

Study on the production of *Aspergillus niger* proteases using whey

María del Pilar Marín-Cortez¹

Armando Robledo-Olivo^{2,5}

Ana Verónica Charles-Rodríguez²

Rosa María Rodríguez-Jasso³

Susana González-Morales⁴

Adalberto Benavides-Mendoza⁵

1 Posgrado en Agricultura Protegida-Universidad Autónoma Agraria Antonio Narro. Saltillo Coahuila, México. CP. 25315.

2 Laboratorio de Fermentaciones y Biomoléculas-Departamento de Ciencia y Tecnología de Alimentos-Universidad Autónoma Agraria Antonio Narro. Saltillo Coahuila, México. CP. 25315.

3 Departamento de Investigación en Alimentos-Facultad de Química-Universidad Autónoma de Coahuila. Saltillo, Coahuila. México. CP. 25280.

4 SECIHTI-Universidad Autónoma Agraria Antonio Narro. Saltillo, Coahuila, México. CP. 25315.

5 Departamento de Horticultura-Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila, México. CP. 25315.

Autor para correspondencia: armando.robledo@uaaan.edu.mx.

Abstract

Whey is a byproduct or waste product of the dairy industry, generally of little use, with a high protein content that can induce fungal synthesis of proteolytic enzymes. This study aimed to evaluate the sustainable production of proteolytic enzymes of *Aspergillus niger* using whey as a substrate in liquid fermentation. Fermentation was carried out in flasks with 50 ml of whey culture medium and glucose concentrations in the Laboratory of Fermentations and Biomolecules in 2024. Fermentation was performed at 30 °C with orbital shaking at 150 rpm for 48 h. The fermentation extracts were analyzed for peptide content and protease activity. The results showed that the enzyme extract without added glucose produced the highest protease activity after 24 h. The use of this extract at a pH of 3.5 resulted in a higher release of peptides from the whey. Whey, being a complex substrate that contains sugars, proteins, fats, and minerals, can influence the growth of microorganisms and the production of enzymes. The valorization of this agro-industrial waste provides an effective and sustainable method to produce biomolecules for agri-food and agro-industrial use.

Keywords:

enzymes, fermentation, peptides, protease, proteins.



Introduction

Proteases are a set of enzymes (proteinases, peptidases, and amidases) that catalyze the breakdown of proteins, releasing peptones and amino acids (Liu *et al.*, 2024). They are classified depending on their optimal pH range to perform hydrolysis: acidic, basic, and neutral (Cho *et al.*, 2015). Their industrial importance lies in their high specificity and efficiency, as they cover 60% of the enzyme production market and account for 40% of global enzyme sales (Naveed *et al.*, 2021). The industrial applications of proteases encompass various areas, including detergent, leather, biotechnology and food industries.

The biochemical characteristics of proteases, such as speed, biodegradability, high specificity, and reduced residue generation, give this group of enzymes a high market value. The protease market during 2025 is forecast at 3.73 trillion dollars and is expected to reach 5.02 trillion by 2030, growing at a compound annual growth rate of 6.1% during the forecasted period (2025-2030) (Mordor Intelligence, 2025).

In particular, *Aspergillus niger* proteases have been identified for their efficacy in the hydrolysis of complex protein structures, including those found in whey (Li *et al.*, 2020; Crament *et al.*, 2024; Nouri *et al.*, 2024). The production of these proteases can be optimized under specific conditions, making them a potential, environmentally friendly, and cost-effective alternative to chemical protein hydrolysis processes.

Whey is a waste from agribusiness, which is obtained from the coagulation of milk casein, generating approximately 9 L of whey when producing 1 kg of fresh cheese (Fernández-Gutiérrez *et al.*, 2017). The cheese-making process causes a problem in the treatment of this waste, which generates pollution and damage to the environment (Dainka *et al.*, 2019), since only 50% of the whey generated in Mexico is processed (Mazorra and Moreno, 2019).

By its nature, whey provides nutrients such as sugars, proteins, fats, and minerals, which are essential for the growth of microorganisms and the production of enzymes. The protein content of whey can be broken down into peptides and amino acids by enzymatic hydrolysis, this being a key to improving digestibility and reducing protein allergenicity, thus facilitating the absorption of bioactive peptides. Recent research highlights that this enzymatic process generates products with functional properties with potential for use in food, agricultural biostimulants, among others (Boscaini *et al.*, 2020; Zamana *et al.*, 2023).

