Bioutilization of Chicken Feather Wastes by Newly Isolated Keratinolytic Bacteria Into Protein Hydrolysates With Improved Functionalities

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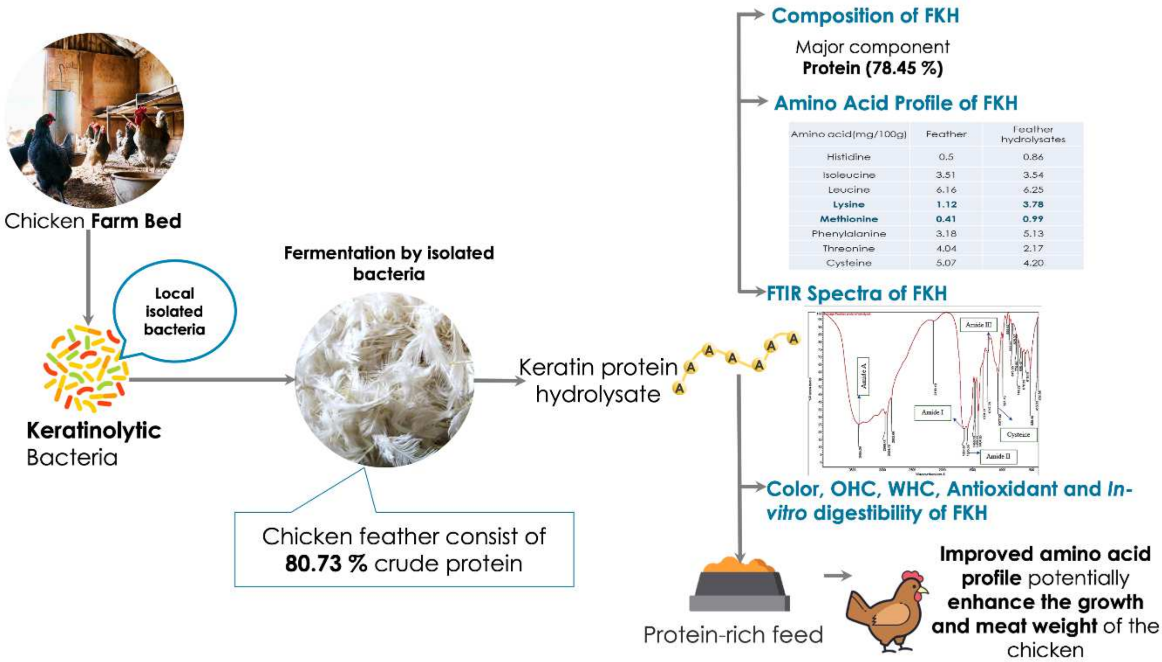
Highlights

1. Keratinolytic bacterial isolate (KB1) was screened from a chicken farm bed.
2. KB1 was able to degrade chicken feather waste into protein hydrolysates.
3. Protein hydrolysates demonstrated good functional and bioactive properties.
4. Feather Protein hydrolysates from poultry waste can be used in feed applications.

Graphical Abstract



Bioutilization of chicken feather wastes by newly isolated keratinolytic bacteria into protein hydrolysates with improved functionalities

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Abstract

In this study, a novel feather-degrading bacteria *B. amyloliquefaciens* KB1 was isolated from chicken farm bed (CFB), identified by morphological, physico-biochemical tests followed by 16s rDNA analysis. Among observed isolates, bacterial isolate (KB1) showed the highest degree of feather degradation (74.78 ± 2.94 %) and total soluble protein (205 ± 0.03 mg/ g). Using the same species of bacteria, the optimum fermentation condition was found at 40 ^{o}C, pH 9, and 1 % (w/v) feather concentration that produced 260 mg/ g of soluble protein and 86.16 % feather degradation using response surface methodology in a Box-Behnken design space. The obtained hydrolysates exhibited bioactive properties. The amino acid profile showed the increase in concentration of essential amino acid compared with feather meal broth. The selection of safe screening source of this new bacteria in CFB produced hydrolysates with enhanced bioactivity applicable for food, feed, and cosmetic applications along with environmental remediation.

