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Production and Optimization of Feather Protein Hydrolysate from *Bacillus* Sp. MPTK6 and Its Antioxidant Potential

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Abstract: The production of feather protein hydrolysate (FPH) by *Bacillus* sp. MPTK6 was investigated for its *in vitro* digestibility and antioxidant potential. The effects of feather concentrations (15-60 g/I) and initial pHs (6-11) on the production of amino acids, peptides and soluble proteins were evaluated for their performance in FPH production medium. The strain MPTK 6 showed maximum keratinase and caesinase activity of 2.4 U/ml and 2.1 U/ml in the initial screening process. The strain MPTK6 showed the ability to degrade raw feather to FPH under optimal conditions (30 g/l chicken feathers, pH 10.0 and 72 h of fermentation). The DPPH free radical-scavenging activity and reducing power showed the antioxidant potential of FPH of the test isolate. The FPH showed very high *in vitro* digestibility compared to that of the raw feathers.

Key words: Feather Protein Hydrolysate · *In vitro* Digestibility · Antioxidant · Keratinase

INTRODUCTION

Feathers represent 5-7% (w/w) of the total weight of mature chickens and are generated in large amounts as a waste product in commercial poultry-processing plants, reaching millions of tons per year worldwide [1]. Poultry feathers were discharged into the environment after the processing which leads to the environmental pollution. Discarded feathers cause various human ailments including chlorosis, mycoplasmosis and fowl cholera [2]. Although the feathers are considered as wastages, it contains large amount of protein and this protein can be converted into animal feedstuffs which helps to reduce the protein shortage and cost effects. Poultry feed production plays an important role in protein supply and in agricultural economy [3].

The protein shortage for food and feed leads us to look for a new protein sources from wastage products like feather wastes [4,5]. Feathers are a significant source of protein for livestock because of their high protein content (>85% CP) [6]. Feathers contain large amounts of cysteine, glycine, arginine and phenylalanine [7, 46]. Raw feathers, however, are very poorly digested by non-ruminant animals because they contain a high proportion of keratin protein that has cysteine disulfide bonds [8]. The indigestible structure of raw feather must be hydrolyzed to be used as a feed ingredient for non-ruminant species. Though keratin can be completely dissolved by reducing agents like copper sulphate, mercapto acetate, iodoacetic acid, amino, sodium sulphite, sodium tetrathionate [9-11] these methods are not suitable for the large scale application. In order to overcome these limitations the use of microbial enzymes which improves the nutritional value of feather wastes have been implemented in recent years.

Alkaline proteases are among the most important industrial enzymes; they are primarily employed as detergent additives, accounting for 40-60% of the global enzyme market. Alkaline proteases are group of proteolytic enzymes that are able to hydrolyze insoluble hard proteins more effectively than other proteases such as trypsin, pepsin and papain [12-14]. The genus *Bacillus* provides most of the alkaline proteases with commercial value, offering the advantages of being easy to culture and maintain and they are potentially valuable in the bioconversion of keratinous wastes; in the detergent, fertilizer, biopolymer, pharmaceutical and animal feed industries; and in leather processing and hydrolysis of

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prion proteins as well [15]. Therefore, such microorganisms and enzymes have been the focus in several studies [16-20] and *Bacillus* alkaline proteases are reported to be among the most efficient keratin degraders [21, 22].

Against these backdrops, this study aimed to investigate the production of feather protein hydrolysate (FPH) by *Bacillus* sp., isolated from local poultry industry. The *in vitro* digestibility and the antioxidant potential of feather protein hydrolysate were also investigated.

MATERIALS AND METHODS

Feathers and its Processing: Chicken feathers (CF), supplied by a local poultry industry were washed threefold with tap water and finally with distilled water. The washed feathers were dried at 90°C for 22 h and then stored at room temperature prior to microbial treatment [23].