This study aimed to explore the effect of glucose concentration on the production of *Aspergillus niger* proteases and their subsequent ability to hydrolyze whey proteins to generate soluble peptides. By investigating the production process of proteolytic enzymes and their efficacy in the breakdown of whey proteins, this research seeks to improve the value of whey as a resource and contribute to sustainable solutions in food processing and biotechnology.

Materials and methods

The research was conducted at the Laboratory of Fermentations and Biomolecules in the Department of Food Science and Technology of the Antonio Narro Autonomous Agrarian University (UAAAN), for its acronym in Spanish.

Raw material procurement and analysis

The whey was obtained from the production of asadero cheese and was kept in glass bottles at refrigeration at 3-5 °C for 24 h until use. The proximate analysis of the whey was performed by drying the whey samples in an oven at 60 °C for 48 h, in order to obtain powder. Subsequently, the percentage content of total ash, protein, and fat of the whey powder was determined with the methodology described by the AOAC (1980).

Total carbohydrate quantification was performed with the phenol-sulfuric acid method, following the method by Dubois *et al.* (1956). A calibration curve was generated using reactive-grade glucose (Fermont, Mexico) from 20 to 100 $\mu\text{g ml}^{-1}$ ($y = 60.71x + 6.645$; $R^2 = 0.99$).

Inoculum and fermentation conditions

The strain used was the *Aspergillus niger* M4 strain (access code KY825168.1) from the collection of the Laboratory of Fermentations and Biomolecules. The cryopreserved strain was propagated in plates with PDA medium (Dibico, Mexico) at 35 °C for five days and then the plates were kept refrigerated at 4 °C until use. Conidia were harvested from the completely sporulated plates, adding 30 ml of Tween 80 (0.1% w/v) (Hyce, Mexico).

A Neubauer chamber (Marienfeld, Mexico) was used to count conidia ml^{-1} for the inoculation of the medium. The liquid culture medium consisted of whey enriched with mineral salts in the following proportion: 5 g L^{-1} of KH_2PO_4 (Jtbaker, Mexico), 0.5 g L^{-1} of MgSO_4 (Jtbaker, Mexico), 0.5 g L^{-1} of KCl (Jtbaker, Mexico), and different concentrations of glucose (Fermont, Mexico) (0%, 7.5%, 15% and 30%). To ensure quality control and maintain the purity of the medium, the same batch of enriched medium was used for fermentation tests.

In order to obtain the protease enzyme, liquid fermentation was carried out in 250 ml Erlenmeyer flasks with a working volume of 50 ml of culture medium. The controlled fermentation conditions were at 30 °C, pH of 5.5, orbital shaking at 150 rpm (Innova 44 Orbital Incubator, New Brunswick), a maximum time of 144 h, and an inoculum of 1×10^6 spores of *A. niger* M4 per ml of medium.

For each glucose level (0%, 7.5%, 15%, and 30%), a completely randomized design was developed, where each flask was considered as an experimental sample reactor, and each experimental time was performed in triplicate, for a total of 84 reactors in times of up to 144 h. The 0% glucose level was considered a negative control. After the fermentation process, the fermentation extracts (FEs) were recovered by centrifugation (Hermle laborTechnik, model Z 32 HK) at 4 000 rpm for 15 min at 4 °C. The supernatant was filtered through a 0.45 μm membrane (Luzuren, China), and the filtrate was considered the FE for future analysis.

Determination of protease activity

A protease activity assay was performed on the obtained EFs, using the methodology by Kunitz (1946), modified by Johnsvesly *et al.* (2002). Casein (Fagalab, Mexico) at 1% was prepared and dissolved in a phosphate buffer at 50 Mm at a pH of 7.0. In the test tube, 0.95 ml casein was added. Subsequently, it was incubated at 30 °C for 15 min and 0.05 ml of FE was added; then, it was left to react for 15 min in a temperature-controlled bath at 30 °C. The reaction was stopped with 1.5 ml of trichloroacetic acid (TCA) (Fagalab, Mexico) at 5% and centrifuged (Hermle laborTechnik, model Z 32 HK) at 4 500 rpm for 20 min, taking the supernatant as an enzymatic extract (EE).

A unit of proteolytic activity (U) was defined as the amount in μmol of tyrosine released per minute, under the conditions of the assay. Tyrosine was determined by the method by Lowry *et al.* (1951). The calibration curve was performed in the range of 0 to 156 $\mu\text{g ml}^{-1}$ tyrosine (FAGALab, Mexico) in 1 M hydrochloric acid (Jalmek, Mexico) ($y = 0.0037x + 0.0308$; $R^2 = 0.9854$). To quantify soluble peptides, EEs were used, and ultraviolet absorption was quantified at 280 nm (Thermo Fisher Scientific, model G10S).