Keywords: *protein hydrolysates, chicken farm bed, bacteria, feathers.*

1. Introduction

Intense growth and development of food processing industries have led to a huge amount of waste as a by-product that is mostly discharged into the environment. Chicken feather remains one of the significant by-products from the poultry industry, mainly due to keratin protein that is hard to degrade [1]. In general, each bird has up to 125 g of feathers and taking into account the daily processing of chicken at 400 million/ day worldwide; this waste reaches five million tons of dry feathers per day [2]. However, chicken feathers are excellent reservoirs of biomolecules with more than 82% crude protein, out of which 91% is keratin, predominantly β-keratin [3]. The higher amount of protein in keratinous waste presents great potential as a source of protein and amino acids for feed, food, and cosmetic applications.

Keratin is generally characterized by its ability to resist common proteolytic enzymes and mechanical stability to chemical, hydrothermal and thermo-chemical treatments under high steam. Currently, the industrial process for feather meal involves high temperature and thus the process is costly and energy-intensive. It also results in denaturation and significant loss of essential amino acids producing low-quality protein products [4]. Alkali pretreatments using KOH, NaOH, $Ca(OH)_2$ increase the extraction and yield but possess threats in dealing with toxic effluents [5]. Land dumping and incineration are other methods that are likely to result in environmental vandalism. The generation of toxic air emissions from burning feathers is higher than that generated from coal combustion plants [2].

Biotechnological methods have been employed recently to biologically degrade feather keratins as it is considered cost-effective and environment-friendly [6-8]. Various microorganisms producing keratinase enzyme have been known to degrade chicken feathers, mainly keratin, including fungi [9], actinomycetes [10], and *Bacillus* species [4, 11]. Microorganisms produce an abundance of metabolites that can break down the keratin

protein into peptides and amino acids. The screened microorganism can produce keratin hydrolysate; this process benefits in having superior control over the hydrolysis process for the yield. Protein hydrolysates produced from feathers keratin will be cheaper and useful raw materials for animal feed, compostable films, nitrogen-rich fertilizers, reinforced fabrics, and biodegradable materials. Other commercial applications of protein hydrolysates include an effective component of detergents, personal care products, medical treatments of psoriasis and acne, nail treatments, and prion proteins degradation [12, 13].

Studies have recently been carried out to isolate and identify keratinolytic bacteria from chicken feather dumpsite [14, 15], forest soil [15] and chicken slaughter shops [16]. In all these studies, the authors had reported successful identification of such bacteria with a very high (80-90 %) feather degrading potential within a week of cultivation. To date, there are no reported studies on the screening of keratinolytic bacteria from chicken farm bed. Chicken farm bed (CFB) was chosen as a potential source for isolating the most desirable keratinolytic bacterial species. Furthermore, the natural selection of poultry habitat as CFB as a potential source of keratinolytic bacteria will eliminate the chances of isolating pathogenic bacteria to chicken and humans. In the present study, bioutilization of feather waste was carried out initially by using newly isolated keratinolytic bacteria from CFB. Then, the same isolated bacteria was used in the culture conditions and optimized for protein hydrolysate production in order to enhance the efficiency of feather degradation.

2. Materials and methods

2.1 Bed and feather samples

Chicken farm bed (**CFB**) soil sample and chicken feathers (**CF**) were supplied by Charoen Pokphand Foods Ltd (CPF) (Chonburi, Thailand). CF was washed two-fold with tap water and finally with distilled water to remove extraneous matters. Similarly, lipid content was removed by immersing the feathers into the solution (chloroform: methanol, 1:1). The washed feathers were dried at 50 ºC for 2 days and stored at room temperature prior to microbial treatment [17]. All other chemicals and used reagents during the study were of analytical grade.

