of the extracts and the protein fractions of fetal bovine serum (FBS) and bovine serum albumin (BSA).

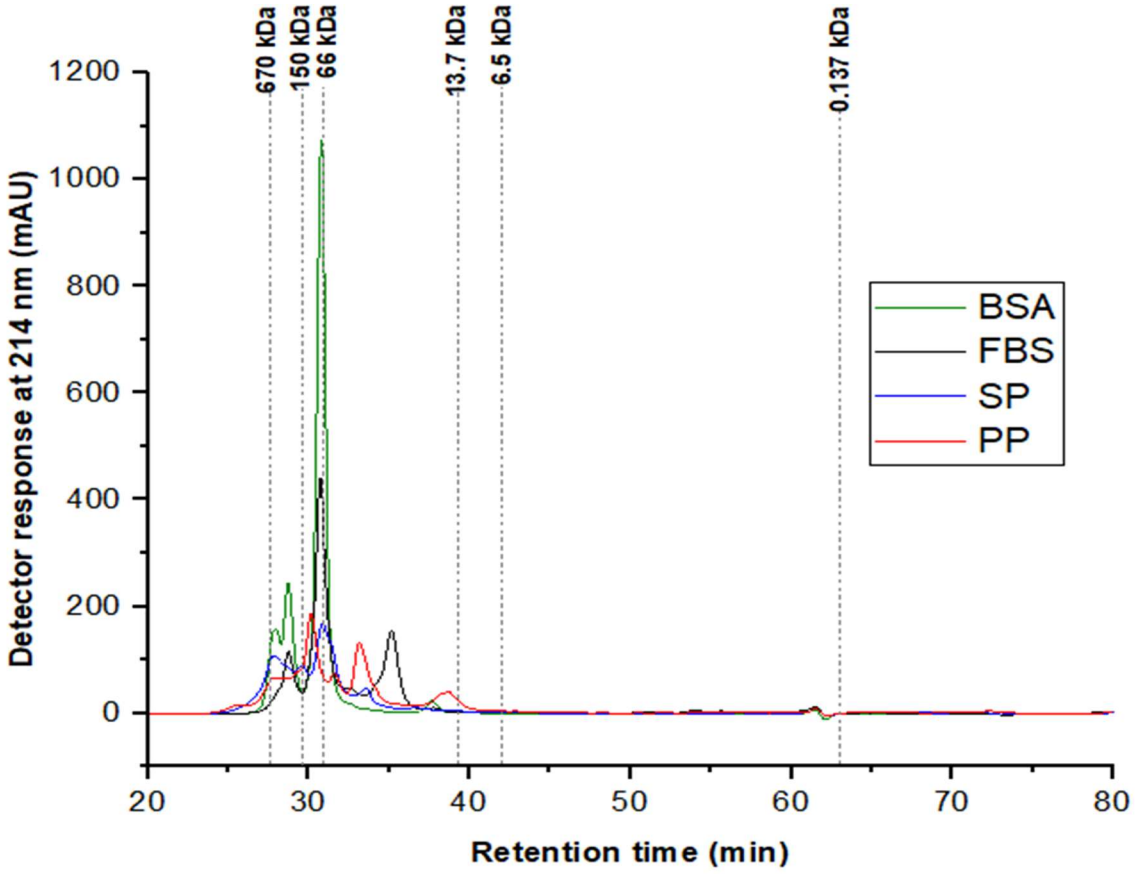


Figure 2. Chromatogram of extracts of soybean meal protein extract (SP), peanut meal protein extract (PP), fetal bovine serum (FBS), and bovine serum albumin (BSA) obtained by size exclusion chromatography.

The percentage of each molecular weight fraction was expressed as the fraction of the relative area concerning the total area of the chromatogram, according to Table 2.

Table 2. Protein distribution profile by weight range molecular

Size ranges (kDa)	Fetal Bovine Serum (FBS) (%)	Bovine Serum Albumin (BSA) (%)	Soybean meal protein extract (SP) (%)	Peanut meal protein extract (PP) (%)
> 670	0.72	3.02	10.05	8.01
670 – 150	13.45	24.42	27.45	17.31
150 – 66	24.59	35.09	18.52	20.91
66 – 13.7	55.01	37.43	42.39	47.47
13.7 – 6.5	0.51	0.04	1.10	2.89
6.5 – 0.137	3.87	-	-	2.01
< 0.137	1.85	-	0.49	1.40

From Figure 2 and according to Table 2, the highest percentage fractions of molecular weight distribution for all analyzed samples are concentrated in the range between

670 and 13.7 kDa. Comparing the percentage distribution of molecular weight for the extracts, a proximity between the values is observed, indicating a proportion of the chromatogram area for the regions of integration between the extracts, as shown in Table 2. The first peak detected for protein extracts includes proteins with an approximate molecular weight of 670 kDa. The peaks found in the sequence include proteins between 150 kDa and 66 kDa. And the last peaks of the extracts have molecular weights between 66 kDa and 13.7 kDa. Thus, it can be observed that the protein extracts, although coming from different agro-industrial matrices, presented a similar molecular weight distribution.

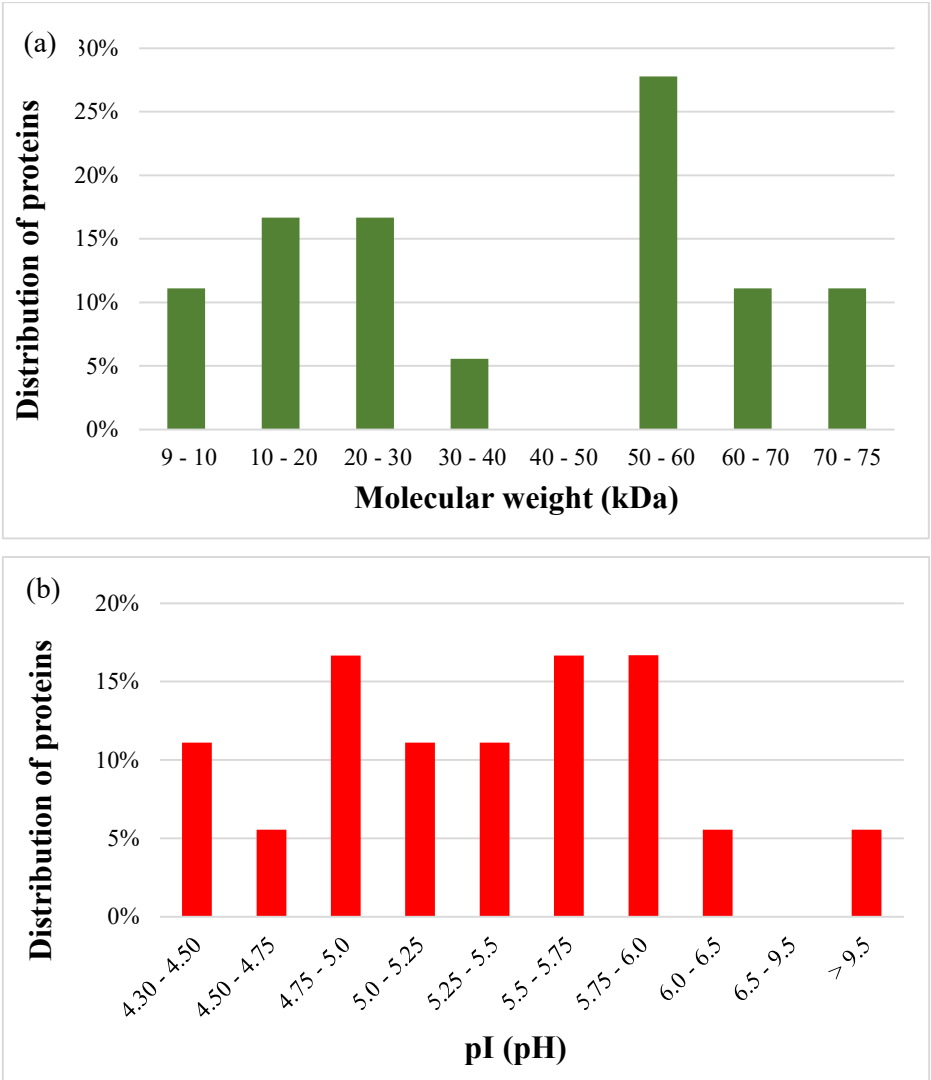
The soybean meal protein extract (SP) showed a protein fraction with the most intense peak in the region of 66 kDa, very close to the highest intensity peak of fetal bovine serum (FBS). This peak of greater intensity observed for the soybean meal protein extract can be explained by the presence of the protein β-conglycinin, which has subunits in the region from 45 to 72 kDa. β-conglycinin, together with glycinin, makes up the most important protein fraction found in soy. Glycinin, on the other hand, can explain the presence of small peaks between 66 kDa and 13.7 kDa, since it is composed of acidic and basic polypeptides, weighing around 38 kDa and 20 kDa, respectively. The peak observed in the region of 670 kDa may be related to the quaternary structure of Glycinin as well as to protein aggregates (González-Pérez & Arellano, 2009).

Peanut protein extract (PP) had its most intense protein fraction with a molecular weight above 66 kDa, that is, slightly displaced from the highest intensity fraction found for fetal bovine serum (FBS). The proteins found in peanut bran protein extract may be related to the protein subunits present in peanuts, peaks greater than 50 kDa may be associated with conarachin and smaller peaks with arachin and other low molecular weight proteins (Bianchi-Hall et al., 1993).

The protein fractions found in fetal bovine serum (FBS) were also compared with bovine serum albumin (BSA) to identify the highest intensity peak, as shown in Figure 2. For fetal bovine serum (FBS), three relevant peaks can be observed. The one with the highest intensity, located in the 66 kDa region, coincides in shape and retention time with the main peak of bovine serum albumin (BSA). The peak in the region between 670 kDa and 150 kDa may be related to globulins (158 kDa) and the peak in the region between 66 kDa and 13.7 kDa with Fetuin (48.4 kDa), the second largest group of proteins in terms of fetal bovine serum composition (Cartellieri et al., 2002; Kiyasu et al., 2000). The combination of the three major protein groups in fetal bovine serum (FBS) makes the percentage distribution of molecular

weights different from the percentage distribution of bovine serum albumin (BSA), as shown in Table 2.

Next, the soybean meal protein extract, with the highest protein content seen in previous studies of this group, was analyzed for proteomic sequencing. Untargeted exploratory proteomic analysis of soybean meal protein extract detected 18 protein clusters from UniProt (Swiss-Prot/TrEMBL) specific database reviewed, with significant coverage percentages, only 3 groups below 75%. This analysis showed a series of differences between the protein groups identified in the soybean meal protein extract. The main characteristics and biological functions of the detected groups are shown in Figure 3.