For the assessment of EE in terms of acidic or alkaline proteolytic activity on whey, enzymatic activities were evaluated at pH 3.5 and 7.0, respectively. Peptide quantification was performed using a tyrosine standard curve (Fagalab, Mexico; $y = 4.0721x$; $R^2 = 0.9913$).

Statistical analysis

The experiments were based on a randomized experimental design, with two factors (glucose and time) at different levels (4 and 7, respectively), considering protease activity as the response variable. The arithmetic mean of the collected data $\pm$ its standard deviation is reported, with three replications per sampling time. Regarding the analytical determinations, the arithmetic mean of three replications for each repetition $\pm$ its standard deviation is reported.

The experimental treatments were applied under controlled conditions, keeping the environmental and operational variables constant. The experimental design and statistical analyses were performed with the Minitab 17 statistical software, using a Shapiro-Wilkins normality test, a one-way analysis of variance with *post-hoc* tests of comparison of means, Fisher (LSD) ($p < 0.05$) and Dunnett ($p < 0.05$). A significance level of $\alpha = 0.05$ was established to minimize the risk of type I errors, guaranteeing the reliability of the results obtained.

Results and discussion

The primary purpose of performing the proximate analysis of whey in this study was to validate the carbohydrate and protein contents present (Table 1), in order to identify the carbon-nitrogen (C/N) ratio. The contents found were as follows: crude protein ($9.58\% \pm 0.04$), fat ($6.27\% \pm 0.58$), ash ($6.09\% \pm 0.53$), and total carbohydrates ($78.05\% \pm 1.04$). The above data were used to calculate the C/N ratio of whey, finding a value of 20.37. These chemical compounds serve as a substrate for the growth of microorganisms and the synthesis of protease-like enzymes.

Table 1. Composition and percentage nutritional value on a dry basis of asadero cheese whey.

Composition	Percentage on a dry basis (%)	
Ash	6.09	± 0.53
Crude protein	9.58	± 0.04
Fat	6.27	± 0.58
Total carbohydrates	78.05	± 1.04

The figure in numbers represents the meaning ($n = 3$) $\pm$ the standard deviation of the samples.

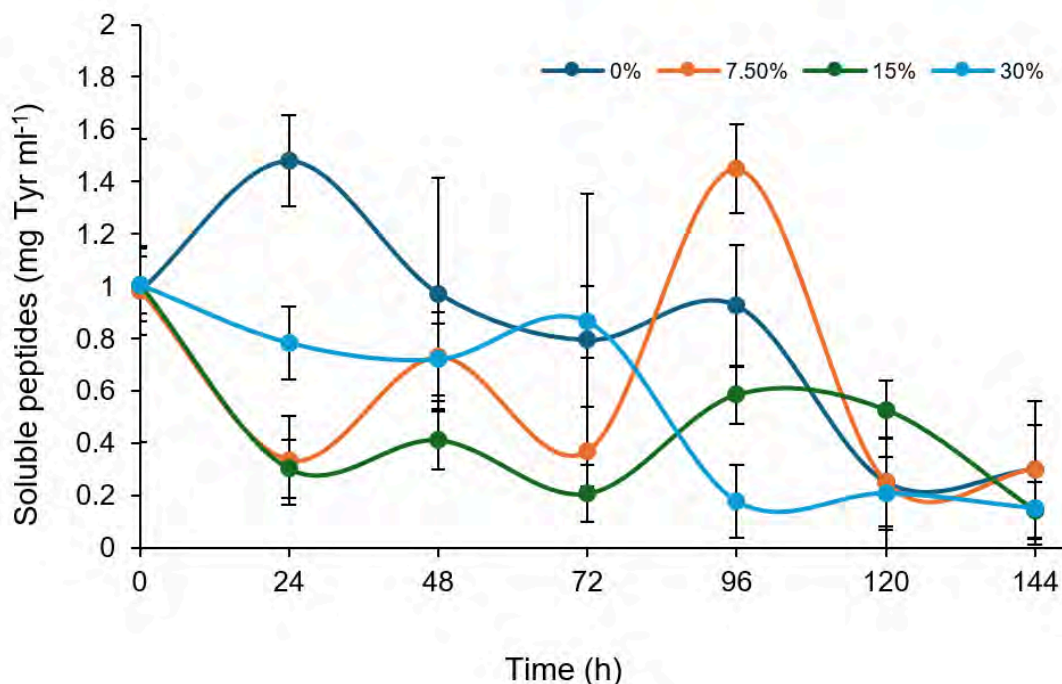
Recent studies have shown that whey protein is a rich source of bioactive peptides, which can improve metabolic health and immune function (Quintieri *et al.*, 2025). In addition, the presence of carbohydrates in high concentrations can influence the stability and solubility of the peptides generated during enzymatic hydrolysis reactions (Tang *et al.*, 2025).

