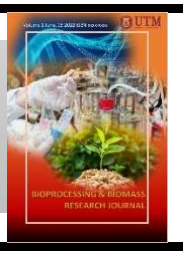
Bioprocessing and Biomass Technology

Journal homepage: https://bioprocessing.utm.my



Research Article

Physical Parameter Screening of Feather Protein Hydrolysate Production by *Aspergillus terreus* UniMAP AA-1 using Submerged Fermentation

S.N.A. Zaki[a], S.M. Puzi[a*], M.F. Tompang[a], N.A.H. Abdullah[a], S.Z. Kamal[a], Z. Zakaria[a], H. Hassan[a], and S.A. Adnan[a], N.H.A. Zaidi[a], Z.M. Idris[a], S.K.M. Rozi[a], N.I.W. Azelee[b], M.N.I. Jamaludin[c]

[a] *Faculty of Chemical Engineering & Technology, Universiti Malaysia Perlis, Perlis, Malaysia*
[b] *Faculty of Chemical & Energy Engineering, Universiti Teknologi Malaysia, Johor, Malaysia*
[c] *Institute of Autonomous System, Universiti Teknologi Petronas, Perak, Malaysia*

ARTICLE INFO

Article History:
Received 12 April 2024
Received in revised form 11 October 2024
Accepted 15 October 2024
Available online 31 December 2024

Keywords:
Chicken feather,
Protein hydrolysate,
Submerged fermentation,
Aspergillus terreus UniMAP AA-1,
Fractional factorial design

ABSTRACT

Feathers, a significant by-product of the poultry industry, pose considerable waste management challenges due to their abundance and resistance to degradation. This study explores the potential of utilizing chicken feather waste as a sustainable protein source through microbial fermentation. We employed *Aspergillus terreus* UniMAP AA-1, a keratinolytic fungus for fermentation aimed at degrading feather keratin. Various fermentation conditions, including initial pH, fermentation time, and inoculum size were tested using a fractional factorial design to optimize protein yield. The highest protein hydrolysate concentration achieved was 5.92 mg/mL, obtained with an inoculum size of 5%, a fermentation duration of 10 days, and an initial pH of 10.0. These results underscore the effectiveness of *Aspergillus terreus* UniMAP AA-1 in transforming feather waste into valuable protein hydrolysate. This research contributes to the advancement of eco-friendly and economically viable methods for valorizing poultry by-products, addressing environmental issues while providing innovative protein sources for livestock feed formulations.

INTRODUCTION

With the poultry farming industry generating a substantial by-product—feathers, accounting for approximately 10% of total chicken weight and exceeding 7.7 x10^8 kg annually (Grazziotin et al., 2006), the challenge of managing this waste stream becomes increasingly pressing (Tesfaye et al., 2017). Improper disposal of feathers poses environmental concerns due to their recalcitrance to degradation through conventional means (Grazziotin et al., 2006). Consequently, exploring alternative utilization avenues for insoluble feather waste has garnered attention, particularly in livestock feed formulations, where rising costs and sustainability imperatives drive the search for novel protein sources (Martínez-Alvarez et al., 2015; Sugiharto & Ranjitkar, 2019).

Feathers, comprising 91% keratin protein, 8% water, and 1% lipid (Tesfaye et al., 2017; Kamarudin et al., 2017), are renowned for their durable and resilient structure. Keratin, a predominant protein found in feathers, constitutes the skeletal tissues and epidermal layers, forming a robust fibrous matrix. Its resistance to enzymatic digestion, attributed to its insolubility and intricate molecular arrangement, stems from hydrogen bonds, hydrophobic interactions, and disulfide bridges (Sinkiewicz et al., 2018).

Due to their keratin content, these feathers offer significant potential as a protein source. However, traditional methods of processing feather waste into feather meal require energy-intensive procedures and chemical treatments to enhance its digestibility (Khalel, 2020; Saber et al., 2010). Despite its potential as a protein source, feather meal's poor digestibility in mammals and birds remains a challenge (Williams et al., 1991).