Isolation and Screening of Alkaline Keratinolytic Bacteria: The test organisms used in this study were isolated from feather composting soil near local poultry farm in Chennai, India. 1 g of soil sample was added to the keratin enrichment medium which contains (%): NH_4Cl-0.05, NaCl-0.05, K_2HPO_4-0.03, KH_2PO_4-0.04, $MgCl_2{\cdot}6H_2O$-0.024, yeast extract-0.01 and 1% feather keratin substrate 1 at pH 8.5 for 3 d under shaken condition. The enriched sample (1 ml) was suspended in 9 ml of sterilized water and was spread on the same feather enriched medium by addition of 1.7% agar and incubated at 37°C for 72 h. After incubation the plates ware stained with Coomassie blue for the observation of clear zones around the margins of colonies and were picked up as alkaline keratinase producers. The positive isolates were then screened for keratinase [24] and caseinolytic activity [26] in liquid culture medium using keratin and casein as substrates at pH 8.5. The strain which showed maximum activity was selected for further study. The stock culture of the strain was maintained on glycerol stocks (50%, v/v) and stored at -20°C.

Characterization and Molecular Identification of Bacteria: The preliminary characterization of the isolated strain was done using Bergey's manual of systemic bacteriology [27] and 16S rDNA analysis was used for the authentication of the strain. The genomic DNA was isolated from the bacteria by the method described by Hosek*et al*. [27]. The highly purified DNA was then amplified in a thermocycler at conditions: 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min which amplifies the 16S rRNA sequences specifically by using the primers [28].

Forward (16F27):5′-AGAGTTTGATCCTGGCTCAG-3′
Reverse(16R1522):5′-AAGGAGGTGATCCAGCCGCA-3′

DNA sequencing was performed in a highly automated gene sequencer. These sequences were read in Genbank databases (BLAST) and compared with the other sequences to analyze the bacterial class and its phylogeny.

Feather Protein Hydrolysates (FPH) Production: The strain MPTK 6 was grown in Luria-Bertani broth medium which contains 10 g/l peptone, 5 g/l yeast extract and 5 g/l NaCl. The initial medium used for feather protein hydrolysates production composed of 20 g/l chicken feather powder (CFP), 0.5 g/l-KH_2PO_4, 0.5 g/l-K_2HPO_4, 2.0 g/l-NaCl, 0.1 g/l-KCl, 0.1 g/l-$MgSO_4{\cdot}7H_2O$ at pH 8.5 autoclaved at 121°C for 20 min. Then the test isolate was inoculated in the medium and incubated for 3 d at 30°C and 250 rpm with a working volume of 100 ml. The cultures were centrifuged at 12,000rpm for 15min at 4°C and the cell-free supernatants were used for determination of keratinolytic activity, caseinolytic activity, soluble protein and amino acid concentration. Productivity was determined as soluble protein concentration per time to reach 3 d concentration (mg/l/h) according to the method of Fakhfakh *et al*. [29].

Effect of Physico-Chemical Culture Variables on FPH: The effects of feather concentrations (15-60 g/I) and initial pH (6-11) on the production of amino acids, peptides and soluble proteins were investigated by *Bacillus* sp. MPTK6 were evaluated for their performance in feather protein hydrolysate production.

Enzyme Activity: Keratinolytic and Caesinolytic activities were determined using azokeratin or azocasein (Sigma Co., USA), respectively, as substrates. Azokeratin was synthesized as described elsewhere [30]. The reaction mixture contained 100 ml of enzyme preparation and 100 ml of 10 mg ml^{-1} azokeratin (or azocasein) in 20 mMTris-HCl buffer, pH 8.0. The mixture was incubated for 30 min at 37°C; the reaction was stopped by adding 500 ml of 10% (w/v) trichloroacetic acid. After centrifugation (10,000 × *g* for 5 min) of the reaction mixture, 800 ml of the supernatant were mixed with 200 ml of 1.8 M NaOH and

the absorbance at 420 nm was measured. One unit of enzyme activity was considered as the amount of enzyme that caused a change in absorbance of 0.01 units at the above assay conditions.

Analytical Assays: Dry matter was determined by oven-drying at 105°C to constant mass [31]. Protein content (dry weight basis) was analyzed according to the Kjeldahl method [32]. Soluble protein and peptide concentrations were determined by the method of Biuret [33] using ovalbumin as the standard. Concentration of amino acids and peptides was determined by the ninhydrin method [34]. Fat content was determined by Soxhlet extraction with hexane for 8 h at boiling point of the solvent. The ash content was determined by combustion of the sample at 550°C for 8 h. Total protein content was measured according to Lowry's method [35]. The amino acid present in the sample were analyzed by ion exchange chromatography and its concentration were estimated by ninhidrin method [34]. Nitrogen content in the sample was estimated according to the method of Deivasigamani and Alagappan [36].

