Production of feather protein hydrolyzed by *B. subtilis* AMR and its application in a blend with cornmeal by extrusion

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ABSTRACT

The poultry industry produces approximately 6 million tons of feathers as a by-product annually. The major constituent of feather is protein (80—90% of which is keratin). In order to use feather protein in animal feedstuffs, it must be hydrolyzed by chemical, physical or enzymatic processes. The aim of this study was to improve the production of chicken feather hydrolysates using keratinolytic *Bacillus subtilis* AMR and evaluate the effectiveness of a blend of this hydrolysate with cornmeal produced by extrusion. The addition of sucrose (0.5 g/L) to the feather medium increased the keratinolytic activity of *B. subtilis* AMR by 1.3 fold. The highest enzymatic activity and production of soluble protein were achieved at pH 8.0. The fermented feather medium presented large amounts of protein with low molecular weight that provided greater nutrient bioavailability. The physicochemical properties of the extrudates showed that the addition of hydrolyzed feather improved the ash and total N content. All essential amino acids were detected in the corn—feather hydrolysate extrudate. This work represents the first study that describes the use of microbial feather hydrolysate in a blend with cornmeal to make an extruded product. These results demonstrated the potential use of this hydrolysate as a supplement in animal feed.

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1. Introduction

Chicken is a major protein source for humans. Currently, the worldwide production of chicken is approximately 96 million tons per year. The consumption of poultry meat has reached a world average of approximately 14 kg person per year (FAO 2015). Feathers correspond to between 5% and 7% of the total weight of a chicken (Onifade, Al-Sana, Al-Musallan, & Al-Zarban, 1998); therefore approximately 6 million tons of feathers are produced as a by-product of the poultry industry. Disposing of or processing this by-product is a challenge.

The main constituent of feather is keratin (80—90%). Keratins are proteins characterized by a high content of cysteine cross-linked by disulfide bonds. These bonds make keratins mechanically stable and resistant to enzymatic lysis by common peptidases such as trypsin, pepsin, and papain. Besides the cysteine content, the resistance of keratins is associated to its compact structure of such as α-helices (α-keratin) or β-sheets (β-keratins). This resistance to lysis has been a major obstacle in processing keratin (Brandelli, Sala, & Kalil, 2015). Moreover, the disposal of feather waste has severe implications of environmental pollution, nowadays.

One alternative to reduce the accumulation of feather waste would be to use feather protein in animal feedstuffs. The search for new materials with lower cost by the feed industries has led the industry to study the use of feathers as a source of protein. One approach is to apply biologic and/or enzymatic treatments to degrade feather for the production of feed ingredients. However, the use of native feather protein as a dietary protein supplement has been carried out only on a limited basis (Jayathilakan, Sultana, Radhakrishna, & Bawa, 2012). Feathers are currently converted to feather meal using steam pressure-cooking and chemical treatments to make them more digestible; however, these processes are expensive and require significant amounts of energy, which does not represent a sustainable solution for feather processing.

Additionally, the hydrothermal treatment destroys some essential amino acids such as lysine, methionine, and tryptophan, and forms non-nutritive amino acids such as lysinoalanine and lanthionine (Onifade et al., 1998). The final product of hydrothermal treatment is feather meal, a product with poor digestibility and variable nutrient quality, making it difficult to use as a component for feedstuff. Additionally, the deficiency in methionine, histidine, and tryptophan limits the use of feather meal as an ingredient in animal feed (Grazziotin, Pimentel, de Jong, & Brandelli, 2006).

Alternatively, a nutritional upgrade of feathers through treatment with microbial keratinases might significantly increase the amino acid availability of feather keratin. Keratinolytic enzymes have been studied from a vast variety of bacteria, actinomycetes, and non-pathogenic fungi. Keratinases from *Bacillus* spp., particularly *B. licheniformis* and *B. subtilis*, have been studied extensively due to their effectiveness in degrading feathers (Lin, Lee, Casale, & Shih, 1992; Mazotto et al., 2011; Abdel-Naby, Ibrahim, & El-Refai, 2016). Biodegradation by these organisms represents an improvement and a sustainable method for the conversion of keratin waste into useful products.

Extrusion technology has been used in the food industry for a long time. Extrusion is a continuous mixing, kneading, and shaping process of high productivity, versatility, and low cost. Additionally, it is associated with the production of high quality products (Berrios, Ascheri, & Losso, 2013). Starch-based food materials, such as corn, rice, and semolina, are widely used in extrusion processes for the production of breakfast cereals, baby foods, feedstuffs, pet foods, pasta, and confectionary products. However, no extruded product using hydrolyzed feather as a protein source has yet been developed.

In a previous work we described that keratinase producing *Bacillus subtilis* AMR had the capacity to degrade hard keratin such as human hair (Mazotto et al., 2010). In the present study we show that keratinolytic *B. subtilis* AMR is able to degrade chicken feather producing a feather hydrolysate that is rich in low molecular peptides and amino acids. The hydrolyzed feather obtained by a microbial process represents a potential alternative for protein enrichment of extrudate food. Finally, a blend of corn meal, a starch-based food material commonly used in feed, and feather hydrolysate was tested.

2. Material and methods

2.1. Chemicals

Gelatin, casein and solvents were obtained from Merck (Darmstadt, Germany). Reagents used in electrophoresis and molecular mass standards were acquired from Sigma Chemical Co. (St. Louis, MO, USA) and BioRad (California, USA), respectively. All other reagents were of analytical grade.

2.2. Feather substrate

Feathers were obtained from a local poultry industry, washed with detergent (linear alkylbenzene sulfonic acid), exhaustively rinsed with tap water, and dried overnight at 60 °C. Cleaned feathers were delipidated by immersion in methanol:chloroform (1:1) solution for 1 h, dried at 60 °C, and stored at room temperature in plastic bags.