2.2 Analytical methods

Moisture content was determined by oven-drying (SLW115TOP, Gibthai, Thailand) at 105 °C to a constant mass (AOAC official method no. 934.01). Fat content was determined using the Soxhlet extraction method (Model 64826, Merck, Germany) with hexane to the solvent's boiling point at 8 h (AOAC, official method no. 920.39). Crude protein content was determined according to the Kjeldahl method (K1100F, Hanon, China) (AOAC official method no. 981.10) following the AOAC standard methods [18].

2.2 Isolation of keratinolytic bacteria from chicken farm bed sample

Initially, the spread plate technique was used to obtain the proteolytic bacteria from CFB. CFB (1 g) was serially diluted up to 10^{-8} in a normal saline (0.8 mg/ 100 mL). Diluted samples (100 µL) from each dilution were spread on the skim milk agar plates (pH 7) and incubated at 37 ºC for 24 h. Bacteria with the visible zone of hydrolysis from 10^{-8} dilution were selected and further streaked to obtain the pure colonies. Feather meal broth (pH 7) was used to study the keratinolytic activities of isolated colonies according to Daroit *et al.* (19) by allowing the growth of bacteria for 18 h that would be used as an inoculum for feather

fermentation. Then, inoculation (1% (v/v), 10^7cfu/ml) was performed in test tubes containing sterile fermentation media (10 mL) and incubated for 7 days at 37°C. On the seventh day, the tubes were visually observed for the degradation of feathers. Further, tubes with observed feather degradation were chosen to check their degree of feather degradation (DFD) in 250 mL Erlenmeyer flask with minimal growth medium MGM broth (100 mL) (1% NaCl; 0.05%; 0.07% K_2HPO_4; 0.14% $MgSO_4.7H_20$) containing processed sterilized feather (1 g) at 37°C incubation temperature. Then, the supernatant was obtained by centrifugation (Centrikon T-324, Germany) at 6000 × *g* for 20 min. Morphological characterization and biochemical tests (shape, size, gram staining, spore, methyl red, citrate utilization, casein hydrolysis, gelatin hydrolysis, and motility) were carried out to identify the genus of the isolate.

Then, the isolate's growth pattern was studied in nutrient broth for 72 h in a shaking flask (150 rpm at 40°C). The inoculum was prepared by sub-culturing the bacteria for 24 h. It was then diluted to get the inoculum size of 10^7 CFU/ mL. The inoculum (10% v/v) was added to sterilized nutrient broth (500 mL) for the study, where turbidity method was used for the measurement of optical density (OD) at 600 nm using UV-Vis Spectrophotometer (Shimadzu UV-1800, Bara-Scientific Co. Ltd., Thailand) at different time intervals (0-70 h).

2.3 Identification of the feather degrading bacteria

2.3.1 Morphological and biochemical tests

Various morphological (form, culture characteristics) and biochemical tests (methyl red test, citrate utilization test, casein hydrolysis, gelatin hydrolysis, and motility) were carried out according to the standard protocols developed in the biotech lab at AIT for the identification of genus of the feather degrading bacteria.

2.3.2 Identification and molecular phylogenetic studies

The identification of the Genomic DNA of feather degrading bacteria was based on 5' 16S rDNA gene sequence comparison. This DNA was amplified with universal 16S rDNA primers under following PCR (T100^{TM} Thermal Cycler, Bio-Rad Laboratories, Inc., Thailand) conditions: 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and elongation at 72 °C for 3 min. PCR product was amplified using forward primer; 20F (5'-GAG TTT GAT CCT GGC TCA G-3') and reverse primer; 1500R (5'-GTT ACC TTG TTA CGA CTT-3'). The nucleotide sequences obtained from all primers were assembled using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), followed by deposition of this sequence into the NCBI GenBank (https://www.ncbi.nlm.nih.gov/). The identification of closest phylogenetic neighbors was performed using the BLASTN program against the 16S rDNA sequence from previous prokaryotes' database collection. The pairwise sequence similarity with the highest value was calculated using the Global alignment algorithm.