In recent developments, microbial-based approaches have emerged as viable alternatives for feather utilization, leveraging enzymatic hydrolysis to enhance nutritional value and product yield under mild reaction conditions (Grazziotin

*Corresponding Author
E-mail address: syazwanimahmad@unimap.edu.my
DOI address
ISBN/©UTM Penerbit Press. All rights reserved

et al., 2008). Keratinolytic enzymes, pivotal in degrading feather keratin, are prevalent among diverse microbial species including bacteria, actinomycetes, and fungi (Raveendran et al., 2018). Notably, certain fungi, such as *Aspergillus*, exhibit robust keratinolytic activity, offering promising avenues for bioprocessing (Gupta & Ramnani, 2006).

Thus, this study aims to investigate the feasibility of utilizing chicken feather waste as a sustainable protein source through microbial-based approaches, specifically by enzymatically hydrolyzing the feathers with *Aspergillus terreus* UniMAP AA-1 to generate protein hydrolysate. Additionally, the study evaluates the efficiency of screening fermentation parameters (including initial pH, fermentation duration, and inoculum size) using a two-level fractional factorial design (FFD) to determine the key parameters influencing the maximum protein yield. By addressing this objective, the research aims to contribute to the development of eco-friendly and economically viable methods for valorizing feather waste in the poultry industry.

MATERIALS AND METHOD

Preparation of Chicken Feather Powder

Waste of chicken feathers was collected from a local chicken market in Gelugor (Penang, Malaysia). The raw feathers were washed with warm tap water and subsequently air-dried for three days under sunlight. Following drying, the feathers were ground into a fine powder with a particle size of 100 µm.

Preparation of Microorganism Stock

The biodegradation of chicken feather waste in this study involved the use of *Aspergillus terreus* UniMAP AA-1, a glucose oxidase (GOx) producing strain (Anas & Arbain, 2012) for fermentation. The fungal strain was maintained on 3.9% (w/v) of potato dextrose agar (PDA) slants, subcultured once a month at 30 °C incubation and stored at 4 °C.

Fungal Inoculum Preparation

The strain *A. terreus* UniMAP AA-1 was cultivated on PDA medium in petri dishes at a temperature of 30 °C for 7 days. To prepare the spore suspension inoculum, the fungal culture was grown on PDA plates. Sterile distilled water (8-10 ml) was gently poured onto each cultured agar plate, and the spores were carefully scraped off using a sterile glass rod. The suspended fungal spores were subsequently filtered using Whatman number 1 filter paper into an Erlenmeyer flask. The spore concentration (spores/ml) was determined by counting the number of spores with a hemocytometer to maintain consistent strength. The spore concentration in the inoculum was maintained at 10^8 spores/ml.

Fermentation

The fermentation process was conducted in a 250 ml Erlenmeyer flask containing 100 ml of basal medium with the compositions of (in 1 liter): NaCl 0.5 g, K_2HPO_4 0.3 g, KH_2PO_4 0.4 g, $MgSO_4$ 0.1 g, feather powder 10 g and yeast extracts 0.1 g. Initial pH of this medium was adjusted (pH 5.0 - 10.0) with hydrochloric acid. Then, the fungus *Aspergillus terreus* UniMAP AA-1 was inoculated (1 – 5% v/v) into the fermentation medium and subsequently incubated at 30 °C with 150 rpm agitation for 4 – 10 days. The following parameters were evaluated in this study: (1) initial pH medium (5.0 – 10.0), (2) fermentation time (4 – 10 days), and (3) inoculum ratio (1 – 5 % v/v for 10^8 spore mL^{-1}).

Recovery of Feather Protein Hydrolysate

After fermentation, the fungal biomass was separated using filter paper (Whatman, No.1) while the supernatant, which contains the protein hydrolysate was recovered and stored in 4 °C chiller.