2.3. Bacterial strain and inoculum preparation

The *B. subtilis* AMR used in this study was isolated from feather agro-industrial residues and identified as describe by Mazotto et al. (2010). *B. subtilis* AMR was propagated in yeast extract medium (yeast extract 5 g/L, peptone 5 g/L, sucrose 20 g/L, KCl 20 g/L) and incubated with shaking at 26 ± 2 °C (150 rpm) for 72 h. The cells were centrifuged and washed twice (at 1900 g for 20 min) with a sterile saline solution (8.5 g/L NaCl). Resuspended cells were used in an inoculum of 10^8 CFU/mL for the production of hydrolyzed feather.

2.4. Effect of pH on hydrolyzed feather production

The inoculum was added to 100 mL of feather medium supplemented with 0.1 g/L of yeast extract at different pH values in 250 mL flasks. The following buffers were used in the feather medium: citric acid buffer (0.1 M citric acid and 0.2 M Na_2HPO_4, at pH 5), phosphate buffer (0.06 M $Na_2HPO_4.7H_2O$ and 0.04 M KH_2PO_4, adjusted to pH 6.0, 7.0, and 8.0), and aminoacetic acid buffer (0.2 M aminoacetic acid and Na_2HPO_4 0.2 M at pH 9.0). This inoculated feather medium was incubated for 8 days in an orbital shaker (Marconi, MA 140/SC) at 150 rpm and 26 ± 2 °C. One 5 mL aliquot was removed daily to determine the keratinolytic activity, feather degradation, and soluble protein concentration. These evaluations are described below.

2.5. Effect of additional substrates on hydrolyzed feather production

The inoculum was prepared as described above and inoculated in 250 mL flasks containing 100 mL of feather medium (10 g/L of feather in phosphate buffer: 0.06 M $Na_2HPO_4.7H_2O$ and 0.04 M KH_2PO_4 adjusted to pH 8.0) supplemented with 0.1 g/L or 0.5 g/L of an additional substrate source. The additional substrates tested were glucose, sucrose, corn steep liquor, casein, gelatin and yeast extract. The control sample was a medium without supplementation. The cultures were incubated for 6 days at 26 ± 2 °C in an orbital shaker at 150 rpm. Aliquots of 5 mL were harvested daily, centrifuged (at 1900 g for 20 min), and the soluble protein concentration and keratinolytic activity were recorded.

2.6. Microorganism-hydrolyzed feather production kinetics

Growth, hydrolysate production, keratinolytic activity and feather degradation were determined by measuring the CFU, soluble protein concentration, and feather degradation daily over a 12-day period. *B. subtilis* AMR was inoculated in 2 L flasks containing 500 mL of the feather medium under the best conditions previously determined. To evaluated the growth, serial dilutions of culture were performed in sterilized saline (NaCl 8.5 g/L) and 10 μL of dilutions was inoculated in agar GYP medium (glucose 20 g/L, yeast extract 5 g/L, peptone 10 g/L, agar 20 g/L). After 24 h of incubation at 28 °C, and the number of CFU was calculated.

2.7. Microorganism-hydrolyzed feather production

In order to produce the feather hydrolysate, *B. subtilis* AMR was inoculated in 3 L flasks containing 1 L of the feather medium under the best conditions previously determined. After 6 days at 26 ± 2 °C of incubation in an orbital shaker at 150 rpm, the culture was centrifuged (at 1900 g for 20 min) and used as the microorganism-hydrolyzed feather.

2.8. Determination of soluble protein

Soluble protein concentration was determined using the Lowry method (Lowry, Rosembrough, Farr, & Randall, 1951) with bovine serum albumin as the standard.

2.9. Feather degradation determination

Feather degradation was measured by percentage of dry weight loss. After cultivation for a determined incubation time, all medium content was filtered through a Whatman filter paper (N°1) and fragments of feathers retained on the filter were washed with distilled water and dried together with the filter at 60 °C until constant weight. The filter paper was weighed on an analytical balance (Shimadzu AY220) before filtration and after filtration and drying. The excess weight corresponds to non-hydrolyzed feathers.

2.10. Keratinolytic activity determination

The keratinolytic activity was evaluated as describe by Mazotto et al. (2011). One unit of keratinolytic activity was defined as the amount of enzyme required to produce an increase of 0.01 absorbance unit at 280 nm under standard assay conditions (1 h at 37 °C).

2.11. SDS-PAGE

Culture supernatant was concentrated in dialyzing membranes (9 KDa cut off) against polyethylene glycol 4000 overnight at 4 °C. Eighty microliters of the concentrated supernatant were mixed with 20 μl of sample buffer, heated at 100 °C for 5 min and loaded to 15% polyacrylamide gel (Laemmli, 1970). The molecular mass standards used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

2.12. Zymography

The concentrated culture supernatant was mixed with a peptidase sample buffer in a sample:buffer ratio of 6:4. Samples (20 μl) were applied in 125 mg/mL SDS-PAGE with 1 mg/mL of co-polymerized gelatin (Heussen & Dowdle 1980; Cedrola et al., 2012).

2.13. MALDI-TOF

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) was used to verify the presence of low molecular mass peptides in the supernatant of the culture. The assay was performed as described by Mazotto et al., 2010.