2.3 Production of feather protein hydrolysate

Keratinolytic bacteria with the highest feather degradation was used as an inoculum for fermenting the raw chicken feather. The bacteria was cultured in nutrient broth for 24 h at 37°C. Then, an inoculum 1% (v/v) containing 10^7 CFU/ml was added to a 250 mL flask containing whole feather (1 g) and MGM (100 mL) as a basal medium for 7 days with shaking incubator at 150 rpm (M2019, Velp Scientifica, Europe). After, every 24 h, sample (5 mL) was harvested, filtered (Whatman filter paper No. 1, GE Healthcare UK), and centrifuged (Centrikon T-324, Germany) (6000 × *g* for 15 min). The supernatant was used to detect the total soluble protein (TSP) and pH. The degree of feather degradation (DFD) was determined from the residual feather on the seventh day. The broth was passed through

filter paper (Whatman filter paper No. 1, GE Healthcare UK), followed by washing to remove the cell debris and finally dried in a hot-air oven (SLW115TOP, Gibthai, Thailand) at 60ºC for 24 h.

Then the percentage of feather degradation was calculated using equation 1.

$$DFD\ \% = \frac{initial\ feather\ weight - residual\ feather\ weight}{initial\ feather\ weight} \times 100 \quad (1)$$

2.3.1 Box-Behnken design

For the optimum fermentation conditions, interactive independent effects of feather concentration (1%, 3% and 5% w/v) (X1), initial pH (6, 7.5, and 9) (X_2) and fermentation temperature (30, 40, and 50 ºC) (X3) as independent variables for the fermentation of chicken feather were varied using Box-Behnken design and response surface methodology (RSM) as a statistical tool according to the Bernal *et al.* (20) with some modifications. Total Soluble protein (TSP) and degree of feather degradation (DFD) were measured as the response variables. The complete experimental design provided by design expert software (trial version 7.0) contained 15 runs with three replicates at the center point. The data were analyzed using a quadratic polynomial regression model, as shown in equation 2.

$$Y = \beta + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_{ii} 2 + \Sigma\beta_{ij}\ X_i X_j \quad (2)$$

where Y represents protein concentration as a response variable, β is a constant identity; independent variables are denoted as X_i and X_j, respectively, and so on.

2.3.2 Determination of total soluble protein and pH content

Feather protein hydrolysates were assayed for total soluble protein using Bradford assay (1976) at 595 nm using a UV-Vis spectrophotometer (UNICAM, Alva, UK) [21]. Bovine serum albumin (BSA) (Sigma Aldrich, USA) was used as a standard to calculate the protein

content in the sample, expressed in mg/ mL. Finally, the protein content of broth was converted in mg soluble protein per gram of feather. During fermentation, the change in the pH of the fermentation media was determined by using a portable digital pH meter (Model 3510, Jenway, UK), calibrated to pH 7 using buffer by directly dipping the electrode in the sample until a constant reading is displayed.

2.3.3 Partial purification of feather protein hydrolysates

Ammonium sulfate (70% w/v) precipitation was used to partially purify protein hydrolysates, according to Jain and Anal (22). The clear supernatant was taken in a glass beaker and stored at 4 ^{o}C. Salt solution (700g/ L) was added dropwise with constant stirring (600 rpm) while maintaining 4 ^{o}C throughout the purification process. The keratin protein was precipitated, and the solution was centrifuged (Centrikon T-324, Germany) at 12,000 × *g* for 15 min (4 ^{o}C). The solid pellet contained the precipitated partially purified protein. The protein concentration was dried using a freeze dryer into powder at -55^{o}C until further use.

2.4 Feather keratin hydrolysate characterization

2.4.1 Proximate composition

Crude protein, fat, and moisture content of feather keratin hydrolysate was performed as earlier described according to AOAC standards [18].