Determination of Soluble Protein Concentration

The quantification of soluble protein present in the protein hydrolysate was carried out using bovine serum albumin (BSA) at a concentration ranging from 0–3 mg/mL as the calibration standard described by Lowry et al. (2014). Spectrophotometric measurements were done at 750 nm.

Parameter Screening by Fractional Factorial Design (FFD)

Screening was carried out through FFD to determine the significant experimental parameters (initial pH, fermentation time and inoculum size) and their interactions which affect the production of protein hydrolysate from chicken feathers. The experimental design was generated based on the first-order model (**Eqn. 1**):

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i x_i \quad \text{(Eqn. 1)}$$

where Y is the response (protein hydrolysate, mg/ml), β_0 is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable. Each parameter was represented at two levels; low and high denoted by coded value (which generated by the software) of (-1) and (+1), respectively. Central points were added to provide an additional level for lack of fit testing and degree of freedom for pure error estimation (Rezende et al., 2018). The actual values which refer to the values of experimental condition were used with respect to the coded value (**Table 1**).

Table 1 Fractional factorial design levels

Factor	Unit	Low (-1)	High (+)	Central (0)
pH	-	5	10	7.5
Fermentation time	day	4	10	7
Inoculum size	% v/v	1	5	3

Table 2 The experimental design of FFD which consisted of 15 runs and the yield production of feather protein hydrolysate as response.

Run	(A) Initial pH medium		(B) Fermentation time (day)		(C) Inoculum size (ml)		Protein hydrolysate (mg/ml)
	Actual	Coded	Actual	Coded	Actual	Coded	
1	7.5	0	7	0	3	0	5.784091
2	10	1	4	-1	1	-1	5.321023
3	7.5	0	7	0	3	0	5.764205
4	5	-1	10	1	1	-1	4.826705
5	10	1	10	1	5	1	5.747159
6	10	1	10	1	5	1	5.852273
7	5	-1	10	1	1	-1	5.181818
8	10	1	10	1	5	1	5.920455
9	5	-1	4	-1	5	1	3.724432
10	5	-1	10	1	1	-1	4.988636
11	5	-1	4	-1	5	1	4.275568
12	5	-1	4	-1	5	1	4.764205
13	10	1	4	-1	1	-1	5.514205
14	7.5	0	7	0	3	0	5.721591
15	10	1	4	-1	1	-1	5.363636

The effect of each parameter was determined by the following equation (**Eqn. 2**):

$$E_{(x_i)} = \frac{2(M_{i+} - M_{i-})}{N} \quad \textbf{(Eqn. 2)}$$

where $E_{(xi)}$ is the effect of the tested variable M_{i+} and M_{i-} is the protein hydrolysate production from the trials where the variable (x_i) measured was present at high and low concentrations, respectively and N is the number of trials. A 2^{3-1} half-factorial design consisting of 15 runs which includes triplicate of center points and trials were performed for all the parameters, as shown in **Table 2**. Design Expert software (StatEase, Minneapolis) v 13.0.1 was used to develop the design and analyze the data.

RESULTS AND DISCUSSION

Production of Protein Hydrolysate from Fermentation

Chicken feather waste was utilized as the main substrate to produce protein hydrolysate through batch fermentation by locally isolated fungus *Aspergillus terreus* UniMAP AA-1. According to Kim (2003), *Aspergillus terreus* is one of the keratinolytic fungal species which has the ability to degrade keratin of chicken feather. Specific proteolytic enzyme such as keratinase is secreted by this fungus to aid keratin hydrolysis for soluble protein production more efficiently than other proteases (Riaz et al., 2024).