2.14. Thermoplastic extrusion

The assay aimed to incorporate microorganism-hydrolyzed feather to cornmeal by extrusion. Blends were prepared by mixing 1 kg of cornmeal and 260 mL of hydrolyzed feather (26%). To determinate if hydrolyzed feather inclusion in cornmeal extrudate could improve the nutritional value, a control was prepared with water replacing the hydrolysate. Distilled water was used in control to reach a similar moisture level between control and samples. Moisture content was measured only at the end of the processes. The Reference Table for chicken feed formulae used in Brazil specifies diets with 26% or 18% of crude protein, as described by D'Agostini, Gomes, Albino, Rostagno & Sá. (2004). In our study, we used 26% of hydrolysate as the crude protein. Protein present in control was only the cornmeal protein, no other protein was added. The blend was extruded in a single-screw extruder (Brabender, mod 20DN, Duisburg, Germany) using the compression rate of 3:1. The die diameter was 3 mm, and the feed rate was approximately 5 kg/h. The temperature zones were: 1, 2, and 3 at 50, 100, and 130 °C respectively. The extrudate was collected after each process reached equilibrium. The extrudate was cut into pellets approximately 4.0 cm long followed by dehydration at 75 °C for approximately 24 h, until reaching a final moisture content of 3–7%.

2.15. Physical and chemical characterization of extrudates

The sectional expansion index (SEI) of the extrudates was evaluated by measuring the extrudate diameter using a 150 mm manual caliper (Vonder®, Curitiba, Brazil). A total of 20 readings were registered for each sample and SEI was calculated according to Equation (1) (Alvarez-Martinez, Kondury, & Harper, 1988).

$$SEI = (D/D_0)2 \tag{1}$$

Where D is the extrudate diameter after cooling and D_0 is the diameter of the insert hole. Physicochemical analysis was performed on the milled extrudates. Moisture content was determined by measuring the weight difference between extrudate before and after dehydration at 105 °C for 20–24 h, until constant weight. The Kjeldahl method, which is a standard method for estimating the protein content in foods (Moore, DeVries, Lipp, Griffiths, & Abernethy, 2010), was applied to determine the protein in this blend of cornmeal and hydrolysate (AACC, 1999) using Na_2SO_4 and $CUSO_4$ as catalysts. AOAC (2000) methods were used to determine ash content. Lipids were extracted with ethyl ether for 5 h followed by evaporation. The resulting residues were dried at 105 °C until constant weight. Lipid content was determined according to AOAC (1990).

2.16. Amino acid determination

The protein hydrolysis was performed according to the official AOAC method, and derivatization of amino acids for chromatographic analysis followed the method of Cohen and Michaud (1993).

2.17. Statistical analysis

The experiments were performed in triplicate using three independent experimental sets. Each independent experimental set was conducted as follows: three flasks with fresh medium were inoculated with fresh inoculum and the experiment was conducted as describe above. Each experimental set was repeated (three times). The statistical significance of the terms in the regression equation was examined by analysis of variance (ANOVA) in Microsoft Excel 2016. Significance was accepted at $p < 0.05$.

3. Results

3.1. Effect of pH on keratin hydrolyzed production

To determinate the effect of pH on keratinase activity and feather degradation, the initial pH of 5.0 was increased to 9.0. The optimal pH value was found to be 8.0, for the highest enzymatic activity and production of soluble protein.