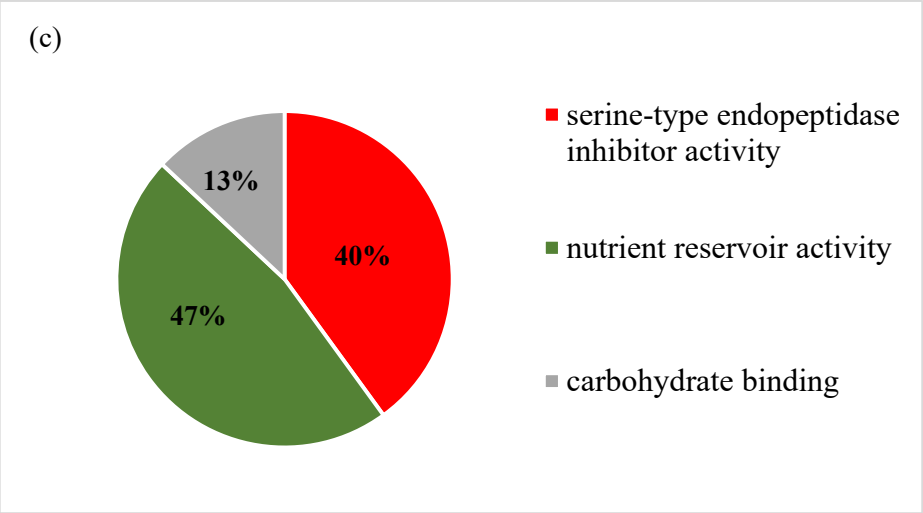


Figure 3. Molecular weight distribution (a), isoelectric point distribution (b), molecular function distribution (c) of proteins identified in soybean meal protein extract.

From the data listed in Figure 3 (a), it is observed that the molecular weight range corresponding to 50 – 60 kDa concentrates the largest number of protein groups identified for protein extract from soybean meal. The other groups identified are distributed between 9 and 75 kDa, showing a good correlation with the values of molecular weight of soy proteins described in the literature (González-Pérez & Arellano, 2009). These results also corroborate with the result found in the size exclusion chromatography (SEC).

As for the isoelectric point, Figure 3 (b), the distribution of proteins occurred more effectively in the pH range ranging from 4.75 to 6.0. According to the functional categorization, Figure 3 (c), the proteins extracted from soybean meal showed an important role in the reserve of nutrients and as serine-type endopeptidases inhibitor activity. Most of the proteins listed with serine-type endopeptidase inhibition activity show trypsin inhibition. Trypsin inhibitors are used in cell culture to inhibit tryptic activity during cell dissociation reducing cell damage and death (Sigma Aldrich, 2023). These protease inhibitors can also prevent the degradation of other types of proteins needed for animal cell growth (Mols et al., 2005).

Of the sequenced groups in the soybean meal protein extract, five groups are related to soy glycinin and its subunits (G1, G2, G3, G4, G5), with more than 85% coverage of the analyzed molecules. Glycinin belongs to the globulin family and constitutes the main protein reserve of soybean seeds (González-Pérez & Arellano, 2009). The molecular weight of this protein group ranges from 54 to 64 kDa and the average isoelectric point occurs around the equivalent pH 5.5. Several effects and functions of glycinin have been reported in the literature, including its performance as a natural antibacterial agent, free of cytotoxicity on the viability of human embryonic kidney cells (Zhao et al., 2018). Glycinin peptides are also highlighted

with anti-inflammatory and anticancer potential in human colon cancer cells (González-Montoya et al., 2018). In addition to the biological aspects, Glycinin also contributes to the sensory properties of meat during storage and inhibits microbial growth (Li et al., 2016).

Another group detected in the soybean meal protein extract was the 2S Albumin storage protein, with an approximate molecular weight of 18.5 kDa, isoelectric point equal to 5.0, and 87.34% molecule coverage. Although albumins are a group of proteins of interest for animal cell growth, 2S albumins from mono and dicotyledonous plants have been associated with causing food allergies, therefore their performance as an input for cultured meat should be evaluated (Moreno & Clemente, 2008).

In general, the soybean meal extract proteins have characteristics of interest for the composition of inputs for the animal's culture medium, however, the applicability must be verified in aspects of allergy and cytotoxicity for cultured meat.

3.3 Characterization of the hydrolysates

Firstly, the hydrolysates were analyzed for protein content. The same analysis was performed for fetal bovine serum for comparison purposes. The results obtained are shown in Table 3.

Table 3. Protein content on a dry basis of hydrolysates and fetal bovine serum

Compound	Protein Content (%)
Soybean meal hydrolysate	69.43 ± 0.04
Peanut meal hydrolysate	54.23 ± 0.14
Fetal bovine serum	69.80 ± 0.03

The results displayed in Table 3 indicate protein predominance in the composition of hydrolysates and equivalent protein concentrations between the residual matrix hydrolysate of soybean meal and fetal bovine serum. Despite the similarity of the protein content of the hydrolysates, mainly in the case of the hydrolysate of the residual matrix of soybean meal with the fetal bovine serum, it must be taken into account that the proteins of the fetal bovine serum are composed mainly of proteins of high molecular weight (van der Valk et al., 2018). On the other hand, the hydrolysates, when undergoing the cleavage of the enzyme Alcalase^{TM} 2.4L, present a protein content distributed in peptides of low molecular weight. Thus, further investigations regarding the protein composition of hydrolysates may increase the discussion about the potential for hydrolysates to act as inputs for replacing the protein portion of fetal

bovine serum in the growth of animal cells, thus enabling a new route for obtaining inputs for cultured meat.

Sequentially, the quantification of the free amino acids generated in the hydrolysates of the residual matrices of the evaluated wastes was carried out to identify possible correlations between their availability and the animal cell requirements described in the literature. Although fetal bovine serum presents high complexity and variability between batches, for initial comparative purposes, its amino acid profile was calculated on a dry basis from data adapted from Büntemeyer et al. (1991). The free amino acid composition obtained from the hydrolysis of a residual matrix of soybean meal and peanut meal, as well as fetal bovine serum is shown in Table 4.

Table 4. Free amino acid composition of soybean meal hydrolysate and peanut meal hydrolysate (g/100 g)

Amino Acids		Soybean meal hydrolysate	Peanut meal hydrolysate	Fetal Bovine Serum[a]
Essential Amino Acids (EAAs)	Isoleucine (Ile)	0.07 ± 0.0012	0.23 ± 0.005	0.09
	Leucine (Leu)	1.05 ± 0.0393	1.42 ± 0.016	0.18
	Phenylalanine (Phe)	0.17 ± 0.0004	0.57 ± 0.005	0.11
	Lysine (Lys)	0.15 ± 0.0031	0.18 ± 0.002	0.12
	Histidine (His)	0.13 ± 0.0061	0.23 ± 0.003	0.07
	Threonine (Thr)	0.08 ± 0.005	0.11 ± 0.003	0.16
	Valine (Val)	0.15 ± 0.0039	0.34 ± 0.00004	0.19
	Methionine (Met)	0.10 ± 0.0016	0.16 ± 0.003	0.02
	Tryptophan (Trp)	0.05 ± 0.0008	0.08 ± 0.002	0.07
Non-Essential Amino Acids (NEAAs)	Aspartic acid/Asparagine (Asp, Asn)	0.05 ± 0.0004	0.09 ± 0.002	0.05
	Glutamic acid/Glutamine (Glu, Gln)	0.13 ± 0.0007	0.24 ± 0.001	0.27
	Serine (Ser)	0.07 ± 0.0012	0.13 ± 0.001	0.16
	Glycine (Gly)	0.05 ± 0.0016	0.13 ± 0.005	0.29
	Arginine (Arg)	0.03 ± 0.0001	0.01 ± 0.001	-
	Alanine (Ala)	0.22 ± 0.0009	0.40 ± 0.00001	0.53
	Proline (Pro)	0.29 ± 0.0293	0.47 ± 0.006	-
	Tyrosine (Tyr)	0.08 ± 0.0058	0.14 ± 0.002	0.09
	Cysteine (Cys)	0.09 ± 0.0032	0.12 ± 0.003	-
	Total Amino Acids	**2.96 ± 0.02**	**5.05 ± 0.020**	**2.40**

[a]Dry basis, adapted from Büntemeyer et al. (1991).

From the data in Table 4, it was possible to observe that the content of free amino acids in the peanut meal residual matrix hydrolysate is more significant than of the fetal bovine serum, being the content of the soybean meal residual matrix hydrolysate close to the latter.

These results indicate a significant group of amino acids in the hydrolysates that, as in fetal bovine serum, can provide adequate nutritional support to cells during culture.

The free amino acid profiles obtained between the two evaluated hydrolysates were slightly different. The peanut meal residual matrix hydrolysate showed a free amino acid composition 1.7 times greater than the free amino acid composition of the soybean meal residual matrix hydrolysate. This result can be explained by the protein content of the hydrolysate and the degree of hydrolysis obtained since the protein content in the hydrolysate of the peanut bran residual matrix was lower than the soybean meal content and the degrees of hydrolysis for both matrices were very close to each other. In this way, the efficiency of Alcalase^{TM} 2.4L in breaking peptide bonds in the peanut residual matrix was greater, as there was less protein available, thus promoting a greater release of free amino acids. However, Alcalase^{TM} 2.4L is an endoprotease, if hydrolysis was promoted using exoproteases, which cleave the peptide bonds at the ends of the chain, the amount of free amino acids could have been even more significant (Pacheco et al., 2006).

Ala and Gly are the two most expressive amino acids for fetal bovine serum, computing 34.17% of free amino acids on a dry basis. Arg, Pro, and Cys did not have their content quantified in the described analysis. And the non-essential amino acid content is higher than the essential amino acid content.

For the soybean meal residual matrix hydrolysate, the prevalent amino acids were Leu, Pro, and Ala, and Leu, Phe, and Pro, for the peanut meal residual matrix hydrolysate, accounting for 52.7% and 48.7% of free amino acids found respectively. The amino acids found in hydrolysates with more significant amounts in the free form, Leu, Pro, Phe, and Ala, are considered hydrophobic amino acids. This result may be related to the high specificity of Alcalase^{TM} 2.4L in the cleavage of hydrophobic peptides, increasing their exposure and release in the reaction medium (Sinthusamran et al., 2020).

The Arg content was the lowest highlighted for both hydrolysates. Arg is one of the main nutrients required in the composition of cell culture medium (Sinke et al., 2023). Although high levels of this free amino acid are not found in the hydrolysates, the presence of Arg in the composition of peptides with lower molecular weight should be considered, since the initial levels of this amino acid in plant matrices were significant.

The essential amino acid content is very similar between the hydrolysates, adding only 0.13% more for the hydrolysate of the residual soybean meal matrix. Among the essential amino acids required, branched-chain amino acids, such as Val, Leu, and Ile, are also required in large amounts by various types of animal cells, such as human fibroblasts and mouse

myeloma cells. The supplementation of branched amino acids in the culture medium can improve animal cell growth results (Yao & Asayama, 2017). Thus, the application of the obtained hydrolysates, which have significant amounts of these amino acids, can promote beneficial effects on animal cell proliferation for the cultured meat industry.

Among the non-essential amino acids, the supply of Glu/Gln is fundamental for providing energy and biosynthetic material to cells (Yao & Asayama, 2017). Although a high Glu/Gln content had been verified in the starting vegetable matrices, the same was not observed in the hydrolysates. In this case, the application of hydrolysates as an input of the culture medium for animal cells with lower nutritional requirements offers lower cytotoxicity risks due to the formation of ammonia from the decomposition of Glu/Gln (Yao & Asayama, 2017). For cells with higher nutritional requirements, the hydrolysates obtained may be deficient in Glu/Gln, requiring supplementation.