The potential of *Aspergillus terreus* UniMAP AA-1 to hydrolyze feather keratin was examined at various fermentation time i.e., 4 to 10 days. For this purpose, the fungal strain was cultivated in a media containing feather as substrate in a flask incubated at 30 °C and agitated at 150 rpm in an incubator shaker. Feathers were added in basal media as shown in **Figure 1 (a)**. Since the feathers are light in weight and insoluble in water, they were observed to be at the surface of media at the beginning of the fermentation process. No significant changes in feather structure were observed within 24 hours, consistent with the findings of Riaz et al. (2024) using *B. subtilis*.

After 2 days of incubation, feather pieces were observed to be soluble and changed the culture media from clear to turbid (bright yellow) showing the keratin was degraded with the gradual growth of fungus, as shown in **Figure 1(b)**. The media was proceeded for filtration and clear yellowish protein hydrolysate were recovered (**Figure 1(c)**) after each fermentation process has been completed.

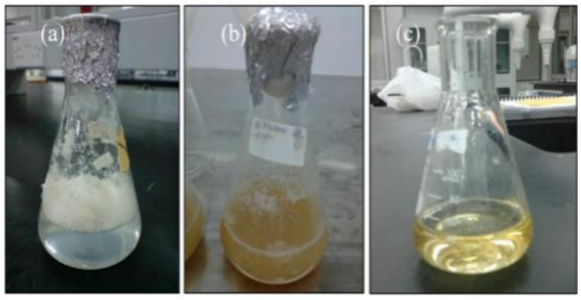


Figure 1 Characteristics of *Aspergillus terreus* UniMAP AA-1 culture media before fermentation (a), after fermentation (b), and the protein hydrolysate following the filtration process (c).

Screening for Significant Factors Affecting Feather Protein Hydrolysate Production

In order to obtain maximum protein hydrolysate from the fungal biodegradation process, several fermentation parameters such as initial pH, fermentation time and inoculum size were screened to investigate their significance and effect through FFD. **Table 3** shows the statistical analysis of the responses and main effects of the selected variables. The FFD model used in this study demonstrates significance, with a Model F-value of 21.17 and a p-value of 0.0001, indicating strong evidence that the selected variables have a significant impact on protein hydrolysate production.

Figure 2 illustrates the production of protein hydrolysate, as indicated by the t-value effect on the left vertical axis. Analysis of the parameters A and B (initial pH medium and fermentation time respectively) demonstrated that their optimization is sufficient to mitigate the influence of other factors.

Table 3 Statistical analysis of fractional factorial designs screening process.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	4.08	3	1.36	21.17	0.0001
A-pH	2.95	1	2.96	46.05	<0.0001
B-time	1.05	1	1.05	16.39	0.0023
C-size	0.069	1	0.069	1.08	*0.3234
Cor Total	5.68	14			

R^2 = 0.8640, *Values of p-values greater than 0.05 indicating the model terms are not significant

Establishing an appropriate parameter range is crucial for maximizing protein hydrolysate production and thus, a range of 1 to 5% was identified as the optimal inoculum size to minimize negative effects on hydrolysate yield.

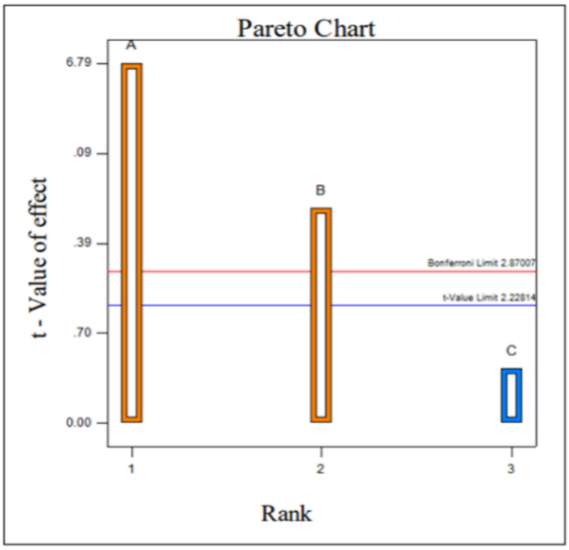


Figure 2 Significant data based on the rank shown on the hypothetical data.