2.4.2 In vitro protein digestibility

In vitro protein digestibility of feather protein hydrolysates were carried out according to Fakhfakh *et al.* (23). Two enzymes; Pepsin (EC 3.4.23.1, from the porcine stomach, Sigma Aldrich, 3000 IU/g), and Pancreatin (EC 232-468-9, from porcine pancreas, Sigma Aldrich, 1400 IU/g) were used for the digestibility studies. Freeze-dried protein hydrolysate (1 g) resuspended in Milli-Q water (1:1) (1 mL) and feather (1 g) were taken in a glass beaker, and

it was dissolved with 2 mg/ mL of pepsin prepared with 2 M HCl and incubated for 2 h at 37ºC. By the end of the incubation period, the pH was changed to 8 with 2 M $NaHCO_3$. Then, pancreatin (2 mg/ mL) prepared with 2 M HCl was added, and incubation was carried for further 16 h. After completion of digestion, the mixtures were centrifuged (Centrikon T-324, Germany). The solubilized protein content in the supernatant was determined by the Kjeldahl method, and % protein digestion was calculated.

$$\% \, protein \ digestion = \frac{protein \ content \ in \ supernatant \ by \ digestion \ of \ 1 \ g \ sample}{protein \ content \ in \ 1 \ g \ of \ sample \ before \ digestion} \quad (3)$$

2.4.3 Color

The color spectra of dried protein hydrolysates were determined by using the Hunter-Lab spectrophotometer colorimeter (Color Flex: 45/0, USA). The sample (10 g) was loaded in the sample holder in a light source and covered with a black lid. Mean values from 10 observations of L^*, a^*, and b^* were used to calculate the whiteness index of keratin hydrolysate, according to Raungrusmee *et al.* (24) using equation 3.

$$Whiteness \ index = 100 - ((100 - 1^2) + (a^2) + (b^2))^{1/2} \quad (4)$$

2.4.4 Oil holding capacity (OHC)

The oil holding capacity of the protein hydrolysates was determined according to Jain and Anal (22). Keratin hydrolysate (100 mg) was dissolved in soybean oil (10 mL) and vortexed for 1 min. They were then centrifuged (Centrikon T-324, Germany) at 2500 × g for 30 min. Free oil was removed, and the adsorbed oil weighed. OHC was calculated as the weight of oil adsorbed per gram of sample.

2.4.5 Water holding capacity (WHC)

The water holding capacity of the protein hydrolysates was determined according to Raungrusmee and Anal (25). A centrifugal tube (15 mL) was taken and weighed. Keratin

hydrolysate (400 mg) was loaded in the centrifugal tube in which distilled water (10 mL) was added, stirred (5 min), and then centrifuged (Centrikon T-324, Germany) at 5000 × g for 30 min. The unabsorbed water was removed by decantation after centrifugation, and the final weight of the tube was recorded. Finally, the amount of water absorbed (g) was calculated per gram of protein hydrolysates.

2.4.6 Chemical fingerprinting by FTIR spectra

Using FTIR spectrophotometer (Bruker Vertex 70, Billerica, MA, USA), the structural and functional groups present on the keratin hydrolysate were evaluated, and all spectra were collectively attenuated in the frequency range of 4000-400 cm^{-1} using 16 scans and 2 cm^{-1} resolution [26]. The lyophilized sample (2 mg) was pressed into the carver hydraulic press after mixing with KBr (100 mg). The spectra were analyzed for the structural characteristics of the protein hydrolysates.

2.4.7 Amino acid profile

The amino acid composition of the feather protein hydrolysates was analyzed according to Dhakal *et al.* (27). The protein hydrolysates (50 mg) was treated with HCL (6N) at 110 °C for 24 h. To remove the residual HCl, the sample was evaporated in a rotary evaporator (Büchi rotavapor R-144, Switzerland). The evaporated sample was dissolved in 10 ml of 0.2 M sodium citrate buffer (pH 2.2). The sample was filtered through a 0.45 mm membrane filter (Titan, Switzerland) and injected into an amino acid analyzer (Biochrome 30, Cambridge, UK) using ninhydrin as a color reactant and on a single ion-exchange resin column. The amino acid composition was converted into mg amino acid per 100 g of protein in feather protein hydrolysates and compared with the raw chicken feather meal based on previous studies.