Likewise, the fat present in whey can affect the emulsification and availability of bioactive peptides, characteristics that are relevant for its application in nutritional supplements and functional products (Lam *et al.*, 2019). The proportion of ash is also an indicator of the presence of essential minerals, which can influence the enzyme activity and stability of compounds. These findings reinforce the importance of asadero cheese whey as a valuable source of chemical and biochemical compounds, hence the interest in its use in processes and products with application in agribusiness (Benítez, 2024).

The soluble peptide content (mg of Tyr ml^{-1}) in each sample during the fermentation process of *A. niger* M4 in whey is shown in Figure 1. The degradation of proteins to peptides by an enzymatic reaction over time was observed. At time zero, all samples start with a similar baseline ($1 \text{ mg of Tyr ml}^{-1}$), but clear differences emerge over time. It should be noted that the treatment at 0% glucose (control) shows a peak of peptides at 24 h, with $1.48 \text{ mg of Tyr ml}^{-1}$ and the treatment of 7.5% glucose accumulates a concentration of $1.45 \text{ mg of Tyr ml}^{-1}$ at 96 h.



Figure 1. Graphical representation of the means of peptides released in mg of tyrosine per mL as a function of time (h). The different lines represent the concentration of glucose at 0%, 7.5%, 15%, and 30%.



The 15% and 30% treatments exhibit a lower concentration of peptides over the fermentation time, with the 30% glucose treatment accumulating 0.86 mg of Tyr ml⁻¹ after 72 h. These patterns may suggest that moderate glucose supplementation (7.5%) encourages peptide release during fermentation until it peaks at 96 h, while higher glucose supplementation (15% and 30%) appears to inhibit this process.

This could be due to an effect in which excessive glucose levels inhibit proteolytic activity, possibly due to the growth of the fungus and a higher requirement for structural nitrogen; therefore, the microorganism begins to degrade the amino acids present in whey (Rojas and Martínez, 2023). This results in a decrease in the concentration of the peptides. Kashung and Karuthapandian (2025) mention that low to moderate concentrations of glucose in substrates can stimulate enzyme activity and peptide release in microbial fermentations, whereas higher concentrations can disrupt enzyme access by inducing substrate aggregation.

Similarly, Li *et al.* (2019) observed that the efficiency of enzymatic hydrolysis peaks at intermediate concentrations in substrates, above which steric impediment or inhibitory interactions can reduce enzymatic efficacy. The initial increase in the control (0%) could reflect native enzyme activity adapted to the environment, but it is transient and less sustained compared to the enzyme activity resulting from moderate glucose supplementation (7.5%).

The decrease in soluble peptides at higher levels of glucose addition is consistent with the findings by Wang *et al.* (2019), who highlighted the importance of an optimal enzyme-substrate ratio to maximize protein hydrolysis. Therefore, future research may focus efforts on improving the extraction and stability of bioactive peptides to maximize their benefits in human health and agribusiness applications.

The analysis of protease activity (U ml⁻¹) in whey fermentation extracts reveals variations in the efficiency of protein hydrolysis under different glucose concentrations. Correlation analyses showed