In this way, the presence of free amino acids is required in the production of inputs for animal cell culture medium, their absence is a limiting factor in animal cell growth (Freshney, 2011). Since the results obtained are close to or higher than the values described in the literature for fetal bovine serum, a comparison restricted to the concentrations of free amino acids shows that the hydrolysates obtained can act as new inputs in the supplementation of culture media for cultured meat. However, studies indicate that the addition of free amino acids generates different and less significant effects on animal cell growth when compared to the application of peptides obtained from protein hydrolysates (Chabanon et al., 2008). Thus, the role of free amino acids in animal cell growth must be evaluated in addition to their nutritional functionality.

To understand the distribution of the peptides present in the hydrolysates, the influence of the degree of hydrolysis, and the protease used on the size of the peptides, a size exclusion chromatography (SEC) was performed. Peptides were separated by molecular weight ranges, as shown in Figure 4.

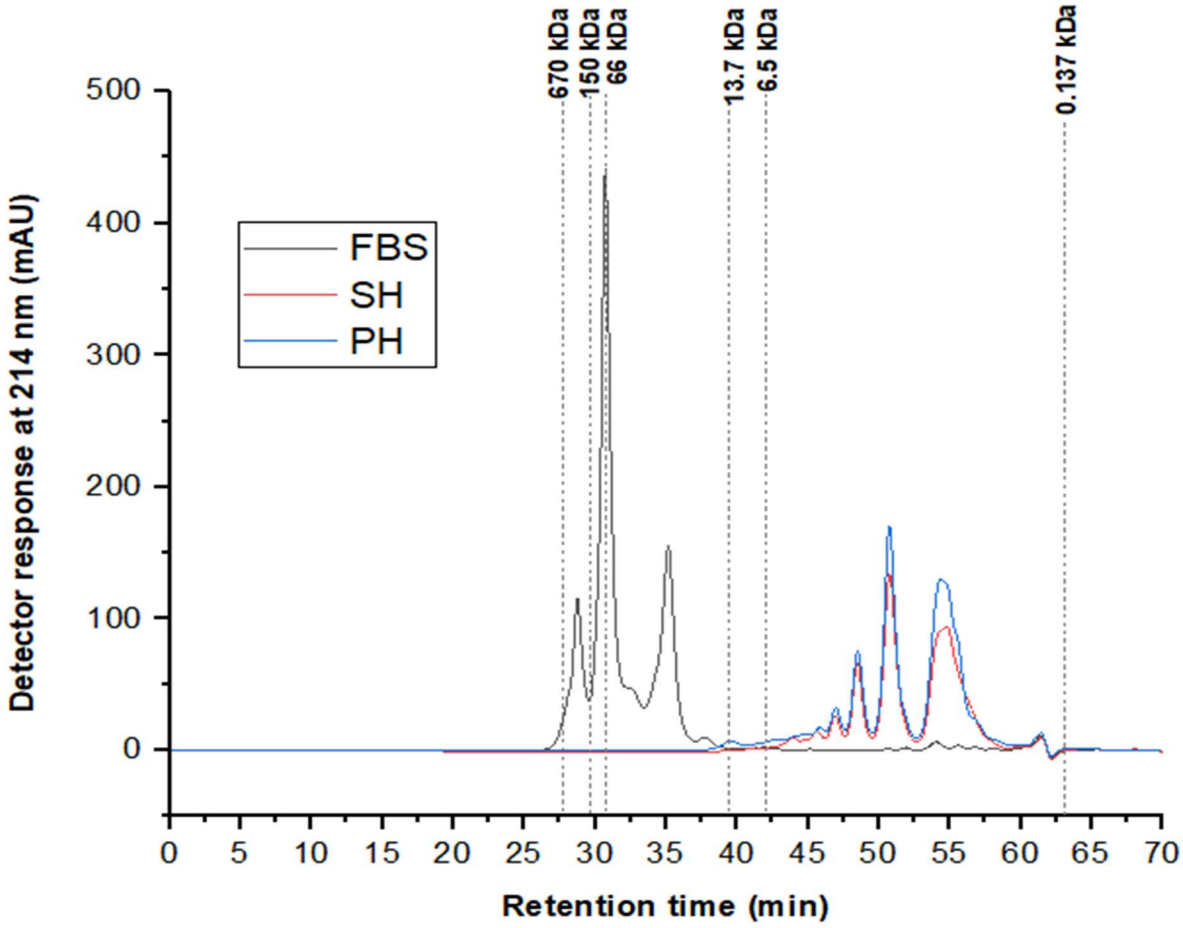


Figure 4. Chromatogram of the protein hydrolysates of soybean meal (SH), peanut meal (PH), and fetal bovine serum (FBS), obtained by size exclusion chromatography.

The percentage of each molecular weight fraction was expressed as the fraction of the relative area concerning the total area of the chromatogram, according to Table 5.

Table 5. Peptide distribution profile by weight range molecular

Size ranges (kDa)	Soybean meal hydrolysate (SH) (%)	Peanut meal hydrolysate (PH) (%)
> 670	-	-
670 – 150	-	-
150 – 66	-	-
66 – 13.7	-	0.70
13.7 – 6.5	0.38	2.27
6.5 – 0.137	99.62	96.47
< 0.137	-	0.56

Comparing the profiles of hydrolysates (SH and PH) with the profile of fetal bovine serum (FBS) in Figure 4, a difference in molecular weight distribution is observed. Fetal bovine

serum (FBS) has a higher concentration of peaks above 13.7 kDa, indicating the predominance of high molecular weight peptides (proteins), whereas hydrolysates (SH and PH) have peaks below 6.5 kDa, highlighting the presence of low molecular weight peptides. This is justified by the enzymatic cleavage performed on hydrolysates, but not performed on fetal bovine serum (FBS).

From the data presented in Table 5, it is observed that the proportion of peptides with molecular weight between 6.5 and 0.137 kDa is predominant in the hydrolysates for the respective degrees of hydrolysis obtained. This result confirms that the protease used for the enzymatic hydrolysis, Alcalase^{TM} 2.4L, is extremely efficient in breaking the peptide bonds of the analyzed hydrolyzed matrices. Furthermore, it can be observed that the performance of the protease is extremely determinant in the hydrolysis of these proteins since the chromatographic profiles shown in Figure 4 for the hydrolysate of soybean residual proteins (SH) and for the hydrolysate of peanut residual proteins (PH) are similar. F

The results obtained can be considered favorable since different studies indicate that low molecular weight peptides have positive nutritional effects on animal cell growth (Franek, 2004), and higher molecular size peptides may have interesting bioactive effects on the environment culture, such as anti-apoptotic effects and mimicry of cell survival and growth factor signals (Chabanon et al., 2008). In addition, another positive factor regarding the application of low molecular weight peptides in the cell culture medium comes from the uptake mechanisms that are energetically more efficient than the uptake mechanisms of free amino acids (Chabanon et al., 2008). Therefore, the peptide composition of the hydrolysates (SH and PH) can guarantee significant bioactive advantages over fetal bovine serum (FBS) in animal cell growth *in vitro*.

Although the chromatographic profiles shown in Figure 4 are very similar for the hydrolysates of residual proteins from soybean meal and peanut meal, as well as the total composition of amino acids already described, the performance of these hydrolysates on animal cell growth cannot be previously approximated or compared. Studies carried out by Chabanon et al. (2008) presented rapeseed hydrolysates, obtained from Esperase^{TM} 7.5L and Neutrase^{TM} 0.8L enzymes, with very close peptide distributions but with distinct bioactive effects on animal cell growth, in addition to the verified nutritional factor.

However, information about the influence of the size and nature of peptides on transport across the animal cell membrane is still scarce. Investigations in this line need to be carried out so that the promoting effects of animal cell growth can be correlated with the

molecular characteristics of the peptides, favoring the application of peptides as inputs for cultured meat (Chabanon et al., 2008).

Finally, the proteomic sequence was performed for the hydrolysate with the highest protein content and the highest degree of hydrolysis determined in previous studies. In this case, the hydrolysate of the residual matrix from the protein extraction of soybean meal was analyzed. For the hydrolysate, an exploratory untargeted proteomic sequencing was performed to identify the peptides generated from the cleavage caused by Alcalase™ 2.4L. In this analysis, 204 peptide sequences related to 25 protein groups were identified with mean square error (RMS) below 10%. However, the coverage percentages of the identified protein groups were lower than the percentages verified for the extracts, since the identification was performed from the fragmented peptides by a non-specific enzyme in the hydrolysis. Of the 204 identified sequences, 54 were repeated in different groups of proteins. This analysis also showed the molecular characteristics of the verified amino acid sequences.

The molecular weight distribution of the peptides from the hydrolysate of the residual soybean meal matrix can be seen in Figure 5.

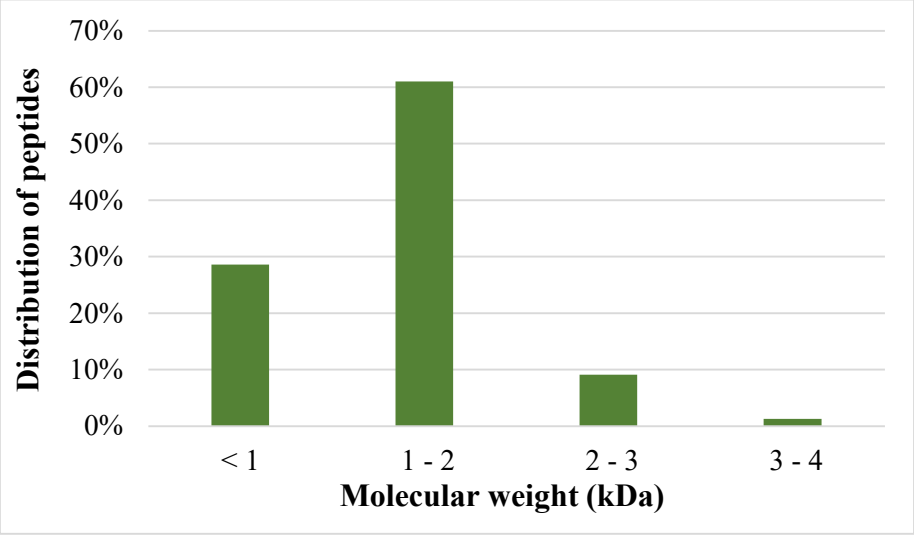


Figure 5. Molecular weight distribution of peptides identified in the hydrolysate of soybean meal residual matrix.

From the data in Figure 5, it is observed that the molecular weight range corresponding to 1 – 2 kDa concentrates the highest number of identified peptides. This result shows the efficiency of the enzyme (Alcalase™ 2.4L) used in breaking the peptide bonds of the residual matrix of protein extraction from soybean meal, favoring the application of the hydrolysate obtained as an input for an application in the culture medium for animal cells, which

requires peptides with low molecular weights for adequate nutritional use (Heidemann et al., 2000).