2.4.8 Antioxidant activity (DPPH assay)

Feather protein hydrolysates were assessed to analyze its ability to reduce the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) (D9132, Sigma-Aldrich, USA) by measuring its absorbance decrease at 517 nm. DPPH solution was made by using DPPH powder (0.004 g in 100 mL 95% ethanol), according to Garrido *et al.* (28). Then stock sample solution of 3 mg/ mL was prepared and diluted to different concentrations (0.125, 0.250, 0.50, 1 and 2 mg/ mL) with distilled water. DPPH solution was then mixed with a sample solution (1:1) in an opaque glass test tube. A blank solution was prepared with DPPH solution (1 mL) and distilled water (1 mL). The samples were incubated (30 min) in the dark, and the absorbance was read at 517 nm. The DPPH inhibition activity was determined by using equation (4).

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{A_b - A_s}{A_b} \times 100 \qquad (5)$$

Where, Ab and As are the absorbance of blank and keratin, respectively. The IC_{50} value, which is the half-maximal concentration of feather keratin hydrolysate to inhibit a substance, was determined using the Graph Pad Prism 7.

2.5 Statistical analysis

All the experimental tests were carried out in triplicates. The results were expressed as the mean of the replicas with the standard deviation. Similarly, IBM SPSS statistics 21 was used to analyze the Analysis of Variance (ANOVA). Tukey's method was used as a post-hoc to analyze the significant difference among the samples at 95% confidence level.

3. Results and discussion

3.1 Isolation of keratinolytic bacteria from chicken farm bed (CFB)

CFB was selected as the source for the isolation of keratinolytic bacteria. The bed soil sample (1 g) was serially diluted to 10^{-8}. The highest dilution showed 3×10^{10} CFU/ mL bacterial population after spreading (100 µL) sample on the skim milk agar (SMA) (incubated at 37 °C for 24 h). Each distinct colony was streaked on the SMA plates to get pure culture. A single colony from each of the thirty plates were then tested to observe their ability to degrade the feather in a test tube containing 10 mL of MGM broth and a single feather piece as a sole source of carbon and nitrogen. The initial pH was maintained 7.5 and incubated for 7 days at 37°C. Out of thirty test isolates, only eight were found to show the feather degradability after 7 days of hydrolysis, and thus the isolate was named KB1, KB2, KB3, KB4, KB5, KB6, KB7, and KB8.

3.1.1 Measurement of the degree of feather degradation and total soluble proteins of isolates

The isolates were grown in an Erlenmeyer flask (250 mL) with MGM broth (100 mL) and chicken feathers (1 g). **Table 1** illustrates the degree of feather degradation and total soluble proteins released by these bacterial isolates during feather degradation. The maximum (74.78 ± 2.94 %) and minimum (11.1 ± 1.23 %) degradation of the whole feather in the broth were shown by the isolates KB1 and KB2, respectively. The same isolates produced maximum soluble protein KB1 (205 ± 0.03 mg/ g of the dry feather) and KB2 (39 ± 0.06 mg/ g dry feather). Based on these findings, keratinolytic bacterial isolate (KB1) was chosen as appropriate. **Fig. 1** illustrates the degradation of a feather (a) control (in a feather meal) (b) solubilized due to isolated bacteria KB1.

Table 1 here

Fig 1. here

3.1.2 Morphological characterization and biochemical test of the isolate

Morphological studies and biochemical tests for isolate KB1 showed that the isolate was gram-positive, endospore-forming, and a motile bacillus. Similarly, the culture study in nutrient agar showed that the colonies were creamy white in color, mucoid, raised, translucent, and exhibits the entire margin. The isolate showed negative results to the methyl red test, citrate utilization test, and positive results to casein and gelatin hydrolysis.

3.1.3 Optimal growth conditions

The growth of the bacterial isolate KB1 was studied in nutrient broth for 72 h, which showed the initial lag phase of 2 h and onset of log-phase till 42 h of growth (Supplementary file figure S1). Then, the optical density started to fall, exhibiting the decline phase. The optical density (OD) measured as absorbance at 600 nm drops gradually with no visible stationary phase. This is because the identified isolate was endospore former, which means due to the depletion of nutrient and accumulation of toxic substances, vegetative cells of endospore former start to undergo spore formations which are smaller in size than vegetative cells [29].