Antioxidant Activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for the determination of the free radical scavenging activity of the extracts (AED and MED) by the method of Koleva*et al.* [37]. For each extract and standard, sample solutions of different concentrations (0.5-3.5 mg/ml) were prepared in methanol and added separately to an equal volume of 100 µM DPPH solution in methanol. The reaction mixture was kept at room temperature for 15 min. Then, the absorbance of the reaction mixture was recorded at 517 nm using a UV-visible spectrophotometer [Shimadzu UVPC-3200 (Kyoto, Japan)]. Gallic acid (GA) was used as standard. Free radical scavenging activity was calculated using the following formula:

$$\% \text{ of free redical scavenging activity} = \frac{\text{Control} - \text{Sample 0D}}{\text{Control 0D}} \times 100$$

The extract concentration having 50% radical inhibition activity (IC_{50}) was calculated from the graph of the free radical scavenging activity (%) against the extract concentration. Three replicates were performed for each sample concentration to check the reproducibility of the experimental result and to get more accurate result. Results are represented as IC_{50} ± standard deviation.

Reducing Power Assay: The iron reducing ability of the hydrolysates was analyzed by Yildirim *et al.* [38]. It was done by preparing different concentrations of hydrolysates and then adding 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide to 1 ml sample of each hydrolysate. Then it was kept at 50°C for 30 min, followed by addition of 2.5 ml of 10% (w/v) trichloroacetic acid. After incubation the sample was centrifuged at 15,000 × g for 10 min. The absorbance of the supernatant was done at 700 nm after incubation of the mixture containing 2.5 ml of the supernatant solution with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride for 10 min the sample with high reducing power was identified by increased absorbance.

***In vitro* Digestibility:** *In vitro* digestibility of sample was done by adding pepsin and pancreatin to the sample [39]. It is done by adding 2mg of pepsin/ml to 1g of ground sample and incubated for 2 h at 37°C in 2M HCl. After incubation, the pH was made to 8 by 2M $NaHCO_3$ and then adds 2 mg of pancreatin/ml and again incubates it for additional 16 h [40].

$$D = \frac{\text{Content of protein releasedupon thedigestion of 1g of sample}}{\text{Content of the total protein 1g of food beforedigestion}}$$

RESULTS AND DISCUSSION

Screening of Alkaline Keratinolytic Bacteria: The test organisms used in this study was isolated from feather composting soil in keratin enrichment medium at pH 8.5. The isolated organisms were screened in the feather enriched medium. Seven isolates showed a clear zone and these positive isolates were further screened for production of extracellular alkaline keratinase and caesinase. The strain MPTK 6 showed maximum keratinase and caesinase activity of 2.4 U/ml and 2.1 U/ml respectively (Table 1). Hence, this isolate was selected for further studies.

Characterization and Molecular Identification of Bacteria: From microscopic appearance and the biochemical tests, the isolate was identified as *Bacillus* sp. MPTK6 and further confirmation was done by sequencing the 16S rDNA gene. Upon the amplification of 16S rDNA sequence using specific primer, an amplified product of 1099 bp was obtained (Fig. 1) which was then sequenced and compared with the Genbank databases using the BLASTN program. The 16S rDNA sequence of the isolate revealed a close relatedness to *Bacillus* sp. with 97% similarity. Hence the strain was confirmed as *Bacillus* sp. and the sequence was submitted to Genbank (JQ746528).