The distribution of amino acids in the identified peptide sequences can be seen in Figure 6. The identified peptide sequences were presented from the letter nomenclature of the amino acids: A = Ala; C = Cys; D = Asp; E = Glu; F = Phe; G = Gly; H = His; I = Ile; K = Lys; L = Leu; M = Met; N = Asn; P = Pro; Q = Gln; R = Arg; S = Ser; T = Thr; V = Val; W = Trp; Y = Tyr (Gaigher et al., 2022).

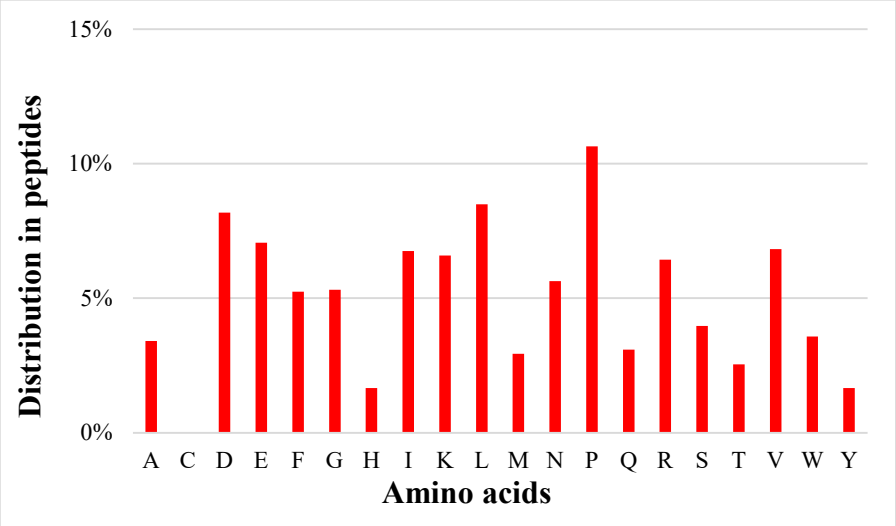


Figure 6. Distribution of amino acids in the peptide sequences identified in the hydrolysate of soybean meal residual matrix.

Figure 6 shows that Pro appears with the highest percentage in the peptide sequences of the hydrolyzed soybean meal, followed by Leu and Asp. Pro and Leu, although not relevantly identified in the total amino acid analysis of soybean meal, were also found in significant concentrations of free amino acids. Asp was detected with high levels of total amino acids in soybean meal. In the case of Cys, the presence of this amino acid is null in the peptide sequences identified in the hydrolysate of the soybean meal residual matrix, since the starting matrix itself is deficient in sulfur amino acids, as verified in the analysis of total amino acids. Although it appears as a portion of free amino acids, Cys is highly required in animal cell growth (Freshney, 2011). In this case, the need for supplementation of this amino acid must be evaluated.

The identified peptide sequences were compared with information from the BIOPEP - UWM virtual database, whose peptide bioactivity is recorded based on experimental data (Minkiewicz et al., 2022). The query to the BIOPEP-UWM database verified a bioactive

peptide sequence (EITPEKNPQLR), which has potential anti-cancer and anti-obesity action. This sequence has been localized to the three groups of β-conglycinin proteins identified - alpha, alpha' and beta.

Another sequence (PLPVLK) located in the Seed linoleate 9S-lipoxygenase-3 group of proteins was identified as part of a longer sequence with bioactivity (LLPLPVLK). The LLPPVLK sequence shows Alpha-glucosidase inhibition activity. Alpha-glucosity inhibitors are widely used in the treatment of type 2 diabetes (van de Laar et al., 2005). However, it cannot be concluded that the identified sequence has the same inhibitory activities as the complete sequence. As for activity related to cell growth, no sequence was initially identified. Currently, the main plant peptide isolated from monocotyledons and dicotyledons with growth factor is Phytosulfokine-α (PSK-α), whose YIYTQ sequence described in the literature was not located in any of the peptides identified in the hydrolysates (Yang et al., 2000).

The sequences were also compared with records from the virtual database CPPsite 2.0 – Database of Cell-Penetrating Peptides (Gautam et al., 2012), in which the peptide mapping allowed the identification of a sequence (SVIKPPTDEQQQRPQEEEEEEEDEKPQ) with cell penetration capacity related to the binding of EEE amino acids. This sequence is part of the G1 subunit of the soy glycinin protein group. Peptides with this intrinsic cell penetration property can help deliver useful conjugated molecules to cells and tissues (Agrawal et al., 2016).

In terms of toxicity, some combinations of amino acids have harmful effects on human consumption: FKKLKL, KKLL, KWK, and CYCD (Chaudhary et al., 2016). Potentially toxic combinations were tested on the soybean meal hydrolysate peptides and no combination was found. Thus, peptides from the hydrolysate of soybean meal residual matrix do not present direct risks to human consumption based on the presented sequences, although the cytotoxicity in animal cells must be tested.

3.4 Extraction and hydrolysis costs

An analysis of input costs was carried out, based on an initial estimate of production. The information obtained, although initial, may provide useful data for the implementation of the process on a large scale in an economical way.

The total production cost of protein extracts and protein hydrolysates was calculated as a function of raw material cost (C_{RM}), equipment cost (C_{Eq}), and operating cost (C_{Op}), based on a hypothetical scenario on a laboratory scale. Table 6 summarizes each of these costs, as well as the final price of the products obtained.

Table 6. Cost analysis of the extracts and protein hydrolysate obtained on a laboratory scale

Final products	C_{RM} [USD/kg]	C_{Eq} [USD/ kg]	C_{Op} [USD/kg]	Total cost [USD/kg]	Total cost [USD/g]
Soybean meal protein extract	1091.20	0.64	20.74	**1112.39**	**1.11**
Peanut meal protein extract	1122.24	0.64	21.05	**1143.75**	**1.14**
Soybean meal residual matrix hydrolysate	57.95	2.58	113.27	**173.80**	**0.17**
Peanut meal residual matrix hydrolysate	91.26	3.84	135.23	**207.11**	**0.21**

Through the data presented in Table 6, it is observed that the costs of protein extracts are very close to each other, however significantly higher than the costs of protein hydrolysates. Although the costs of agro-industrial wastes used are computed in the raw material costs (C_{RM}) only for protein extracts, the use of agro-industrial wastes makes up a minimal portion of the costs of the final product. Therefore, this result is mainly due to the cost and amount of Viscozyme^{TM} L enzyme used in protein extraction. The cost and amount of Viscozyme^{TM} L used in the extraction were considerably higher than the cost and amount of Alcalase^{TM} 2.4L used in the enzymatic hydrolysis of residual matrices. Alcalase^{TM} 2.4L is a commercial enzyme with high activity for enzymatic hydrolysis and relatively low cost, allowing its application on a large scale (Montone et al., 2019). However, Viscozyme^{TM} L still has few applications in the protein extraction market. Thus, the study carried out requires experimental planning to optimize the application of Viscozyme^{TM} L, seeking to maximize protein extraction and the possibility of reducing verified costs.

Thus, for protein extracts, the cost of raw material (C_{RM}) makes up most of the total cost of the final product. However, the same trend is not observed in the cost of protein hydrolysates, where operating costs (C_{Op}) exceed raw material costs (C_{RM}), as shown in Figure 7. The limited capacity of the lyophilizer and the low final yield per batch to obtain the lyophilized hydrolysate favor the expressive increase of this term.

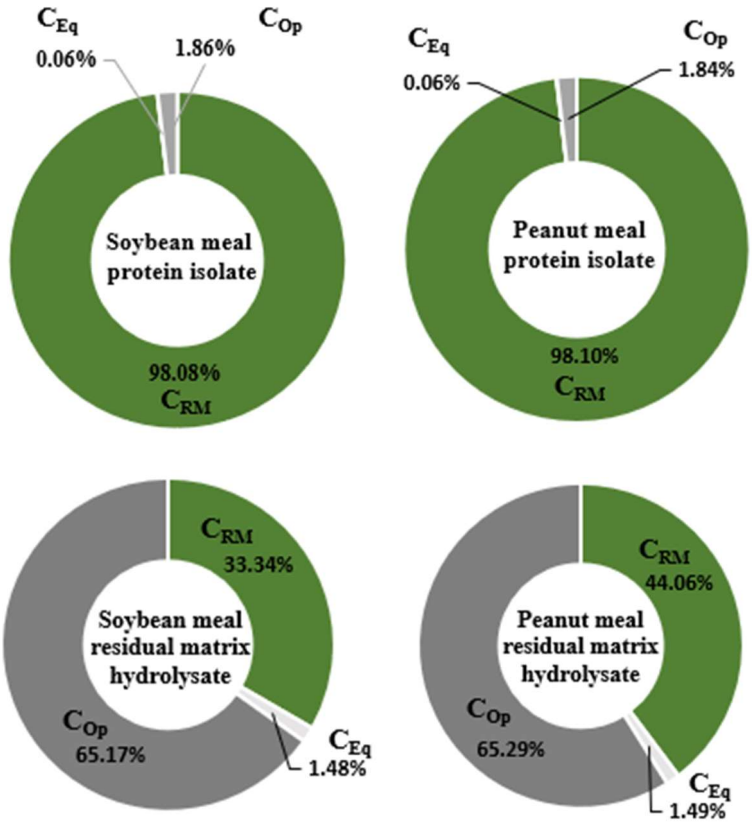


Figure 7. Percentages of costs (C_{RM}, C_{Eq}, and C_{Op}) calculated for laboratory-scale final products.

As shown in Figure 7, C_{RM} accounts for over 98% of protein extract costs. The percentage decreases considerably for protein hydrolysates – 33.34% for soybean meal residual matrix hydrolysate and 44.06% for peanut meal residual matrix hydrolysate – this result shows the influence of enzyme cost on raw material cost (C_{RM}) and the final volume obtained in the operating cost (C_{Op}). However, scale-up in the process of lyophilization of protein hydrolysates can generate reductions in the cost of operation.

Regarding equipment costs, these represent less than 2% of the total costs observed for extracts and hydrolysates. As for the final costs obtained, the production values of soybean meal derivatives were lower than the values of peanut meal derivatives. This trend can be observed due to the slightly increased yield in the extraction of protein from soybean meal and the lower concentration of Alcalase^{TM} 2.4L enzyme required for enzymatic hydrolysis, as determined in previous studies.

Compared to the commercial fetal bovine serum values shown in Table 7, it can be seen that the cost of extracts and hydrolysates was less than the selling price of any of the different fetal bovine serums listed. However, the calculated values are an estimate for

laboratory-scale production and do not take into account other industrial production, labor, packaging, storage, distribution costs, and the profit margin.