Based on this Pareto chart, the most significant factors influencing protein hydrolysate production from chicken feathers by *Aspergillus terreus* UniMAP AA-1 were fermentation time and the initial pH of the medium. This finding is also consistent with Alamnie et al. (2024), who also emphasize the critical role of the initial pH of the medium, particularly within the range of 5 to 9, and incubation period of 60 hours in optimizing hydrolysate yields from chicken feathers by bacteria and yeasts.

Initial pH of medium

As shown in **Figure 3**, an increase in the initial pH of the medium correlates with enhanced protein hydrolysate production. Optimizing the initial pH is crucial for promoting microbial growth during submerged fermentation, as microbes play a key role in determining the appropriate pH conditions. In this study, *Aspergillus terreus* UniMAP AA-1 was utilized to identify the optimal pH for maximizing protein hydrolysate production, highlighting its importance in the fermentation process.

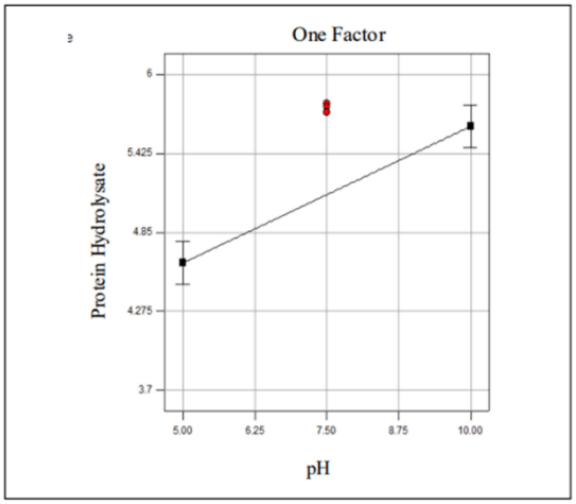


Figure 3 Response of protein hydrolysates to the effect of initial pH medium.

Fermentation time

Fermentation time is a crucial parameter influencing the production of protein hydrolysate. The specific type of microorganism employed plays a significant role in keratinolysis, determining the duration required for complete keratin degradation. In this study, the optimal conditions included an inoculum size of 3.0 mL (10^8 spores/mL) and an initial pH of 7.5, both of which demonstrated a positive correlation with increased hydrolysate production as illustrated in **Figure 4**. Notably, *Aspergillus terreus* UniMAP AA-1 exhibited effective protein hydrolysis over a fermentation period exceeding 10 days, emphasizing the importance of extended fermentation time for maximizing protein hydrolysate yields.

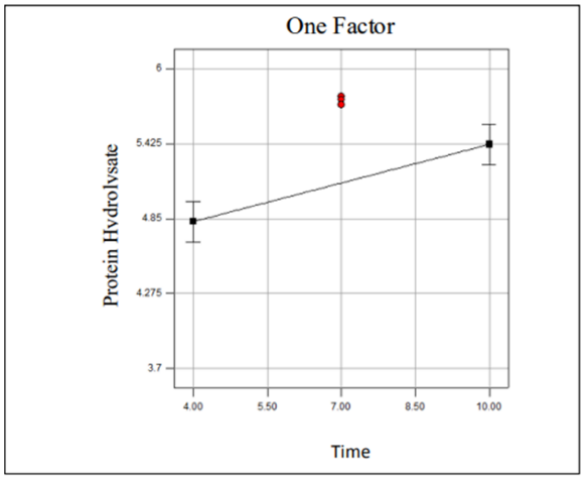


Figure 4 Reaction of protein hydrolysate based on the effect of fermentation time.