After six days of fermentation (144 h), the soluble protein concentration at pH 8.0 was 5.25 mg/mL, 1.32 and 11.18 fold higher than that found at pH 7.0 (3.97 mg/mL) and 9.0 (0.47 mg/mL), respectively (Fig. 1A). Soluble protein produced in culture medium was directly related to feather hydrolysis, as shown in Fig. 1B. Thus, the feather solubilization at pH 8.0 after six days of fermentation was 95%, the highest value reached. The growth of B. subtilis AMR at
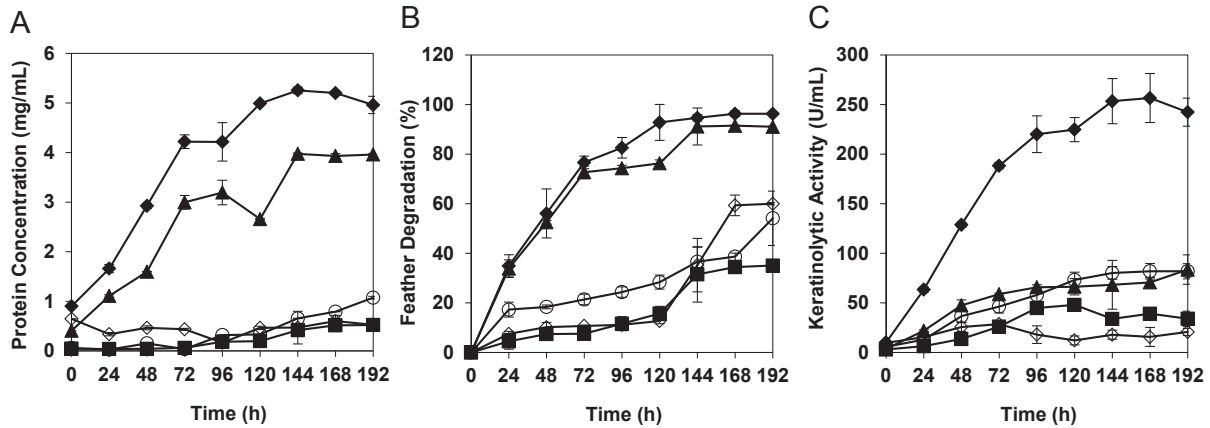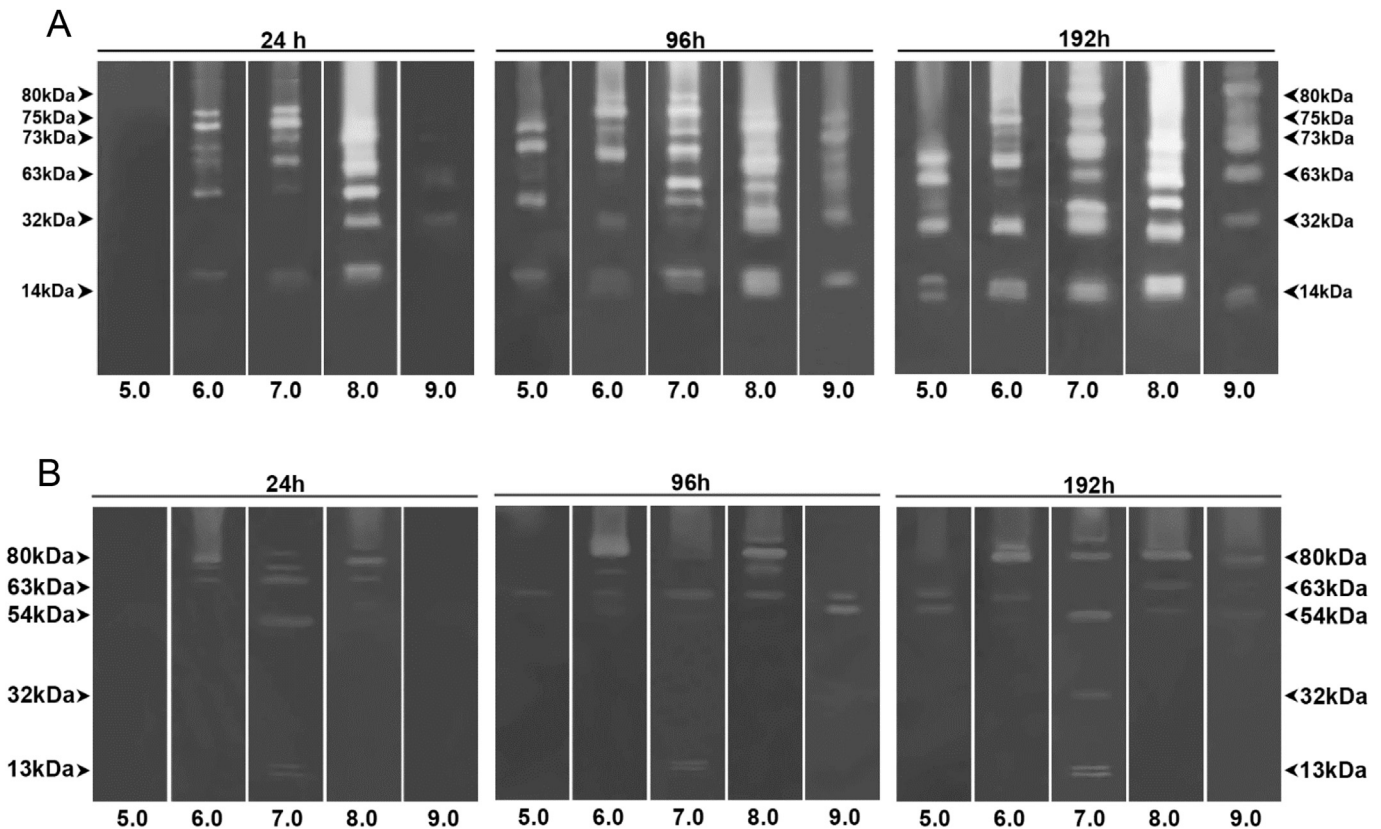
Fig. 1. Soluble protein concentration (A) feather degradation (B), and keratinolytic activity (C) of the culture supernatant by *B. subtilis* AMR in medium supplemented with yeast extract at different pH (5.0–9.0). Feather media were buffered at pH 5.0 (■), pH 6.0 (○), pH 7.0 (▲), pH 8.0 (◆) and pH 9.0 (◇). The experiment was conducted in triplicate.

pH 7.0 and 9.0 on feather leads to 91% and 35% of degradation of feather, respectively (Fig. 1B).

The breakdown of feather protein was performed by the extracellular proteolytic enzyme secreted by *B. subtilis* AMR. At pH 8.0, the keratinolytic activity was significantly higher than that observed at other pH values (Fig. 1C). The pH significantly affected (P < 0.0001) the keratinolytic activity and consequently the feather degradation. Thus, for the strain used in this study, pH 8.0 was the best pH value for hydrolyzed feather production.

The zymographic analysis of extracellular enzymes produced during fermentation in feather media showed a production of multiple peptidases and keratinases (Fig. 2A and B). Corroborating with the keratinases assay, the pH affected the expression of enzymes, particularly keratinases (Fig. 2B). The proteolytic profile at pH 8.0 showed more band intensity (Fig. 2A), which increased during the process. No bands or weak signals were observed at pH 5.0 and 9.0 after 24 h of fermentation in both gelatin gel (Fig. 2A for peptidases detection) and keratin gel (Fig. 2B for keratinases detection). *B. subtilis* AMR produced two keratinases in feather medium at pH 7.0 migrating at approximately 13 kDa. At the same pH, a band migrating at approximately 32 kDa was detected after 192 h. Those bands were not observed at other pH values.

Fig. 2. Extracellular peptidases zymography (A) and extracellular keratinases zymography (B) of the culture supernatant of *B. subtilis* AMR in medium supplemented with yeast extract at different pH (5.0–9.0).