Table 7. Price of different commercial fetal bovine serum

Supplier/ reference	Description	Price* [USD]	Amount [mL]	Final Price [USD/mL]	Final Price (dry basis) [USD/g]
Sigma-Aldrich/F0926	USDA approved, sterile-filtered, suitable for cell culture	705.93	500	**1.41**	**69.80**
Sigma-Aldrich/F1051	Canada origin, sterile-filtered, suitable for cell culture	787.75	500	**1.58**	**78.22**
Sigma-Aldrich/12306C	Non-USA origin, from USDA approved countries, heat inactivated, sterile-filtered, suitable for cell culture	805.14	500	**1.61**	**79.70**
Sigma-Aldrich/F9665	Heat inactivated, non-USA origin, sterile-filtered, suitable for cell culture	875.50	500	**1.75**	**86.63**
Sigma-Aldrich/F4135	USA origin, heat inactivated, sterile-filtered, suitable for cell culture, suitable for insect cell culture, suitable for hybridoma	1606.72	500	**3.21**	**158.91**

* Data from April 27, 2023.

For comparative purposes, it should also be taken into account that the market values of fetal bovine serum are for the commercialized physical form, frozen liquid, with high humidity and low solids content. If the values were compared on a dry basis as presented in Table 7, the differences would be even more expressive.

However, the costs obtained refer only to the protein portion necessary for cell growth, the other components present in fetal bovine serum, such as growth factors, hormones and vitamins, were not verified in the inputs and would require other sources for supplementation, such as genetic recombination techniques.

4 Conclusion

Non-animal agro-industrial wastes subjected to enzymatic extraction and subsequent enzymatic hydrolysis of the residual matrix are seen as good candidates for obtaining inputs for cultured meat. In this sense, soybean meal and peanut meal were the agro-industrial wastes studied to obtain these inputs.

The characterization of protein extracts obtained from soybean meal and peanut meal indicated the presence of standard proteins from these plant matrices, with molecular

weights close to those of proteins present in fetal bovine serum. However, other analyzes are necessary to understand the use and function of these proteins in animal cell growth *in vitro*.

The hydrolysates obtained from a high degree of enzymatic hydrolysis showed significant levels of free amino acids and low molecular weight peptides. For the hydrolysate obtained from the residual matrix of soybean meal, in addition to the favorable nutritional characteristics, the proteomic sequencing demonstrated the presence of bioactive peptides and there was no detection of sequences indicated as toxic for human consumption.

And the hypothetical cost analysis obtained for the production of inputs on a laboratory scale showed values significantly lower than the market values of fetal bovine serum, indicating an alternative low-cost protein route for obtaining inputs for cultured meat. However, it should be taken into account that these inputs may correspond only to the necessary protein portion, requiring the composition of other nutrients for a possible supplementation of the culture media for animal cell growth.

Thus, protein extracts and hydrolysates obtained from non-animal agro-industrial residues are candidates with high nutritional, bioactive, non-toxic and low-cost potential for application as inputs for cultured meat.

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Supporting Information

Table S1. Proteins identified by proteomic sequencing in soybean meal protein extract

Accession	Description	mW (Da)	pI (pH)	Coverage (%)
P01063	Bowman-Birk type proteinase inhibitor C-II	9194	4.3799	91.57
P01064	Bowman-Birk type proteinase inhibitor D-II	9460	4.6670	97.59
P01055	Bowman-Birk type proteinase inhibitor	12083	5.8037	100.00
Q01417	18 kDa seed maturation protein	17595	9.9946	43.93
P19594	2S seed storage albumin protein	18447	5.0156	87.34
P01071	Trypsin inhibitor B	20028	4.459	95.58
P25272	Kunitz-type trypsin inhibitor KTI1	22531	4.7754	62.07
P01070	Trypsin inhibitor A	23990	4.7651	58.80
P05046	Lectin	30908	5.5957	89.82
F7J077	Beta-conglycinin beta subunit 2	50411	5.8228	76.08
P11828	Glycinin G3	54207	5.6265	88.77
P04405	Glycinin G2	54356	5.2983	95.25
P04776	Glycinin G1	55671	5.8257	97.78
P04347	Glycinin G5	57920	5.5137	85.46
Q04672	Sucrose-binding protein	60484	6.4233	80.34
P02858	Glycinin G4	63758	5.0054	96.80
P0DO15	Beta-conglycinin alpha subunit 2	70263	4.9380	82.15
P11827	Beta-conglycinin alpha' subunit	72184	5.3862	84.38

Table S2. Proteins identified by proteomic sequencing of residual matrix hydrolysate from soybean meal protein extraction

Accession	Description	mW (Da)	pI (pH)	Peptides	Coverage (%)
P0DO15	Beta-conglycinin alpha subunit 2	70263	4.938	29	20.9917
F7J077	Beta-conglycinin beta subunit 2	50411	5.8228	20	19.8178
P11827	Beta-conglycinin alpha' subunit	72184	5.3862	17	11.5942
P04776	Glycinin G1 OS=Glycine max	55671	5.8257	16	18.7879
P09186	Seed linoleate 9S-lipoxygenase-3	96697	6.2534	15	7.7013
P13917	Basic 7S globulin OS=Glycine max	46363	8.224	13	6.3232
P04405	Glycinin G2 OS=Glycine max	54356	5.2983	12	7.6289
P08170	Seed linoleate 13S-lipoxygenase-1	94310	5.9312	12	6.5554
P10538	Beta-amylase OS=Glycine max	56107	5.2734	12	10.8871
P02858	Glycinin G4 OS=Glycine max	63758	5.0054	7	9.4139
P09439	Seed linoleate 9S-lipoxygenase-2	97085	6.2725	7	4.7399
P01070	Trypsin inhibitor A OS=Glycine max	23990	4.7651	7	11.5741
P05046	Lectin OS=Glycine max	30908	5.5957	6	5.614
P11828	Glycinin G3 OS=Glycine max	54207	5.6265	5	3.5343
P38417	Linoleate 9S-lipoxygenase-4	96474	5.6279	5	2.3447
P04347	Glycinin G5 OS=Glycine max	57920	5.5137	4	7.3643
P24095	Seed linoleate 9S-lipoxygenase	96757	5.7173	4	2.1991
Q04672	Sucrose-binding protein	60484	6.4233	3	2.6718
P02519	17.3 kDa class I heat shock protein	17335	6.1904	2	7.8431
P62302	40S ribosomal protein S13	17129	10.7842	2	8.6093
P04793	17.5 kDa class I heat shock protein	17534	5.1621	2	7.8431
O48561	Catalase-4 OS=Glycine max	56701	6.8496	1	1.2195
P08298	Urease OS=Glycine max	90653	5.729	1	0.8363
P22895	P34 probable thiol protease	42766	5.6895	1	1.847
Q2PMN5	NAD(P)H-quinone oxidoreductase subunit 6, chloroplastic	19430	4.2451	1	2.8409

Tables S3. Peptides identified by proteomic sequencing of residual matrix hydrolysate from soybean meal protein extraction

Accession	m/z	Charge	Peptide mW (Da)	Delta (Da)	Sequence
	846.1621	4	3380.65	-0.03223	(E)REPQQPGEKEEDEDEQPRPIPFPRPQPR(Q)
	686.5875	4	2742.34	-0.01709	(Q)PGEKEEDEDEQPRPIPFPRPQPR(Q)
	615.8093	4	2459.22	-0.01294	(E)KEEDEDEQPRPIPFPRPQPR(Q)
	583.7868	4	2331.12	-0.00806	(K)EEDEDEQPRPIPFPRPQPR(Q)
	551.5267	4	2202.08	-0.00610	(E)EDEDEQPRPIPFPRPQPR(Q)
	519.2667	4	2073.04	-0.00342	(E)DEDEQPRPIPFPRPQPR(Q)
	384.4683	4	1533.83	0.01038	(E)RQFPFPRPPHQK(E)
	460.2516	3	1377.73	0.00085	(R)QFPFPRPPHQK(E)
	879.4667	1	878.47	-0.00616	(K)NKNPFLF(G)
	780.3901	2	1558.77	-0.00940	(Y)YVVNPDNNENLRL(I)
	836.9291	2	1671.86	-0.01550	(Y)YVVNPDNNENLRLI(T)
	943.9921	2	1885.99	-0.02124	(Y)YVVNPDNNENLRLITL(A)
	447.5952	3	1339.76	0.00110	(T)LAIPVNKPGRFE(S)
	366.8888	3	1097.63	0.00854	(L)AIPVNKPGRF(E)
P0DO15	409.9014	3	1226.68	0.00378	(L)AIPVNKPGRFE(S)
	386.2225	3	1155.64	0.00415	(A)IPVNKPGRFE(S)
	457.7653	2	913.51	0.00153	(I)PVNKPGRF(E)
	522.286	2	1042.56	0.00049	(I)PVNKPGRFE(S)
	659.3953	1	658.39	-0.00256	(S)VIVEIS(K)
	445.2453	4	1776.95	0.00183	(S)SRKTISSEDKPFNLR(S)
	508.7776	4	2031.08	0.00024	(L)GKFFEITPEKNPQLRDL(D)
	567.3036	3	1698.89	-0.00671	(F)FEITPEKNPQLRDL(D)
	442.2468	3	1323.71	0.00232	(F)EITPEKNPQLR(D)
	518.2828	3	1551.83	-0.00073	(F)EITPEKNPQLRDL(D)
	655.8538	2	1309.70	-0.00696	(I)TPEKNPQLRDL(D)
	605.3299	2	1208.65	-0.00720	(T)PEKNPQLRDL(D)
	541.2925	3	1620.85	-0.00073	(F)LSIVDMNEGALLLPH(F)
	754.8871	2	1507.77	-0.01196	(L)SIVDMNEGALLLPH(F)
	474.5886	3	1420.74	0.00378	(S)IVDMNEGALLLPH(F)
	725.275	1	724.27	-0.00317	(Q)FEWDE(S)
	743.3187	2	1484.63	-0.00635	(Q)FEWDESMGIPGAF(Y)
	561.3002	1	560.30	-0.00342	(M)GIPGAF(Y)
P09439	513.3077	2	1024.60	0.00061	(T)IMPLPVVKE(L)
	599.3382	1	598.33	-0.00220	(Q)ALPADL(I)
	457.2481	3	1368.72	0.00208	(H)GDLKDKPWWPK(L)
	442.5859	3	1324.73	0.00513	(L)KDKPWWPKLQ(T)
	561.3002	1	560.30	-0.00342	(F)GIPGAF(Y)
P24095	599.3382	1	598.33	-0.00220	(Q)ALPADL(V)
	874.459	1	873.46	-0.00842	(L)EIWDAIK(T)
	632.3355	1	631.33	-0.00519	(I)WDAIK(T)
	761.399	1	760.40	-0.00543	(A)VSLIDTN(S)
	501.2474	2	1000.48	0.00317	(Q)NQLDQMPR(R)
P11828	435.5584	3	1303.65	0.00647	(Q)NQLDQMPRRF(Y)
	759.3781	1	758.37	-0.00427	(Q)LDQMPR(R)
	646.2961	1	645.29	-0.00226	(L)DQMPR(R)

(continued on next page)