Table 1: Keratinase and caesinase production by bacterial isolates from poultry farm soil

Isolate	Keratinase (U/ml)	Caesinase (U/ml)
MPTK 1	1.9±0.17	1.1±0.13
MPTK 2	1.4±0.11	1.7±0.10
MPTK 3	1.4±0.10	1.3±0.03
MPTK 4	1.2±0.61	1.4±0.02
MPTK 5	0.7±0.17	1.3±0.01
MPTK 6	2.4±0.42	2.1±0.03
MPTK 7	1.4±0.04	1.7±0.23

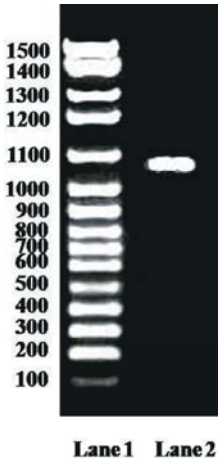

Fig. 1: Photographic representation of PCR amplified product in Agarose Gel (Lane 1, 15 kbp ladder; Lane 2, PCR amplified sample)

Effect of Physico-Chemical Culture Variables on FPH: Considering that feathers are composed by at least 90% of keratin, the use of this protein source can be of great interest. Production of feather protein hydrolysates using keratinolytic bacteria has been considered as an interesting alternative [41]. Thus, the effects of feather concentrations, initial pH on the production of amino acids, peptides and soluble proteins were investigated on *Bacillus* sp. MPTK6.

Effect of Feather Concentrations: The microorganism *Bacillus* sp. MPTK6 was grown in mineral medium containing different amounts of raw feathers as the sole carbon and nitrogen source. The highest levels of proteolytic activities were obtained after cultivation for 72 h and then activities remained constant. The strain exhibited the highest enzyme production (620 U/ml) in culture medium containing 30 g/l of chicken feathers (Fig. 2). The results are in line with those reported by Fakhfakh*et al*. [29] using the strain *Bacillus pumilus* A1.

The highest levels of amino acids and peptides (34±2.0 g/l) and soluble proteins and peptides (5.9±2 g/l) were also obtained in the medium containing 30 g/l of

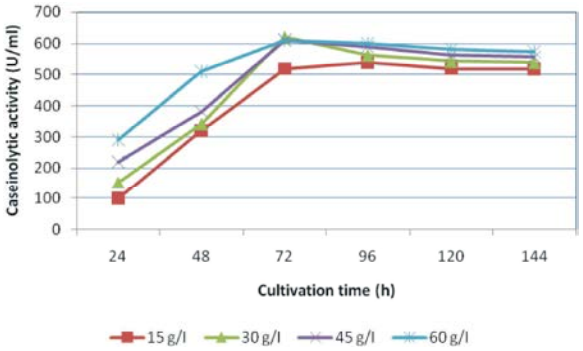

Fig. 2: Effects of feather concentration on caseinolytic activity

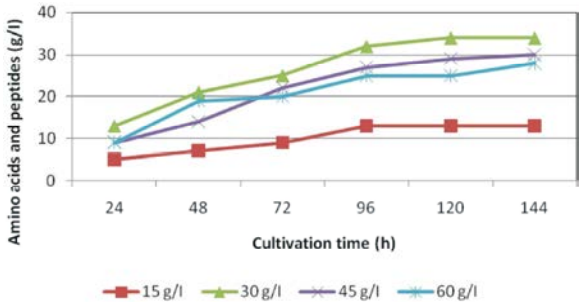

Fig. 3: Effects of feather concentration on amino acids and peptides

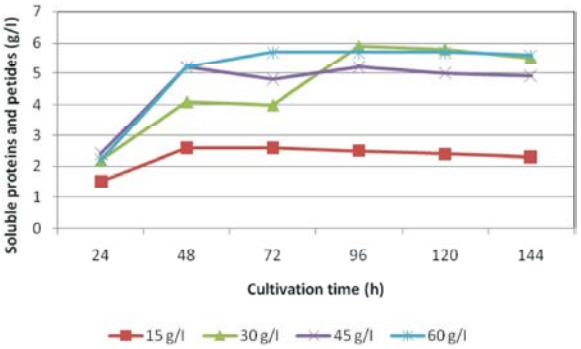

Fig. 4: Effects of feather concentration on soluble proteins and peptides

feathers (Figs. 2 and 3). However, at 60 g/l of feathers, proteases, amino acids, peptides and soluble proteins production were reduced. In fact, it was demonstrated that high feather concentration may cause substrate inhibition or repression of keratinase production, resulting in a low percentage of feather degradation. The highest ratio of soluble proteins and peptides per gram of feathers was reached with 10 g/l of feathers (Fig. 4). Maximum productivity of soluble proteins and peptides were obtained in culture medium containing 30 g/l of feathers (Fig. 4).