3.2. Effect of additional substrates on feather degradation and hydrolysate production

To enhance the keratinolytic activity and the feather hydrolysis, the effect of adding yeast extract, glucose, sucrose, corn steep liquor, casein or gelatin to the formulation was investigated. Although *B. subtilis* AMR is able to grow and degrade feather in medium containing feather as the sole source of carbon, nitrogen, and energy, an additional substrate improved the keratinolytic production of the microorganism and consequently improved the feather hydrolysate concentration. Sucrose at 0.5 g/L was the best additional substrate for keratinase production (360.6 U/mL); when glucose and casein were used as additional substrate, both at 0.5 g/L, the keratinolytic activity was 310 and 304 U/mL, respectively (Table 1). Sucrose at 0.5 g/L was significantly better than other substrates evaluated (P < 0.05), except glucose at 0.5 g/L (P = 0.14). Due to its better performance and lower cost, it was selected as the additional substrate chosen. Comparing the enzyme production in
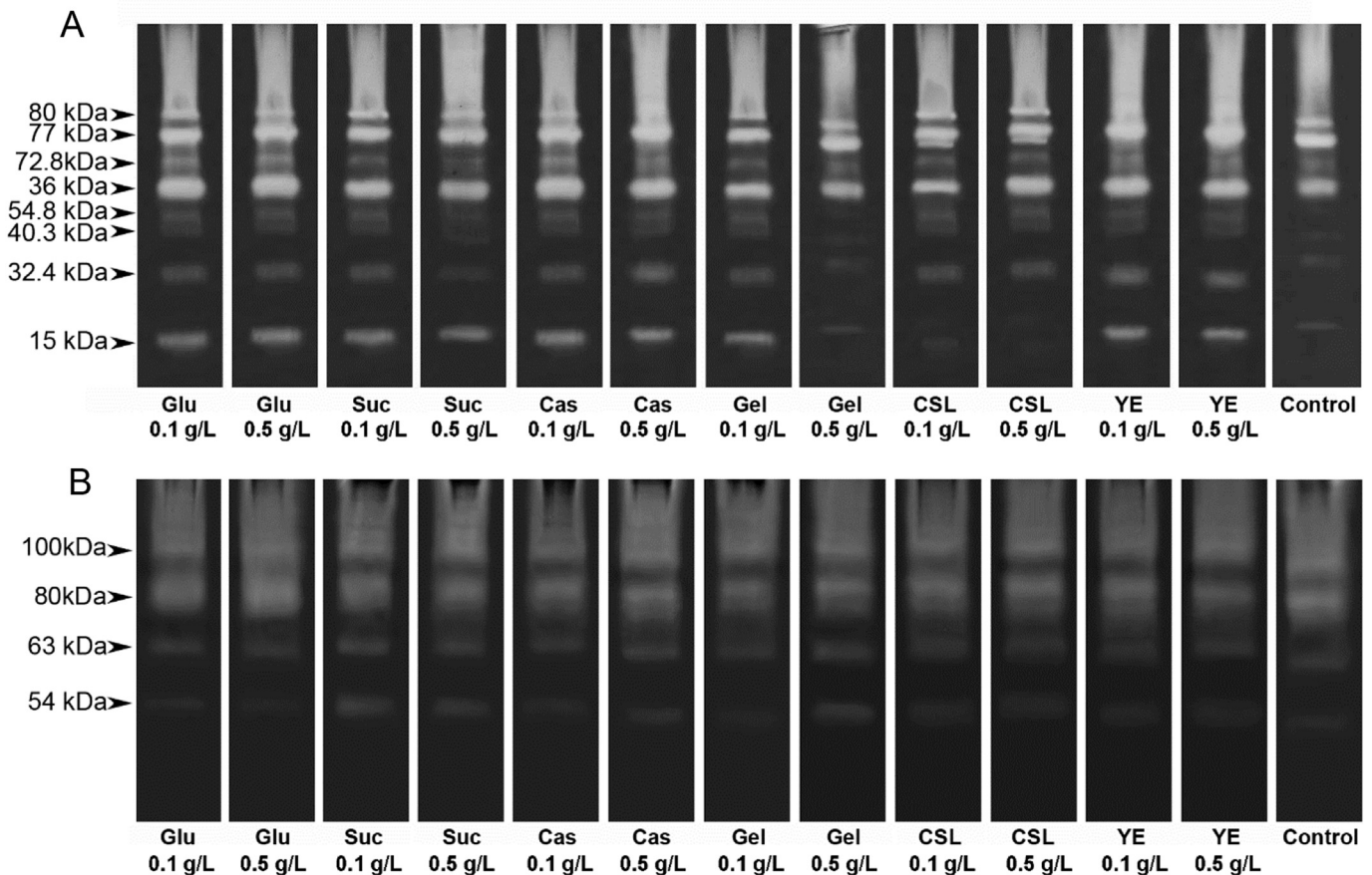
Table 1
Keratinolytic activity and total protein concentration of the culture supernatant and feather degradation by *B. subtilis* AMR in feather medium supplemented with 0.1 or 0.5 g/L of different additional substrate.

Additional Substrate	Keratinolytic Activity (U/mL)	Soluble protein concentration (mg/mL)	Feather degradation (%)	Microbial biomass (10^7 CFU/mL)
Glucose 0.1 g/L	282 ± 4.2	3.75 ± 0.09	95.76 ± 1.62	51.85 ± 21.15
Glucose 0.5 g/L	310 ± 36.16*	3.36 ± 0.12	87.84 ± 2.63	79 ± 1*
Sucrose 0.1 g/L	250 ± 17.78	3.84 ± 0.02	97.37 ± 1.13*	24.8 ± 1.2*
Sucrose 0.5 g/L	360.6 ± 10,26*	3.70 ± 0.20	89.46 ± 2.24	26.3 ± 1.7*
CSL 0.1 g/L	212.67 ± 24.03	3.59 ± 0.07	85.73 ± 2.02	57.75 ± 16.25*
CSL 0.5 g/L	265 ± 35.38	3.59 ± 0.15	86.97 ± 0.87	84.5 ± 4.5*
Casein 0.1 g/L	173 ± 24.33	4.11 ± 0.21*	98.93 ± 1.10*	36.8 ± 1.8*
Casein 0.5 g/L	304.67 ± 24.03	3.72 ± 0.18	91.46 ± 1.01	112 ± 28
Gelatin 0.1 g/L	160.6 ± 11.02	3.77 ± 0.17	89.77 ± 0.08	28.6 ± 3.4*
Gelatin 0.5 g/L	222.34 ± 25.72	3.94 ± 0.05	89.65 ± 0.13	36.4 ± 11.6
YE 0.1 g/L	302 ± 2*	3.61 ± 0,11	98.93 ± 0.90	16.5 ± 0.5*
YE 0.5 g/L	267 ± 0	3.79 ± 0.13	94.12 ± 1.62	47 ± 13
Control	274 ± 34.64	3.74 ± 0.11	90.08 ± 0.22	9.05 ± 0.95

Measurements were made on the 6th day. The experiment was conducted in triplicate.
*p < 0,05 vs control.