Table S3. *(continued)*

Accession	m/z	Charge	Peptide mW (Da)	Delta (Da)	Sequence
F7J077	497.9246	3	1490.75	-0.00134	(L)AIPVNKPGRYDDF(F)
	876.4519	2	1750.90	-0.01599	(L)AIPVNKPGRYDDFFL(S)
	784.3938	2	1566.78	-0.01111	(I)PVNKPGRYDDFFL(S)
	760.3759	2	1518.75	-0.01038	(S)FHSEFEEINRVL(L)
	618.3192	2	1234.62	0.00354	(H)SEFEEINRVL(L)
	574.8029	2	1147.59	0.00293	(S)EFEEINRVL(L)
	510.2788	2	1018.54	-0.00269	(E)FEEINRVL(L)
	659.3953	1	658.39	-0.00256	(G)VIVELS(K)
	445.483	4	1777.90	0.00500	(S)SRKTISSEDEPFNLR(S)
	553.7604	2	1105.50	0.00122	(S)SEDEPFNLR(S)
	759.3781	1	758.38	-0.00488	(N)NFGKFF(E)
	645.3378	1	644.33	-0.00220	(N)FGKFF(E)
	508.7776	4	2031.08	0.00024	(F)GKFFEITPEKNPQLRDL(D)
	567.3036	3	1698.89	-0.00671	(F)FEITPEKNPQLRDL(D)
	442.2468	3	1323.71	0.00232	(F)EITPEKNPQLR(D)
	518.2828	3	1551.83	-0.00073	(F)EITPEKNPQLRDL(D)
	655.8538	2	1309.70	-0.00696	(I)TPEKNPQLRDL(D)
	605.3299	2	1208.65	-0.00720	(T)PEKNPQLRDL(D)
	689.3687	2	1376.73	-0.00818	(S)SVDINEGALLLPH(F)
	760.4193	1	759.42	-0.00513	(D)DVFVIPA(A)
	564.606	3	1690.80	-0.00378	(Y)YVVNPDNDENLRMI(T)
	642.8102	2	1283.61	-0.00574	(Y)VVNPDNDENLR(M)
	510.2526	3	1527.74	-0.00073	(Y)VVNPDNDENLRMI(T)
P11827	447.5952	3	1339.76	0.00110	(T)LAIPVNKPGRFE(S)
	366.8888	3	1097.63	0.00854	(L)AIPVNKPGRF(E)
	409.9014	3	1226.68	0.00378	(L)AIPVNKPGRFE(S)
	386.2225	3	1155.64	0.00415	(A)IPVNKPGRFE(S)
	457.7653	2	913.51	0.00153	(I)PVNKPGRF(E)
	451.2515	3	1350.73	0.00159	(K)FEEINKVLFGR(E)
	659.3953	1	658.39	-0.00256	(S)VIVEIS(K)
	445.2453	4	1776.95	0.00183	(S)SRKTISSEDKPFNLR(S)
	567.3036	3	1698.89	-0.00671	(L)FEITPEKNPQLRDL(D)
	442.2468	3	1323.71	0.00232	(F)EITPEKNPQLR(D)
	518.2828	3	1551.83	-0.00073	(F)EITPEKNPQLRDL(D)
	655.8538	2	1309.70	-0.00696	(I)TPEKNPQLRDL(D)
	605.3299	2	1208.65	-0.00720	(T)PEKNPQLRDL(D)
O48561	736.3975	1	735.40	-0.00592	(N)NLPVFF(V)
P08298	772.4545	1	771.45	-0.00641	(S)FLPVPSL(D)
P22895	796.3665	1	795.37	-0.00641	(K)VTIDGYE(T)
Q2PMN5	670.3572	1	669.35	-0.00323	(D)FFLPF(E)
P04347	494.7656	2	987.51	0.00165	(Q)LDQNPRVF(Y)
	620.2683	2	1238.52	-0.00269	(A)GNPDIEHPETM(Q)
	492.7966	2	983.58	0.00116	(L)NSLTLPALR(Q)
	539.2693	2	1076.53	-0.00208	(Q)GNAVFDGELR(R)
P38417	561.3002	1	560.30	-0.00342	(F)GIPGAF(Y)
	666.4531	1	665.45	-0.00226	(A)PIPVIK(E)
	795.4925	1	794.49	-0.00549	(A)PIPVIKE(I)
	874.459	1	873.46	-0.00842	(L)EIWDAIK(S)
	632.3355	1	631.33	-0.00519	(I)WDAIK(S)

(continued on next page)

Table S3. *(continued)*

Accession	m/z	Charge	Peptide mW (Da)	Delta (Da)	Sequence
P04776	761.399	1	760.40	-0.00543	(A)VSIIDTN(S)
	444.2208	3	1329.63	0.00439	(N)SLENQLDQMPR(R)
	817.4021	2	1632.80	-0.01538	(N)SLENQLDQMPRRF(Y)
	565.7686	2	1129.52	0.00317	(L)ENQLDQMPR(R)
	478.5708	3	1432.69	0.00110	(L)ENQLDQMPRRF(Y)
	501.2474	2	1000.48	0.00317	(E)NQLDQMPR(R)
	435.5584	3	1303.65	0.00647	(E)NQLDQMPRRF(Y)
	759.3781	1	758.37	-0.00427	(Q)LDQMPR(R)
	646.2961	1	645.29	-0.00226	(L)DQMPR(R)
	584.3061	3	1749.90	-0.00598	(L)SVIKPPTDEQQQRPQ(E)
	813.619	4	3250.47	-0.02930	(L)SVIKPPTDEQQQRPQEEEEEEEEDEKPQ(C)
	568.6271	3	1702.86	-0.00598	(N)GERVFDGELQEGRVL(I)
	631.8378	2	1261.67	-0.00659	(L)LNALPEEVIQH(T)
	518.277	2	1034.54	-0.00134	(N)ALPEEVIQH(T)
	417.2295	3	1248.66	0.00366	(R)QIKNNNPFKF(L)
	603.8308	4	2411.31	-0.01514	(R)QIKNNNPFKFLVPPQESQKR(A)
	482.2497	2	962.48	0.00165	(R)SNDVYLPR(D)
	668.4299	1	667.43	-0.00482	(Q)NVLPLL(Q)
	540.7767	2	1079.54	-0.00220	(S)AFDLNFTPR(E)
	497.9531	3	1490.83	0.00146	(L)YSGGIKLPTDIISK(I)
	664.8902	2	1327.77	-0.00610	(Y)SGGIKLPTDIISK(I)
	414.5901	3	1240.74	0.00793	(S)GGIKLPTDIISK(I)
	395.5828	3	1183.72	0.00745	(G)GIKLPTDIISK(I)
P09186	376.5759	3	1126.70	0.00818	(G)IKLPTDIISK(I)
	738.4728	1	737.47	-0.00372	(K)ISPLPVL(K)
	666.4531	1	665.45	-0.00226	(S)PLPVLK(E)
	795.4925	1	794.49	-0.00549	(S)PLPVLKE(I)
	599.3382	1	598.33	-0.00220	(Q)ALPADL(I)
	874.459	1	873.46	-0.00842	(L)EIWDAIK(T)
	632.3355	1	631.33	-0.00519	(I)WDAIK(T)
	478.7372	2	955.46	0.00348	(K)NEPWWPK(M)
	665.3749	1	664.37	-0.00262	(T)PIHIW(D)
	443.7446	2	885.47	0.00269	(T)SLPEWVR(I)
P05046	500.2855	2	998.55	0.00049	(T)SLPEWVRI(G)
	528.7963	2	1055.58	0.00073	(T)SLPEWVRIG(F)
	602.3361	2	1202.64	0.01196	(T)SLPEWVRIGF(S)
	645.8408	2	1289.68	-0.01062	(T)SLPEWVRIGFS(A)
	447.2433	3	1338.70	0.00354	(W)SVVEDLPEGPAVK(I)
	726.8974	2	1451.79	-0.00781	(W)SVVEDLPEGPAVKI(G)
	626.8403	2	1251.67	-0.00610	(S)VVEDLPEGPAVK(I)
P01070	527.7739	2	1053.53	-0.00195	(V)EDLPEGPAVK(I)
	463.2542	2	924.49	0.00122	(E)DLPEGPAVK(I)
	456.8744	3	1367.59	0.00708	(G)ENKDAMDGWFR(L)
	499.2156	2	996.41	0.00317	(K)DAMDGWFR(L)

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Table S3. *(continued)*

Accession	m/z	Charge	Peptide mW (Da)	Delta (Da)	Sequence
P13917	803.4594	1	802.46	-0.00732	(L)VTVPQFL(F)
	748.7162	3	2243.14	-0.01563	(N)AYPSVDLVMDKPNGPVWRIS(G)
	735.3715	2	1468.74	-0.01086	(S)VDLVMDKPNGPVW(R)
	580.3137	3	1737.92	-0.00574	(S)VDLVMDKPNGPVWRI(S)
	609.3257	3	1824.96	-0.00183	(S)VDLVMDKPNGPVWRIS(G)
	863.9431	2	1725.89	-0.01648	(V)DLVMDKPNGPVWRIS(G)
	537.9604	3	1610.86	-0.00244	(D)LVMDKPNGPVWRIS(G)
	500.267	3	1497.78	0.00146	(L)VMDKPNGPVWRIS(G)
	438.2345	3	1311.68	0.00427	(V)MDKPNGPVWRI(S)
	700.3568	2	1398.71	-0.00977	(V)MDKPNGPVWRIS(G)
	591.3218	2	1180.64	-0.00732	(M)DKPNGPVWRI(S)
	634.8381	2	1267.67	-0.00671	(M)DKPNGPVWRIS(G)
P04405	761.399	1	760.40	-0.00543	(A)VSIIDTN(S)
	444.2208	3	1329.63	0.00439	(N)SLENQLDQMPR(R)
	817.4021	2	1632.80	-0.01538	(N)SLENQLDQMPRRF(Y)
	565.7686	2	1129.52	0.00317	(L)ENQLDQMPR(R)
	478.5708	3	1432.69	0.00110	(L)ENQLDQMPRRF(Y)
	501.2474	2	1000.48	0.00317	(E)NQLDQMPR(R)
	435.5584	3	1303.65	0.00647	(E)NQLDQMPRRF(Y)
	759.3781	1	758.37	-0.00427	(Q)LDQMPR(R)
	646.2961	1	645.29	-0.00226	(L)DQMPR(R)
	748.3617	1	747.36	-0.00531	(L)DFPALW(L)
P08170	631.8378	2	1261.67	-0.00659	(L)LNALPEEVIQH(T)
	518.277	2	1034.54	-0.00134	(N)ALPEEVIQH(T)
	707.3099	2	1412.61	-0.00293	(H)FEWDGSMGIPGAF(Y)
	561.3002	1	560.30	-0.00342	(M)GIPGAF(Y)
	774.4206	2	1546.84	-0.00989	(L)YEGGIKLPRDVIST(I)
	497.3409	2	992.66	0.00281	(S)TIIPLPVIK(E)
	446.8175	2	891.62	0.00366	(T)IIPLPVIK(E)
	511.3371	2	1020.66	0.00024	(T)IIPLPVIKE(L)
	666.4526	1	665.45	-0.00281	(I)PLPVIK(E)
	795.4917	1	794.49	-0.00629	(I)PLPVIKE(L)
	599.3382	1	598.33	-0.00220	(Q)ALPADL(I)
	457.2481	3	1368.72	0.00208	(H)GDLKDKPWWPK(L)
	442.5859	3	1324.73	0.00513	(L)KDKPWWPKLQ(T)
	443.4827	4	1769.90	0.00366	(L)NRNGLHLPSYSPYPR(M)
	462.9122	3	1385.71	0.00415	(N)GLHLPSYSPYPR(M)
	406.2117	3	1215.60	0.00818	(L)HLPSYSPYPR(M)
P02858	488.2662	2	974.52	-0.00171	(Q)LDQTPRVF(Y)
	492.7966	2	983.58	0.00116	(L)NSLTLPALR(Q)
	539.2693	2	1076.53	-0.00208	(Q)GNAVFDGELR(R)
	654.8648	2	1307.72	-0.00964	(S)YLKDVFRAIPS(E)