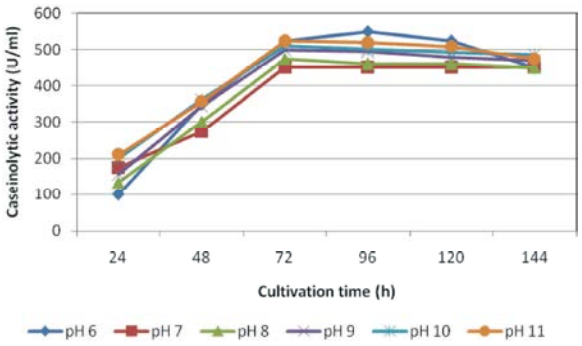

Fig. 5: Effects of medium pH on caseinolytic activity

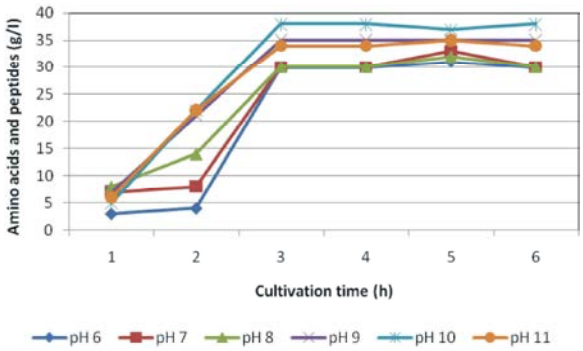

Fig. 6: Effects of medium pH on amino acids and peptides

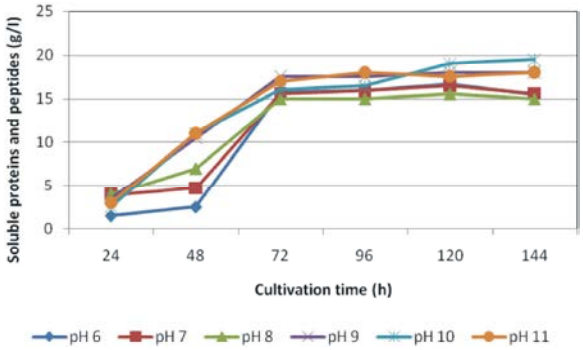

Fig. 7: Effects of medium pH on soluble proteins and peptides

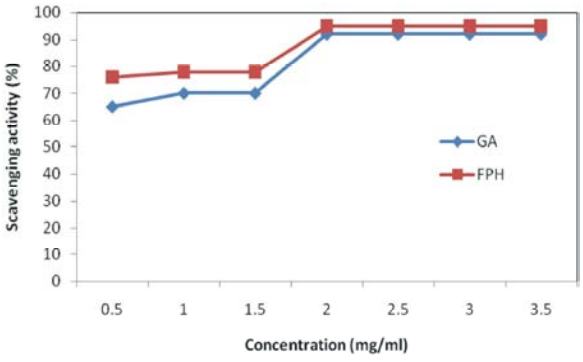

Fig. 8a: Antioxidant activity of feather protein hydrolysate (FPH)

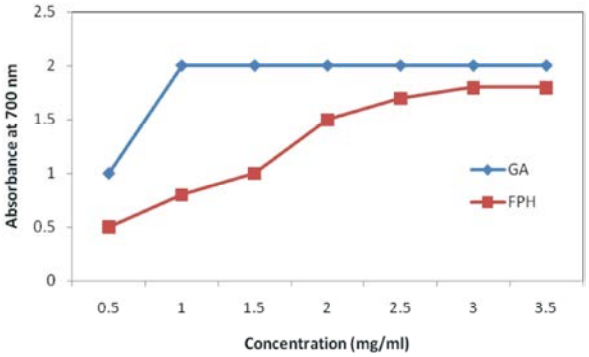

Fig. 8b: Reducing power of feather protein hydrolysate (FPH)

Effect of pH: The effect of the initial medium pH values from 6.5 to 11 on the production of proteolytic enzymes and on feather protein hydrolysate was studied by cultivating the strain MPTK6 in the medium containing 30 g/l of intact feathers. As shown in Fig. 5, the highest protease production level was obtained at pH 6. Nevertheless, at this pH value no complete feather degradation was achieved (evaluated by physical appearance). The optimum initial pH of the strain MPTK6 to produce FPH was observed to be 9-10. The highest amino acids and peptides (38±1.2 g/l) and soluble proteins and peptides (19.5±0.2 g/l) productions were observed at pH 10.0 (Figs. 6 and 7).