3.1.4 Identification of the bacteria by 16s rDNA

The bacterial isolate KB1 was identified using single-strand 16s rDNA sequencing for species characterization phylogenetically. BLAST search engine showed that the species had the highest similarity with the *Bacillus siamensis* and *Bacillus velezensis* 99.78%. The phylogenetic analysis of the bacteria that was observed to be located in the same cluster with *Bacillus siamensis* (**Fig. 2**). Moreover, it showed 99.63% similarity with *Bacillus amyloliquefaciens* and *Bacillus subtilis* sub. *Subtilis*. Fan *et al.* (30) suggested that all the

closest neighbors of KB1, including *B. amyloliquefaciens, B. siamensis,* and *B. velezensis*, do form an "operational group" as *B. amyloliquefaciens* within the *B. subtilis* species complex. Therefore, the isolated bacteria KB1 can be related to *B. amyloliquefaciens*.

Fig. 2 here

3.2 Optimization of fermentation condition using response surface methodology

The identified bacterial isolate KB1 was used for the fermentation of raw chicken feathers in MGM broth. The fermentation process was optimized by using RSM with independent factors; initial feather concentration (1%, 3%, and 5 % w/v): Initial pH (6, 7.5, and 9): fermentation temperature (30, 40 and 50 ºC) against the response variables; total soluble protein (mg/ g) and the degree of feather degradation (%) using Box-Behnken design.

Table 2 illustrates the results obtained after 15 sets of experiments with experimental and predicted values. Regression analysis was performed for the fitting of the response surface model in the given experimental design space. Following this, a multiple regression quadratic equation was obtained that represents an empirical relationship between the responses and the independent variables as shown:

$$Y_1 = 116.82 - 80.43\,X_1 + 9.08\,X_2 - 0.69\,X_3 - 1.36\,X_1X_2 + 0.39\,X_1X_3 - 1.26\,X_2X_3 + 45.61X_1^2 - 5.69\,X_2^2 + 1.21\,X_3^2 \quad (5)$$

$$Y_2 = 74.50 - 14.84\,X_1 + 8.45\,X_2 + 0.088\,X_3 - 0.028\,X_1X_2 + 0.91\,X_1X_3 - 1.12\,X_2X_3 - 4.53\,X_1^2 - 10.41X_2^2 - 1.30X_3^2 \quad (6)$$

Where Y_1 and Y_2 are total soluble protein and degree of feather degradation, respectively. Similarly, X_1, X_2 and X_3 are initial feather concentration % (w/v), pH and fermentation temperature (ºC) respectively.

The effects of each independent variable on the response were determined with the help of F-test (ANOVA), where initial feather concentration (% w/v) and pH had a significant effect on

the production of total soluble protein (mg/ g) and degree of feather degradation (%) ($p < 0.001$). Lack of fit test helps to measure the model's failure to represent predicted and observed data in the experimental design space. The model had a non-significant lack of fit value (p values) of 0.0789 and 0.4136, respectively for total soluble protein and degree of feather degradation, meaning the variation of data fits the actual response variable with the model able to predict values of total soluble protein (mg/ g) and degree of feather degradation (%). The R^2 value for soluble protein and the degree of feather degradation was 0.9866 and 0.9781, respectively, which showed a good fit of the empirical model with the experimental data.

Table 2 here

Fig. 3 (a) and 3 (b) illustrate the response surface (3-D) plots with interactive effects of two independent variables on a single response variable. The soluble proteins were found to be increasing at increasing alkaline condition and decreasing feather condition. Similar findings were observed in the degree of feather degradation. The maximum production of soluble protein (260 mg/ g) was observed at initial pH 9 and 1% (w/v) feather concentration, with 86.16 % feather degradation. At higher initial feather concentration, it was observed that soluble protein release and feather degradation was minimum representing 56.20 mg/ g and 34.19 %, respectively. At higher substrate concentration, the enzyme excretion is lower and hence is the lower degradation of feather and soluble protein production [31]. However, the influence of temperature was not significant for both the production of soluble protein and feather degradation ($p > 0.05$). The Design Expert Software determined the optimum fermentation conditions based on the desirability function (Design Expert). The optimum condition (desirability = 0.976) includes initial feather concentration of 1% (w/v), pH of 9, and fermentation temperature of 40 ºC at which the maximum protein concentration and degree of feather degradation were reported to be 260 mg/ g and 86.16 % respectively.