(continued on next page)

Table S3. *(continued)*

Accession	m/z	Charge	Peptide mW (Da)	Delta (Da)	Sequence
P10538	602.3369	1	601.33	-0.00317	(L)IIDIE(V)
	460.9716	4	1839.85	0.00098	(A)RAGHPEWELPDDAGKY(N)
	807.3592	2	1612.72	-0.01294	(A)GHPEWELPDDAGKY(N)
	597.2814	2	1192.54	0.00720	(E)WELPDDAGKY(N)
	623.3187	2	1244.63	-0.00415	(L)SGGWREDIRVA(G)
	738.7174	3	2213.14	-0.01514	(L)LEATKPTLPFPWLPETDMK(V)
	829.0869	3	2484.26	-0.02344	(L)LEATKPTLPFPWLPETDMKVDG(-)
	701.0257	3	2100.06	-0.00635	(L)EATKPTLPFPWLPETDMK(V)
	791.3936	3	2371.18	-0.01929	(L)EATKPTLPFPWLPETDMKVDG(-)
	658.0106	3	1971.02	-0.00916	(E)ATKPTLPFPWLPETDMK(V)
	748.3748	3	2242.13	-0.03320	(E)ATKPTLPFPWLPETDMKVDG(-)
	634.332	3	1899.98	-0.00806	(A)TKPTLPFPWLPETDMK(V)

Table S4. Equipment and description of materials for cost analysis

Supplier/Reference	Equipment	Description	Price* [USD]	Lifetime [Days]	Capacity	Power [W]
Esco Lifescience Group, Singapore	Thermostatic orbital shaker	OrbiCult IBS-R-25-3 model	9632.73	365	9 x 1 L	790
Hettich Benchtop, Tuttlingen, Germany	Centrifuge	Rotanta 460 model	11717.45	365	6 x 250 mL	1077.85
Terroni Scientific Equipment, Brazil	Lyophilizer	Benchtop Freeze Dryer LS3000	7292.49	365	3 kg	2500
Marconi Laboratory Equipment, Brazil	Dubnoff-type Metabolic Water Bath with Reciprocal Shaking	MA093/1	1628.46	365	9 x 250 mL	1600

* Data from April 27, 2023.

Table S5. Compound and reagent list and prices

Supplier/Reference	Description	Price* [USD]	Amount	Final Price
Nasdaq Index	Soybean meal	427.40	1000 kg	0.43 USD/kg
Brazilian market data	Peanut meal	385.38	1000 kg	0.39 USD/kg
Sigma-Aldrich/V2010	Viscozyme^{TM} L, cell wall degrading enzyme complex from *Aspergillus sp*., lysing enzyme from *Aspergillus sp.*	196.84	50 mL	3.94 USD/mL
Sigma-Aldrich/P4860	Alcalase^{TM} 2.4L, proteinase from *Bacillus licheniformis, Subtilisin A*	173.52	50 mL	3.47 USD/mL

*Data from April 27, 2023.

Table S6. Calculation of the cost of raw materials

Final product	Compound	Price [USD/g]	Quantity [g/kg]	C_{RM} [USD/kg]
Soybean meal protein extract	Soybean meal	0.00043	6635.00	2.84
	Viscozyme^{TM} L (density: 1.2 g/mL at 25 °C - Sigma Aldrich, 2023)	3.28	331.75	1088.36
	Total [USD/kg] - Soybean meal protein extract (86.45% protein)			**1091.20**
Peanut meal protein extract	Peanut meal	0.00039	6820.00	2.63
	Viscozyme^{TM} L (density: 1.2 g/mL at 25 °C - Sigma Aldrich, 2023)	3.28	341.00	1119.62
	Total [USD/kg] - Peanut meal protein extract (76.80% protein)			**1122.24**
Soybean meal residual matrix hydrolysate	Alcalase^{TM} 2.4 L (density: 1.25 g/mL at 25 °C - Sigma Aldrich, 2023)	2.78	20.88	57.95
	Total [USD/kg] - Dry soybean meal residual matrix hydrolysate (69.43% protein)			**57.95**
Peanut meal residual matrix hydrolysate	Alcalase^{TM} 2.4 L (density: 1.25 g/mL at 25 °C - Sigma Aldrich, 2023)	2.78	32.87	91.26
	Total [USD/kg] - Dry peanut meal residual matrix hydrolysate (54% protein)			**91.26**

Table S7. Calculation of the cost of equipment

Final product	Equipment	Price [USD]	Lifetime [Days]	Final Capacity [kg]	Cycles	C_{Eq} [USD/ kg]
Protein extracts	Thermostatic orbital shaker	9632.73	365	2.1	58	0.1517
	Centrifuge	11717.45	365		58	0.1845
	Lyophilizer	7292.49	365		58	0.1148
				Total [USD/kg]		**0.4510**
Soybean meal residual matrix hydrolysate	Lyophilizer	7292.49	365	0.17	126	0.9129
	Centrifuge	11717.45	365			1.4668
	Dubnoff-type Metabolic Water Bath with Reciprocal Shaking	1628.46	365			0.2039
				Total [USD/kg]		**2.5835**
Peanut meal residual matrix hydrolysate	Lyophilizer	7292.49	365	0.15	126	1.0898
	Centrifuge	11717.45	365			1.7511
	Dubnoff-type Metabolic Water Bath with Reciprocal Shaking	1628.46	365			0.2434
				Total [USD/kg]		**3.0843**

The formula used for the cost of equipment is listed below:

$$C_{Eq} = \frac{P}{(Cy * LT * Cap)}$$

where: P = equipment price; Cy = operating cycles; LT = lifetime; Cap = final productive capacity, in this case, limited by the final capacity produced in the lyophilizer, therefore the capacity of all equipment in each cycle was considered the final production of the lyophilizer.

Table S8. Calculation of equipment operating cost

Final product	Equipment	Energy Price [USD/kW·h]	Power [kW]	Operation time [h]	C_{Op} [USD/kg]
Soybean meal protein extract	Thermostatic orbital shaker	0.148	0.790	102.6	5.712
	Centrifuge		1.078	86.5	6.572
	Lyophilizer		2.500	48.0	8.457
				Total [USD/kg]	**20.741**
Peanut meal protein extract	Thermostatic orbital shaker	0.148	0.790	104.9	5.842
	Centrifuge		1.078	88.9	6.755
	Lyophilizer		2.500	48.0	8.457
				Total [USD/kg]	**21.054**
Soybean meal residual matrix hydrolysate	Dubnoff type Metabolic Water Bath with Reciprocal Shaking	0.148	1.600	7.8	10.565
	Centrifuge		1.078	0.5	0.459
	Lyophilizer		2.500	48.0	102.245
				Total [USD/kg]	**113.270**
Peanut meal residual matrix hydrolysate	Dubnoff type Metabolic Water Bath with Reciprocal Shaking	0.148	1.600	7.8	12.613
	Centrifuge		1.078	0.5	0.550
	Lyophilizer		2.500	48.0	122.062
				Total [USD/kg]	**135.225**

The formula used for the cost of operation is listed below:

$$C_{Op} = \frac{EP * P * OT}{Cap}$$

where: EP = energy price; P = power; OT = operation time; Cap = produced capacity, taking into account the final amount generated in the lyophilizer.

DISCUSSÃO GERAL

Com o advento tecnológico da carne cultivada, os desafios para o cultivo de células animais *in vitro* têm ganhado cada vez mais atenção. Muitos esforços têm sido realizados no sentido de tornar a carne cultivada passível de produção em larga escala, em condições economicamente favoráveis. Para isso, o desenvolvimento de meios de cultivo isentos de componentes de origem animal é a chave para superar parte dos altos custos de produção e os obstáculos de expansão (HO *et al*., 2021; RUBIO *et al*., 2020).

Dentre os componentes empregados para suplementação do meio de cultivo de células animais, o soro fetal bovino tem sido o principal, contribuindo para a proliferação e a sobrevivência das células. Contudo, sua utilização vai contra as premissas éticas da produção de carne cultivada, além do seu alto custo e dos riscos de contaminação envolvidos (HO *et al*., 2021).

Nesse contexto, este trabalho estudou rotas de obtenção de insumos proteicos de interesse para carne cultivada, a partir de resíduos agroindustriais livres de derivados animais, empregando técnicas assistidas por enzimas. Os insumos obtidos tiveram sua composição proteica descrita e analisada quanto às aplicações futuras em carne cultivada, uma vez que a complexidade destes tem se tornado a principal barreira com relação à utilização nos meios de cultivo. Além disso, foi realizada a estimativa do custo de produção dos insumos em escala laboratorial, para comparação inicial com o preço de mercado com o soro fetal bovino.

Para tanto, foram selecionados farelo de soja e de amendoim como resíduos agroindustriais para extração e hidrólise enzimática, devido ao alto teor de proteínas, à boa distribuição do perfil de aminoácidos totais e ao baixo teor de lipídios, dispensando o desengorduramento por solvente.

Os resultados das extrações proteicas demonstraram que, dependendo do tipo de matriz de partida e do pH do meio reacional empregado nas etapas de extração e solubilização, a aplicação da enzima Viscozyme^{TM} L é extremamente favorável à obtenção de proteínas. A metodologia assistida pela referida enzima, ajustada com pH igual a 7,0 e temperatura igual a 50 °C para a extração e pH igual a 10,0 e temperatura igual a 30 °C para solubilização das proteínas, possibilitou o aumento significativo das extrações em comparação com o método convencional, para os resíduos estudados. Valores de pH mais distantes do ponto isoelétrico das proteínas presentes nas matrizes geraram maiores rendimentos de extração. No entanto, uma análise mais aprofundada das temperaturas de extração e de solubilização e da concentração de enzima utilizada podem tornar os resultados ainda mais promissores.

Com relação ao conteúdo de proteínas dos extratos, análises iniciais de SDS-PAGE indicaram a presença de globulinas e albuminas a partir da comparação entre os pesos moleculares identificados e os valores descritos na literatura. Estes dois grupos também compõem a maior parcela de componentes proteicos do soro fetal bovino. Não obstante, essa comparação limita-se, inicialmente, a grupos semelhantes e pouco pode ser dito a respeito da atuação das proteínas obtidas dos resíduos vegetais no crescimento celular animal.