Chicken Feather Protein Hydrolysate Yield

In **Table 2**, the highest total soluble protein hydrolysate production from 10 g/L chicken feather powder was 5.92 mg/mL with an inoculum size of 5%, a fermentation time of 10 days and an initial pH medium of 10.0. This production level significantly outperformed conventional methods, as the protein hydrolysate yields from bacterial and yeast sources were only 0.246 mg/mL (Alamnie et al, 2024), while Mazotto (2013) reported yields of approximately 1-2 mg/mL of soluble protein using *Aspergillus niger*. These findings

suggest that optimizing fermentation conditions can substantially enhance protein hydrolysate production.

CONCLUSION

This research successfully demonstrates the feasibility of utilizing chicken feather waste as a sustainable protein source through the microbial fermentation process using *Aspergillus terreus* UniMAP AA-1. The study highlights the significant role of physical parameters, specifically initial pH and fermentation time, in optimizing the production of feather protein hydrolysate. Through a fractional factorial design, we identified that an inoculum size of 5%, a fermentation time of 10 days, and an initial pH of 10.0 yielded the highest protein hydrolysate concentration of 5.92 mg/mL, surpassing traditional methods significantly. The findings underscore the importance of optimizing fermentation conditions to enhance protein recovery from feather waste, addressing both environmental challenges associated with poultry by-products and the demand for alternative protein sources in livestock feed. This research contributes valuable insights to the field of waste valorization and provides a foundation for further studies, including response surface methodology (RSM) for fine-tuning the identified parameters. Overall, the use of *Aspergillus terreus* UniMAP AA-1 represents a promising approach to transforming chicken feather waste into a valuable protein resource, promoting sustainable practices in the poultry industry.

Acknowledgement

This research is through the Fundamental Research Grant Scheme (FRGS/1/2013) which is financially supported by the Ministry of Education Malaysia (MOHE). The researchers and authors for this project express their appreciation to the Faculty of Chemical Engineering & Technology (FKTK), Universiti Malaysia Perlis (UniMAP) for providing laboratory facilities, research materials to carry out research and data analyses.

References

Alamnie, G., Melake, A., Berhanu, Y., Alemu, M., Damtew, B., & Aemiro, A. (2024). Chicken Feather Protein Hydrolysate as a Low Cost Peptone Source for Microbial Cultivation: A Promising Perspective of Economic and Environmental Advantage. *Case Studies in Chemical and Environmental Engineering*, 9, 100741. https://doi.org/10.1016/j.cscee.2024.100741

Anas, N. A., & Arbain, D. (2012). *Aspergillus terreus* Unimap AA-1: A Newly Isolated Extracellular Glucose Oxidase-Producing Strain. *World Applied Sciences Journal*, *17*(1), 86-90.

Grazziotin, A., Pimentel, F. A., de Jong, E. V., & Brandelli, A. (2006). Nutritional Improvement of Feather Protein by Treatment with Microbial Keratinase. *Animal Feed Science and Technology*, *126*(1–2), 135–144. https://doi.org/10.1016/j.anifeedsci.2005.06.002

Grazziotin, Adriane, Pimentel, F. A., Jong, E. V. De, & Brandelli, A. (2008). Poultry Feather Hydrolysate as a Protein Source for Growing Rats. *Brazilian Journal of Veterinary Research and Animal Science*, *45*(supl.), 61. https://doi.org/10.11606/S1413-95962008000700008

Gupta, R., & Ramnani, P. (2006). Microbial Keratinases and heir Prospective Applications: An Overview. *Applied Microbiology and Biotechnology*, *70*(1), 21–33. https://doi.org/10.1007/s00253-005-0239-8

Kamarudin, N. B., Sharma, S., Gupta, A., Kee, C. G., Chik, S. M. S. B. T., & Gupta, R. (2017). Statistical Investigation of Extraction Parameters of Keratin from Chicken Feather using Design-Expert. *3 Biotech*, *7*(2), 127. https://doi.org/10.1007/s13205-017-0767-9

Kim, J. (2003). Keratinolytic Activity of FiveAspergillusSpecies Isolated from Poultry Farming Soil in Korea. *Mycobiology*, 31(3), 157. https://doi.org/10.4489/myco.2003.31.3.157