Fig. 3. Extracellular peptidases zymography (A) and extracellular keratinases zymography (B) of the culture supernatant by *B. subtilis* AMR in feather medium supplemented with 0.1 or 0.5 g/L of glucose (Glu), sucrose (SUC), corn steep liquor (CSL), casein (Cas), gelatin (Gel) or yeast extract (YE). Zymography was performed on the 6th day.

medium without supplementation, the additional sucrose increased the activity 1.31 fold for keratinase. Feather degradation in sucrose supplemented medium reached 97% (Table 1). The supplements did not produce any significant increase in feather degradation; however, the addition of 0.5 g/L sucrose increased the enzymatic activity and was used throughout our experiments.

Zymography of enzymes produced in feather media with different supplementations showed few alterations of the peptidase and keratinase profiles, specifically the intensity of the 15 and 32 kDa bands in gelatin gel (Fig. 3A and B). The results indicate that feather was the substrate that induced the production of peptidases and keratinases, independently of the presence of other substrates.

3.3. Production of feather hydrolysate

Kinetic assays were performed to determine the duration of the fermentation process. The bacterial growth was measured daily for 12 days (Fig. 4A). The cell inoculum was massive and the initial cell concentration was very high, leading to slight variations in counting. However, the cells performed a growth curve. On the first day of incubation, the number of cells decreased slightly, since the cells were grown in a richer medium before being transferred to the feather medium. After that, the cell concentration grew slowly until the 8th day, and then the stationary phase began. The cells remained in the stationary phase for a long period probably due the abundant substrate. (Finkel, 2006).

On the 6th day, when the feather degradation had reached 95%, the release of proteins was at a maximum (Fig. 4B). After the maximum feather degradation, the microorganisms began to use the proteins released into the culture medium, leading to a slight reduction in their concentration. The pH increased from 7.8 to 8.5 possibly due to proteolysis and the subsequent release of ammonia by microbial metabolism (data not shown). Fig. 4C illustrates feathers before and after fermentation.

3.4. Feather microorganism-hydrolysis by B. subtilis AMR produced low molecular weight peptides

Low molecular weight peptides are readily digestible. To determine the presence of low molecular weight peptides in the fermented feather medium, we performed SDS-PAGE and MALDI-TOF. The fermented feather medium presented large amounts of protein with molecular weights lower than 14 kDa (Fig. 5B). Mass spectrometry was used to investigate peptides in a molecular weight range not detected by SDS-PAGE. This procedure revealed peptides with 816–2595 Da (Fig. 5A). These results suggest that microbial hydrolyzed feather may be used as an additional hydrolysate protein source in feed.

3.5. Inclusion of microorganism-hydrolyzed feather in cornmeal

Knowing that peptides of low molecular weight were found in the feather hydrolysate, we investigated adding it (260 mL/kg or 1.5 g of peptides) as a supplementary protein and amino acid source to cornmeal feed.

Cornmeal with and without feather hydrolysate were used as a primary raw material for the production of extrudates. Pure cornmeal and that mixed with the feather hydrolysate were processed in a single screw extruder. The physicochemical analysis of the extrudates is presented in Table 2. The results of the physicochemical analysis showed that the addition of the feather hydrolysate significantly improved (p < 0.05) the ash content. The values of SEI varied from 5.39 to 6.79, as shown in Table 2. The SEI was not affected by the addition of the hydrolysate. The addition of feather hydrolysate in cornmeal also increased the concentration of
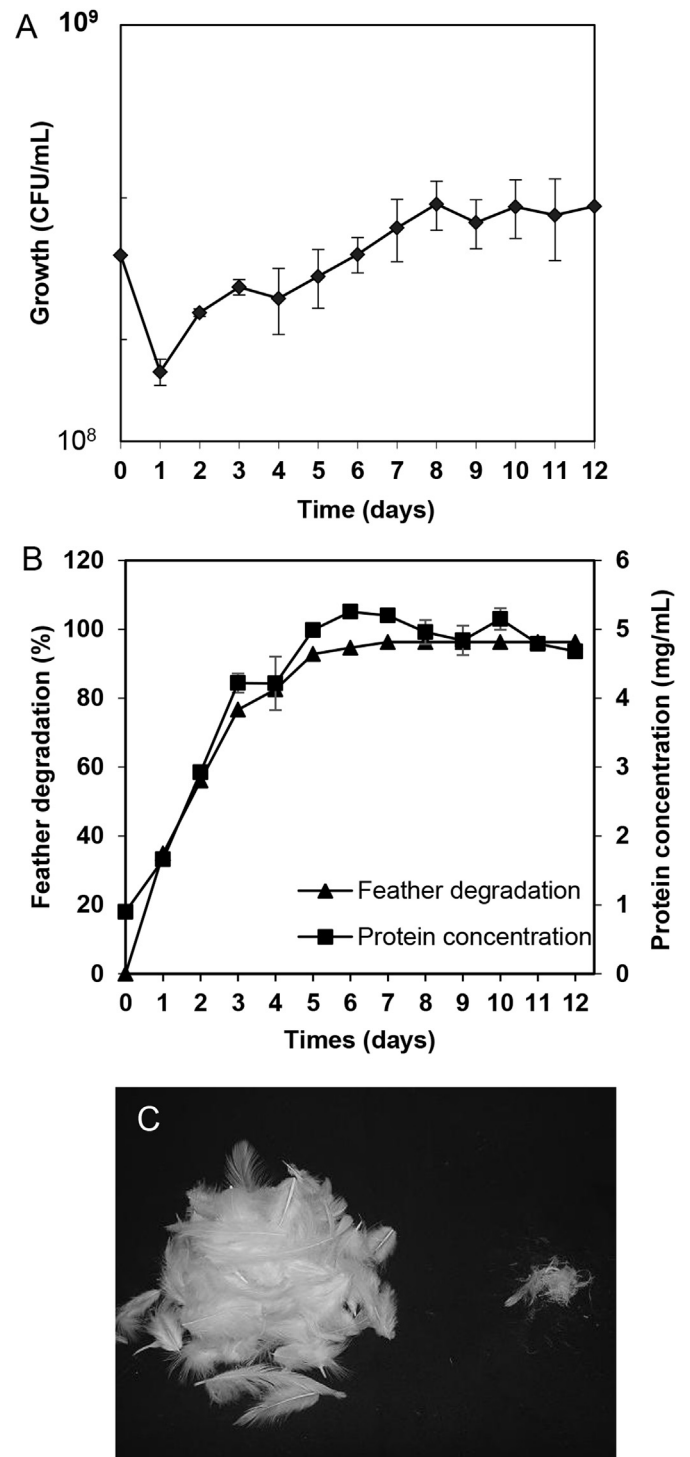