As matrizes residuais da extração proteica foram secas, reaproveitadas e submetidas à ação de proteases comerciais, a fim de obter hidrolisados proteicos com alto grau de hidrólise. Foram realizados estudos comparativos entre as proteases Alcalase^{TM} 2.4L e Neutrase^{TM} 0.8L, bem como aplicações simultâneas e sequenciais destas. Os resultados obtidos indicaram que a utilização individual da Alcalase^{TM} 2.4L apresenta melhor performance na hidrólise proteica das matrizes trabalhadas, comprovando a atuação rigorosa dessa enzima (TACIAS-PASCACIO *et al*., 2020). Embora a análise tenha se limitado à observação do grau de hidrólise, a combinação entre proteases e a limitação do grau de hidrólise podem colaborar na produção de peptídeos com diferentes bioatividades (CEYLAN *et al*., 2022).

No intuito de maximizar os graus de hidrólises para a obtenção de aminoácidos livres e de peptídeos de baixo peso molecular, requeridos nutricionalmente pelas células durante o crescimento celular animal (CHABANON *et al*., 2008), foram realizadas otimizações das condições experimentais para hidrólise enzimática das matrizes residuais com Alcalase^{TM} 2.4L.

As variáveis independentes analisadas foram a temperatura e a concentração de enzimas, tendo sido o pH fixado em 8,0, seguindo definições relacionadas na literatura (SANTOS *et al*., 2020). As condições ótimas determinadas em pH 8,0 para a matriz residual do farelo de soja foram: temperatura a 50 °C e concentração enzima:substrato de 3,5%. Para a matriz residual do farelo de amendoim, a temperatura reacional também foi de 50 °C, porém, pelas diferenças nas conformações proteicas, a concentração enzima:substrato foi de 5,0%. Nestas condições, o grau de hidrólise atingido para cada matriz residual, em um tempo de reação de 5 h, foi superior a 30%, indicando boa clivagem das ligações peptídicas e, consequentemente, conteúdos favoráveis de aminoácidos livres e de peptídeos de baixo peso molecular.

Os extratos e os hidrolisados proteicos obtidos foram, então, caracterizados objetivando a melhor compreensão a respeito de suas composições e complexidades proteicas. Análises de teor proteico e de distribuição de peso molecular foram realizadas, além da verificação da composição de aminoácidos livres para os hidrolisados. Também foi performado

o sequenciamento proteômico para as amostras obtidas a partir do farelo de soja, uma vez que apresentaram maior conteúdo proteico e máximo grau de hidrólise.

Para os extratos foram observados teores proteicos acima de 75% e distribuição dos pesos moleculares em faixas semelhantes aos valores descritos para as principais proteínas do material de origem. Embora o resultado desta análise corrobore o resultado do SDS-PAGE realizado e os picos de maior intensidade tenham sido verificados em faixas próximas aos picos do soro fetal bovino, a simples comparação dos pesos moleculares ainda tem discussão limitada no que diz respeito à atuação dessas proteínas como insumos para carne cultivada.

Em relação aos hidrolisados, as análises também indicaram conteúdos proteicos significativos e próximos à composição centesimal em base seca do soro fetal bovino. Além disso, as disposições dos aminoácidos livres e peptídeos de baixo peso molecular demonstraram o sucesso das hidrólises enzimáticas, uma vez que a distribuição de pesos moleculares dos hidrolisados se concentrou na faixa abaixo de 6,5 kDa, indicando que a maximização do grau de hidrólise foi atingida para cada matriz estudada. Assim, pode-se afirmar que a Alcalase^{TM} 2.4L foi responsável pela extensiva clivagem das ligações peptídicas e a aplicação desses hidrolisados em meios de cultivo livres de soro fetal bovino tem potencial para promover o crescimento de células animais pelas qualidades nutricionais destacadas.

Quanto ao sequenciamento proteômico dos insumos obtidos a partir do farelo de soja, os resultados apresentaram, além das características nutricionais verificadas, outros aspectos relevantes à aplicação desses insumos. Dentre os 18 grupos proteicos identificados no extrato, os principais estão relacionados com atividades de minimização de danos e morte celular *in vitro* e com a atuação como agentes antibacterianos, anti-inflamatórios e não tóxicos para diversos grupos celulares testados em estudos. Por sua vez, a proteômica do hidrolisado apresentou 204 sequências peptídicas, relacionadas a 25 grupos de proteínas. O peso molecular dos peptídeos identificados confirmou os dados obtidos na cromatografia de exclusão por tamanho, estabelecendo a faixa de 1 – 2 kDa como o intervalo de maior concentração. De modo geral, observou-se boa distribuição de aminoácidos nas sequências analisadas, exceto para cisteína, que é um dos aminoácidos requeridos no crescimento celular animal. Em termos de bioatividade, as sequências foram comparadas aos registros do banco de dados BIOPEP-UWM, sendo uma delas catalogada com potencial anticâncer e antiobesidade. Não foram identificadas combinações tóxicas de aminoácidos entre os peptídeos analisados.

Por fim, os resultados da análise de custos em escala laboratorial apresentaram valores reduzidos para a produção dos insumos quando comparados aos valores de mercado do soro fetal bovino, principalmente em base seca. Contudo, deve ser dada atenção especial a esta

análise, uma vez que os insumos só podem ser avaliados em relação à parcela proteica da composição do soro fetal bovino. Fatores de crescimento, hormônios e outros nutrientes não são suplementados por esses insumos, mostrando-se necessária a utilização de outras fontes para tal suplementação. Além disso, os custos calculados também não levaram em conta muitos outros aspectos da produção, como mão-de-obra, embalagem, armazenamento e distribuição.

De modo geral, os resultados apresentaram considerável número de indícios positivos a respeito dos insumos como fonte nutricional e como agentes bioativos no crescimento celular animal para carne cultivada. Não obstante isso, mostram-se necessárias outras investigações acerca da citotoxicidade, do transporte celular e do aproveitamento destes insumos pelas células para que a aplicação seja sedimentada.

CONCLUSÃO

Neste trabalho, resíduos agroindustriais livres de derivados animais foram valorizados para a obtenção de insumos de interesse para carne cultivada, com foco na extração proteica assistida por enzimas e na obtenção de aminoácidos e de peptídeos bioativos de baixo peso molecular via hidrólise enzimática.

Os resultados das extrações proteicas dos resíduos selecionados indicaram que a especificidade da enzima somada às condições aplicadas para a extração e a solubilização das proteínas são fatores preponderantes, que podem aumentar significativamente o rendimento da extração quando comparada às metodologias convencionais.

Quanto à hidrólise enzimática da matriz residual da extração, a utilização exclusiva da Alcalase^{TM} 2.4L apresentou os melhores resultados em termos do grau de hidrólise. A otimização do processo foi performada a partir de duas variáveis independentes: temperatura e concentração de enzima, que maximizaram o resultado.

Os extratos foram obtidos com alto teor proteico e as proteínas relacionadas aos grupos proteicos mais abundantes do material de origem. Das proteínas identificadas no sequenciamento proteômico do extrato do farelo de soja, vários grupos apresentaram funções biológicas favoráveis ao crescimento celular animal, caracterizando-se como insumo proteico potencial para carne cultivada.

Os hidrolisados apresentaram teores proteicos adequados, aminoácidos livres em quantidades significativas e distribuições peptídicas em baixos pesos moleculares. Esses resultados podem favorecer a aplicação nutricional dos hidrolisados em meios de cultivo livres de soro fetal bovino. O sequenciamento proteômico do hidrolisado obtido a partir da matriz residual do farelo de soja possibilitou, também, a identificação de bioatividade para uma das sequências peptídicas analisadas.

Com relação aos custos dos insumos, os valores estimados em escala laboratorial mostraram-se consideravelmente baixos. Todavia, para que haja comparação efetiva com os valores de mercado do soro fetal bovino devem ser considerados outros custos não relatados na análise, bem como a necessidade de utilização de fontes complementares de componentes essenciais ao crescimento celular.

Em arremate, os resultados obtidos neste estudo são animadores, visto que os insumos avaliados apresentam fatores nutricionais e bioativos favoráveis para o crescimento celular animal, o aproveitamento dos resíduos agroindustriais contribui para a redução dos impactos ambientais e o custo obtenção desses insumos em escala laboratorial indica vantagens

econômicas do processo. Dessa forma, tem-se que a realização de novas pesquisas visando à exploração das potencialidades desses insumos constitui terreno fértil não apenas para a produção de carne cultivada, mas também para o desenvolvimento de novas tecnologias de cultivo de células animais.

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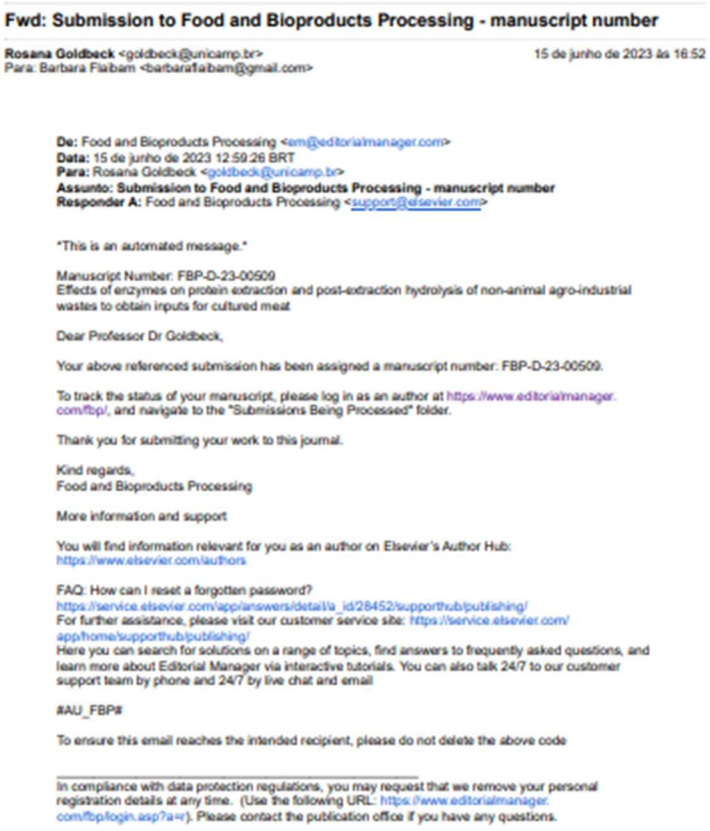
ANEXO – COMPROVANTES DE SUBMISSÃO DOS ARTIGOS

ARTIGO I: Non-animal protein hydrolysates from agro-industrial wastes: a prospect of alternative inputs for cultured meat

Artigo em elaboração para posterior submissão.

ARTIGO II: Effects of enzymes on protein extraction and post-extraction hydrolysis of non-animal agro-industrial wastes to obtain inputs for cultured meat

Artigo submetido e sob revisão na revista “Food and Bioproducts Processing”, revista com fator de impacto de 4.6 (Clarivate Analytics, 2023) e A1 na área de avaliação “Ciência de Alimentos” na classificação de periódicos quadriênio 2017-2020 da Qualis Periódicos CAPES.



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ARTIGO III: Low-cost protein extracts and hydrolysates from non-animal agro-industrial waste: properties and analyzes for application as inputs of interest for cultured meat

Artigo em elaboração para posterior submissão.