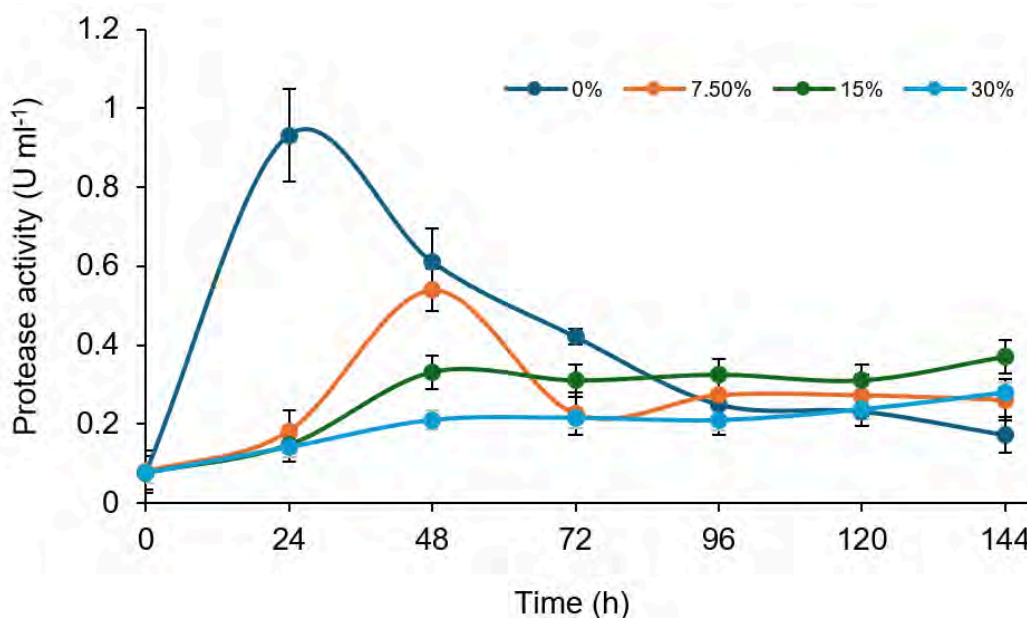
a negative relationship between protease activity and the addition of glucose to the fermentation process ($U\ ml^{-1} = 0.793 - 0.019\ ConcGluc$), with an average production of $0.793\ U\ ml^{-1}$ of protease if glucose was not added. On the other hand, there is a decrease of $0.019\ U\ ml^{-1}$ in maximum protease activity for each percentage concentration of glucose that is added.

The glucose concentration is not significant ($p = 0.112$) for protease production and represents 78.88% of the maximum production of enzyme activity. Pearson's correlation of protease activity and glucose concentration showed a strong negative relationship (-0.888), indicating that the higher the glucose concentration, the lower the protease activity. Dunnett's post-hoc test made it possible to compare the control with other glucose concentration treatments.

The results showed that there is no statistically significant difference ($p = 0.075$) with the 7.5% glucose concentration. The presence of glucose in concentrations above 15% can influence the decrease in enzymatic activity by modifying the fermentation environment and the availability of the substrate for proteolytic action. This caused an adjustment in the metabolic pathway of *A. niger* depending on the carbon sources available in the whey.

This results in growth variations, affecting the protease activity that is used for the hydrolysis of proteins and amino acids present in whey (Li *et al.*, 2022). Figure 2 shows the treatments of enzyme extracts during the fermentation kinetics of *A. niger*. The highest protease activity was obtained with the 0% treatment (control) at 24 h, with $0.93\ U\ ml^{-1}$, whereas the mean glucose supplementation at 7.5% obtained an increase at 48 h ($0.54\ U\ ml^{-1}$). The presence of various carbon sources can affect protease production, as mentioned by Siala *et al.* (2012), who obtained protease values of 3.33 and $1.24\ U\ ml^{-1}$ with glucose and lactose, respectively.

Figure 2. Graphical representation of the arithmetic means of protease activity in $U\ ml^{-1}$ against time in hours. The different lines represent the concentration of glucose at 0%, 7.5%, 15%, and 30%.



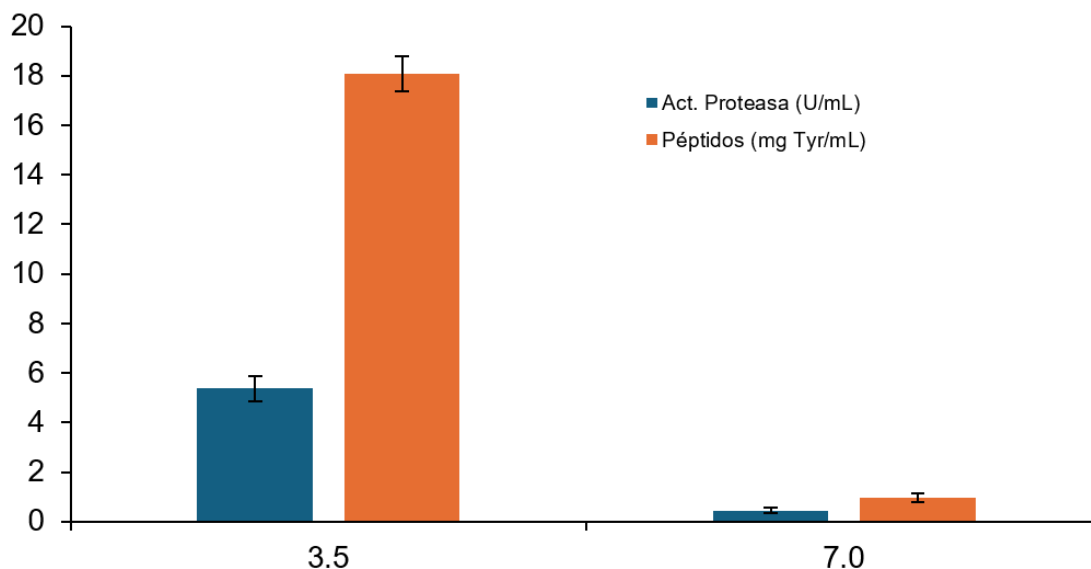
On the other hand, Irazoqui *et al.* (2024) underlined the role of proteases in the production of whey protein hydrolysates, highlighting their potential in the generation of compounds with antioxidant and antimicrobial properties. The results obtained reinforce the importance of protease activity in whey fermentation, especially in the presence of glucose as an influential factor in the decrease of protease activity, affecting the generation of peptides over time.