Fig. 4. Fermentation kinetics of _B. subtilis_ AMR in feather medium. A: soluble protein concentration and feather degradation of culture supernatant by _B. subtilis_ AMR in feather medium; B: growth of _B subtilis_ AMR in feather medium; C feather degradation after 8 days of fermentation.

peptides and amino acids, represented by the increase of total nitrogen (Table 2).

The amino acid composition of the extrudate had high concentrations of glutamic acid, proline, and alanine (Table 3). With the exception of methionine, all essential amino acids, including those essential for avian species (arginine, glycine, and proline),
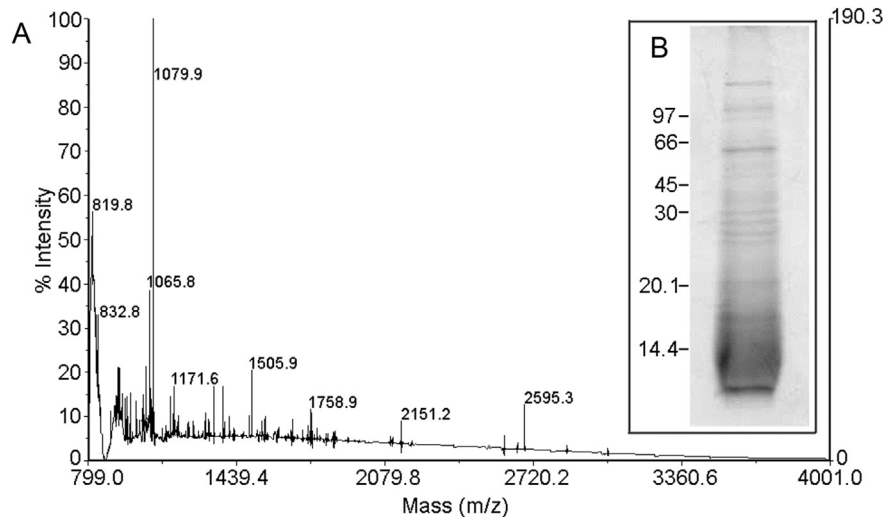
Fig. 5. MALDI-TOF and SDS-PAGE of hydrolyzed keratin from feather. (a) MALDI-TOF and (b) SDS-PAE.

were detected in the corn feather hydrolysate extrudate, supporting its use as a feed ingredient (Table 3). However, methionine and cysteine were not detected in our tests due to the methodological limitations. Thus, it is possible that all amino acids were actually included in the extrudate.

4. Discussion

This study evaluated the use of microorganism-hydrolyzed feather as a source of protein in corn extruded products. The first step was to produce hydrolyzed feather via a sustainable process in which a microorganism with high keratinolytic activity was used. *Bacillus subtilis* AMR was previously described as being able to degrade hard keratin substrate (Mazotto et al., 2010) and was therefore selected for this study. Additionally, *B. subtilis* has been granted the status of Qualified Presumption of Safety by European Food Safety Authority and some enzymes produced by *B. subtilis*, such as peptidases, have GRAS status (Generally Recognized as Safe) by the U.S Food and Drug Administration (FDA).

The addition of only 0.5 g/L of sucrose was able to increase the keratinolytic activity of *B. subtilis* AMR 1.3 fold (Fig. 2). We used a minimal concentration of an additional nutrient source to improve the growth conditions and keep the costs associated to culture medium as low as possible. Other studies have reported the effect of adding supplementary carbon and nitrogen sources for keratinase production. The keratinolytic activity increased up to 1.5-fold with the addition of 10 g/L carboxymethyl cellulose in the fermentation media of *B. weihenstephanensis* PKD 5 (Sahoo, Das, Thatoi, Mondal, & Mohapatra, 2012). Yeast extract, as well as sucrose, tryptone and peptone, had a positive effect on keratinase production by *B. subtilis* KD-N2 (Cai & Zheng, 2009). The effect of an additional carbon and nitrogen source on keratinolytic activity varies according to the species. Thus, it is necessary to investigate the best conditions for each case.