Hydrogen ion concentration is one of the most important factors affecting the growth of the bacteria, keratinase production and percentage of feather degradation. Since cultures were conducted in media containing only chicken feather as nitrogen and carbon source, complete degradation of chicken feather to produce a hydrolysate rich in amino acids and peptides could only be achieved when high keratinolytic activity was attained. Thus, different pH values were tested for proteolytic enzyme and FPH production. Complete feather degradation by the MPTK6 strain was observed at initial pHs of 9.0-11.0. This result could be explained by the fact that the proteolytic enzymes produced by MPTK6 strain are highly active at alkaline conditions. This finding is similar to that published by [42] which shows that complete feather degradation by the strain *Bacillus* sp. FK 46 was reached with initial pH of 9.0. However, Grazziotin *et al*. [43] reported that the strain Vibrio sp. Kr2 produced the maximum level of soluble proteins with initial pH comprised between 6.0 and 8.0. The MPTK6 strain was able to complete feather degradation, indicating its strong keratinolytic activity. Maximum amino acids,

Table 2: Composition and In vitro digestibility of raw feathers and FPH

Composition	Raw feathers	FPH
Protein	83.1±0.2	84.2±0.1
Fat	0.041±0.03	1.7±0.5
Moisture	2.92±0.4	5.8±0.6
Ash	9.8±0.1	0.52±0.02
In vitro digestibility (%)	96±0.5	1.8±0.5

peptides and proteins production as well as more efficient feather degradation were observed under the following conditions (30 g/l of raw feathers; pH 10 and 48 h).

Antioxidant Activity: DPPH is a stable free radical that shows maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the absorbance would be reduced [44]. The decrease in absorbance is taken as a measure for radical-scavenging activity. The DPPH radical-scavenging activity was investigated at different concentrations (0.5-3.5 mg/ml) of the FPH. The results presented in Fig. 8a clearly show that the FPH exhibited an interesting radical scavenging activity with an IC_{50} value of 0.5 mg/ml.

It is worthy to note that the FPH, produced in this study, exhibited high DPPH free radical-scavenging activity which is comparable to that obtained from the findings of Fakhfakh*et al*. [29] using the strain *Bacillus pumilus* A1. The IC_{50} value of FPH produced by MPTK6 strain (0.3 mg/ml) was lower than that of protein hydrolysate from smooth hound (0.6 mg/ml) using DPPH assay. The reducing power of the FPH was investigated at different concentrations and was to be concentration dependent. Its value increased with the higher FPH concentrations as was reported by Zhu *et al.* [45]. The reducing power results revealed that FPH, with high amino acid and peptide contents, could react with free radicals to form stable products.

***In vitro* Digestibility:** The *in vitro* digestibility of the feather protein hydrolysate directed by MPTK6 in the optimized conditions was measured using pepsin and pancreatin. As shown in Table 2, the FPH presents a very high digestibility (96%) compared to that of raw feathers (1.8%). The physico-chemical characterization of the produced FPH was also determined (Table 2).

Proteins represent the major component (83.1%) of the FPH followed by ash (9.8%). The low humidity of this hydrolysate (2.92%) may contribute to the microbiological stability of the product during its storage. The results are in line with those reported by Fakhfakh *et al*.,[29] using the strain *Bacillus pumilus* A1 who recorded 98% of *in vitro* digestibility in his study.

CONCLUSION

These results, from the present study, permit us to conclude that the strain MPTK6 had the ability to degrade raw feather to FPH under optimal conditions (30 g/l chicken feathers, pH 10.0 and 72 h of fermentation). The study also showed the DPPH free radical-scavenging activity and reducing power showing the antioxidant potential of FPH obtained from the test isolate. In addition, it presents a very high *in vitro* digestibility compared to that of the untreated feathers. Future studies should be conducted to evaluate the use of these hydrolysates as feed additive *in vivo*.

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