The optimization of fermentation conditions can improve enzymatic efficiency and the production of bioactive compounds; these findings contribute to the knowledge about the use of a waste such as whey in the area of biotechnology and its potential application in some sectors of the agro-industrial chain.

The hydrolytic activity of proteases can help improve the digestibility of proteins, reducing their allergenicity and generating bioactive peptides. Recent studies have shown that this hydrolytic process can optimize the production of compounds with antioxidant, antihypertensive, and antimicrobial effects (Luparelli *et al.*, 2025). Derived from the above results, the condition of whey fermentation at 0% glucose with a time of 24 h was selected for the use of the enzymatic extract at two pH levels (3.5 and 7.0), to elucidate its effectiveness in the hydrolysis of whey.

The analysis of proteolytic activity in whey showed variations in hydrolysis efficiency under different pH conditions in the enzyme extract (Figure 3). At a pH of 3.5, an activity of 5.22 U ml^{-1} was found, indicating an enzymatic efficiency under acidic conditions, releasing a peptide concentration of $18.06 \text{ mg of Tyr ml}^{-1}$. At a pH of 7.0, the protease activity decreases substantially to $0.97 \text{ mg of Tyr ml}^{-1}$.

Figure 3. Effect of pH values of 3.5 and 7.0 on enzyme activity and peptide concentration during the enzymatic hydrolysis process of whey. The values report the mean ($n=3$) $\pm$ the standard deviation of the samples. The enzymatic extract obtained at 24 h of fermentation with 0% glucose was considered.



Correlation tests revealed a negative relationship between pH and peptide release by enzymatic hydrolysis on whey ($\text{mg of Tyr ml}^{-1} = 35.162 - 4.8852 \text{ pH}$), with a decrease of $4.8853 \text{ mg of Tyr ml}^{-1}$ in peptide concentration for each increase in pH value. pH is significant ($p=0$) for peptide generation and explains 99.97% of peptide production by enzymatic hydrolysis. Pearson's correlation between pH and peptide concentration showed a strong negative relationship (-1), indicating that the higher the pH value, the lower the concentration of peptides by enzymatic hydrolysis.

This suggests that the activity of the protease produced by *A. niger* M4 is much lower under neutral pH conditions compared to acidic conditions. Rojas and Martínez (2023) analyzed the proteolytic activity of *A. niger* in whey, highlighting its ability to generate bioactive peptides with antioxidant properties, evaluating the influence of pH on the stability of the peptides obtained. The optimization of pH conditions in hydrolysis can improve enzymatic efficiency and the production of soluble peptides and thus improve the functionality of whey and its application in high-value-added products, but especially in agribusiness.

Conclusions

The results obtained showed that the production of *Aspergillus niger* M4 proteases in liquid fermentation with enriched whey is regulated by the concentration of glucose added to the culture medium. The fungus *A. niger* M4 is capable of hydrolyzing the proteins present in whey and can be used for the generation of soluble peptides. At a pH of 3.5, the enzymatic extract of *A. niger* M4 obtained a higher protease activity, capable of hydrolyzing the proteins existing in whey to obtain bioactive peptides.

The enzymatic extract obtained in the process could have applications when creating new products for agribusiness. Future research could optimize enzyme production and operating conditions to scale up this process for commercial use.

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proteins

Counts

Figures: 6
Tables: 2
Equations: 0
References: 30