The best pH for hydrolyzed feather production by *B. subtilis* AMR is pH 8.0. This result is in agreement with other data in the literature. Most of the keratinolytic microorganisms present a maximum keratinase production that varies from neutral to alkaline pH (Cai & Zheng, 2009; El-Refai, AbdelNaby, Gaballa, El-Araby, & Abdel Fattah, 2005; Sahoo et al., 2012). We observed that the maximum soluble protein concentration was achieved after 6 days of incubation, coinciding with the highest keratinolytic activity (Fig. 3). For

B. weihenstephanensis PKD-5, the maximum keratinolytic activity was observed after 7 days of fermentation (Sahoo et al., 2012). The optimized keratinase production by *B. pumilus* FH9 reduced the process time to 2 days (El-Refai et al., 2005). Based on our results and these reports on keratinolytic *Bacillus*, the efficiency of feather degradation by *B. subtilis* AMR can be improved by performing detailed optimization studies. Our group is currently exploring such possibilities.

Feather keratin induced the production of at least eight peptidases and four keratinases. These results suggest that several peptidases, including keratinase, participate in keratin hydrolysis. Similar results were observed for another *Bacillus* strain. The zymography analysis of the crude enzymatic extract of *B. pumilus* A1 revealed the presence of at least five keratinolytic enzymes migrating at 14—45 kDa (Fakhfakh-Zouari, Haddar, Hmidet, Frikha, & Nasri, 2010). Several keratinase from the *Bacillus* strains presented molecular weights in the range of 20—40 kDa (Jaouadi et al., 2015). However, keratinases with higher molecular masses have been reported, such as: the keratinase of 97 kDa from *Bacillus subtilis* DP1 (Sanghvi et al., 2016) and the keratinase of >200 kDa from *B. cereus* 1268 (Mazotto et al., 2011).

The results observed by mass spectrometry showed that feather hydrolysates contained peptides with 816—2595 Da (Fig. 4). In agreement with our results, the feather hydrolysis by keratinase produced by *Brevibacillus* sp. strain AS-S10-II generated peptides with 262—1621 Da (Mukherjee, Raia, & Bordoloi, 2011), and by *B. subtilis* SLC with 550—892 Da (Cedrola et al., 2012). These results suggest that microorganism-hydrolyzed feather is an efficient nonaggressive method to produce low molecular weight peptides, which may be used as an additional protein source in feed.

Recently, the benefits of incorporating functional ingredients in extruded products have been proposed to improve nutritional value and functionality (Santillán-Moreno, Martínez-Bustos, Castaño-Tostado, & Amaya-Llano, 2011). Thus, the use of traditional protein, vitamin, and fiber sources have been widely investigated (Santillán-Moreno et al., 2011). However few studies report the protein fortification of corn extrudates using unusual protein sources. To date, protein feather hydrolysate has not been used as an additional nutrient in corn extrudate. The cornmeal and hydrolyzed feather blend may represent an economical approach to protein enrichment of extrudate food. Feather has been used in animal feed, but its poor digestibility limits its use. Microbial

Table 2
Physical and chemical characterization of cornmeal, feather hydrolysate, and the cornmeal-feather hydrolyzed extrudate.

	Cornmeal[b]	Feather hydrolysate (FH)	Corn Extrudate (CE)[b]	CE + FH
Ash (g/100 g)	0.17 ± 0,014	0.57 ± 0		0.75 ± 0
Crude fat (g/100 g)	0.9 ± 0.007	0.3 ± 0.007		1.1 ± 0.07
Total N (g/100 g)	1.08 ± 0	0.26 ± 0.02		1.18 ± 0.04
Moisture (g/100 g)	12.63 ± 0.14	97.69 ± 0.04		10.14 ± 0.05
Sectional expansion index[a]	–	–	6.79 ± 0.05	5.39 ± 0.04

[a] Average of 12 determinations.
[b] Controls.

Table 3
Amino acid concentration (g/100 g) of extrudate obtained by mixture of cornmeal and feather hydrolysate.

Amino acid	CE + FH[a]	Cornmeal extrudate	Corn[b]
Asp	0.37	0.36	0.48
Glu	1.34	1.30	1.16
His	0.27	0.27	0.18
Thr	0.24	0.24	0.19
Pro	0.70	0.66	0.52
Val	0.31	0.31	0.33
Ile	0.24	0.24	0.20
Phe	0.33	0.35	0.32
Ser	0.31	0.31	0.19
Gly	0.17	0.17	0.24
Arg	0.38	0.39	0.28
Ala	0.47	0.46	0.47
Tyr	0.33	0.31	0.06
Lys	0.14	0.18	0.21
Leu	0.87	0.88	0.74

[a] Cornmeal extrudate with feather hydrolysate.
[b] Ulrich, Jakel, Dyer, & Lohrmann, 2002.

keratinase has been suggested as an alternative to hydrolyze feather and improve the digestibility (Brandelli et al., 2015). However, few products were created using this hydrolysate and no report describes its application in a blend with corn meal to produce an extrudate food. Bone and Shannon (1975) in the patent US4006266 A described blends containing an amylaceous ingredient (20–60%) and animal protein (2–25%) source in addition to a vegetable protein source, fat, and sufficient water for processing by extrusion to produce dry pet food. The amylaceous material used was cereal grains or starch and proteinaceous material comprised of collagen, albumen, casein, sodium caseinate, calcium caseinate, ammonium caseinate, potassium caseinate, and magnesium caseinate. The same as in our work, they developed a mixture by extrusion of starch material with animal protein, but they used a conventional protein source, different from our work, which opted to use residual protein from the poultry industry as an alternative. Although corn meal and feather have been used as a component in feedstuff, the blend of corn meal and microbial hydrolysate feather is new and could also be used to improve the nutritional value of animal feed and snack foods, one of the fastest-growing branches of the food industry (Brnčić et al., 2001).

5. Conclusions

In conclusion, the hydrolyzed feathers obtained by microorganism treatment represent a low-cost and sustainable alternative for waste poultry utilization. Additionally, this hydrolysate can be combined with cornmeal to produce, by extrusion cooking, a nutritionally improved feed product.

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