Khalel, A. (2020). Enhancing Plant Growth by Chicken Feather Compost Obtained from Feather Degradation by *Streptomyces enissocaesili*. *Bioscience Biotechnology Research Communications*, *13*(4), 1847–1853. https://doi.org/10.21786/bbrc/13.4/32

Lowry O H, Rosebrough N J, F. A. L. & R. R. J. (2014). Citation Classics. In *Encyclopedia of Quality of Life and Well-Being Research* (Issue 1, pp. 921–921). Springer Netherlands. https://doi.org/10.1007/978-94-007-0753-5_100521

Martínez-Alvarez, O., Chamorro, S., & Brenes, A. (2015). Protein Hydrolysates from Animal Processing By-Products as a Source of Bioactive Molecules with Interest in Animal Feeding: A Review. *Food Research International*, *73*(1069), 204–212. https://doi.org/10.1016/j.foodres.2015.04.005

Mazotto, A. M., Couri, S., Damaso, M. C., & Vermelho, A. B. (2013). Degradation of Feather Waste by *Aspergillus Niger* Keratinases: Comparison of Submerged and Solid-State Fermentation. *International Biodeterioration & Biodegradation,* 85, 189–195. https://doi.org/10.1016/j.ibiod.2013.07.003

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., & Pandey, A. (2018). Applications of Microbial Enzymes in Food Industry. *Food Technology and Biotechnology*, *56*(1), 16–30. https://doi.org/10.17113/ftb.56.01.18.5491

Rezende, C. A., Atta, B. W., Breitkreitz, M. C., Simister, R., Gomez, L. D. & McQueen-Mason, S. J. (2018). Optimization of Biomass Pretreatments using Fractional Factorial Experimental Design. *Biotechnology for Biofuels*, 11(206). https://doi.org/10.1186/s13068-018-1200-2

Riaz, A., Muzzamal, H., Maqsood, B., Naz, S., Latif, F. & Saleem, M. (2024). Characterization of a Bacterial Keratinolytic Protease for Effective Degradation of Chicken Feather Waste into Feather Protein Hydrolysates. *Frontiers in Bioscience (Elite Ed) 16*(3):22. https://doi.org/10.31083/j.fbe1603022

Saber, W. I. A., El-Metwall, M. M., & El-Hersh, M. S. (2010). Keratinase Production and Biodegradation of Some Keratinous Wastes by *Alternaria tenuissima* and *Aspergillus nidulans*. *Research Journal of Microbiology*, *5*(1), 21–35. https://doi.org/10.3923/jm.2010.21.35

Sinkiewicz, I., Staroszczyk, H., & Śliwińska, A. (2018). Solubilization of Keratins and Functional Properties of Their Isolates and Hydrolysates. *Journal of Food*

Biochemistry, *42*(2), e12494. https://doi.org/10.1111/jfbc.12494

Sugiharto, S., & Ranjitkar, S. (2019). Recent Advances in Fermented Feeds towards Improved Broiler Chicken Performance, Gastrointestinal Tract Microecology and Immune Responses: A Review. *Animal Nutrition*, *5*(1), 1–10. https://doi.org/10.1016/j.aninu.2018.11.001

Tesfaye, T., Sithole, B., & Ramjugernath, D. (2017). Valorisation of Chicken Feathers: A review on Recycling and Recovery Route—Current Status and Future Prospects. *Clean Technologies and Environmental Policy*, *19*(10), 2363–2378. https://doi.org/10.1007/s10098-017-1443-9

Williams, C. M., Lee, C. G., Garlich, J. D., & Shih, J. C. H. (1991). Evaluation of a Bacterial Feather Fermentation Product, Feather-Lysate, as a Feed Protein. *Poultry Science*, *70*(1), 85–94. https://doi.org/10.3382/ps.0700