Fig. 3 (a) and 3 (b) here

3.3 Characterization of feather protein hydrolysates

3.3.1 Physico-chemical composition of raw feathers and Feather protein hydrolysates (FPH)

Table 3 illustrates the physico-chemical characterization of FPH compared to raw feathers. Raw feathers consists of 80.73 ± 1.53 % crude protein as a major constituent. Considering that feathers are composed of more than 80 % crude protein (keratin), the use of this protein source can be of great interest to produce protein hydrolysates [3]. Other components analysed include fat (1.27 ± 0.05 %), ash content (0.83 ± 0.07 %), volatile compounds (80.84 ± 0.90 %) and fixed carbon (5.41 ± 0.07 %). Whereas FPH constitutes a remarkable amount of protein (78.45 ± 0.38 %). The reduction in the protein content is due to utilization by bacterial culture to increase biomass; hence the output protein is slightly less than that of a raw feather. The low moisture content (3.54 ± 0.04 %) of the hydrolysate is due to the freeze-drying, which helps extend the product's shelf-life.

Table 3 here

In vitro protein digestibility plays a significant role in the formulation of food and feed products. The *in vitro* protein digestibility of raw feather and feather protein hydrolysates were observed *in vitro* using pepsin and pancreatin and it was observed as 1.75 ± 0.5 % and 82.36 ± 0.62 %, respectively. Raw feathers are primarily composed of keratin protein, commonly resistant to enzymes like pepsin, pancreatin, trypsin, papain, etc. [31]. However, newly isolated bacteria KB1 solubilized the native keratin protein into peptides and amino acids, which are easily digested by the gastrointestinal enzymes. Fakhfakh *et al.* (23) used commercially available *B.pumilus* A1 to produce feather protein hydrolysates with an *in vitro* digestibility of 98 ± 0.7%.

3.3.2 Color of protein hydrolysates

The color parameters of sample hydrolysates were measured with Hunter Colorimeter. The parameters were expressed as L* for darkness to lightness, a* for greenness to redness, and b* for blueness to yellowness. L* a*and b* values were found to be 76.49 ± 0.08, 3.19 ± 0.22, and 23.27 ± 1.63, respectively. The whiteness index of produced protein hydrolysates was found to be 66.77 ± 1.12.

3.3.3 Oil holding capacity (OHC) and water holding capacity (WHC) feather protein hydrolysates

Feather protein hydrolysate exhibited excellent OHC (5.46 g/ g) and WHC (3.35 g/ g) of protein hydrolysate, respectively. The increased concentration of polar groups such as COOH and NH_2 that is caused by enzymatic hydrolysis has a substantial effect on the amount of adsorbed oil and water [22]. Oil and water-holding properties of protein hydrolysates are crucial in food and feed formulation. These properties directly affect the texture, color, appearance, and the shelf-life of the final product. The higher the water holding capacity, the more the energy to reduce the moisture content and even reduce shelf life. However, it can help to solubilize the water-soluble component in the food matrix (Jain & Anal, 2017). Similarly, higher the oil holding capacity, the product's palatability will be increased with a soft texture and higher fat-soluble nutrients. However, the products will have a lesser shelf-life as the product may face rancidity. The water-holding capacity is due to the hydrophilic moiety of proteins. The oil holding capacity results from a lipophilic and non-polar moiety of protein [32].

3.3.4 Fourier- transform infrared spectroscopy (FTIR) of feather protein hydrolysates

The FTIR spectra of feather protein hydrolysates exhibiting different peaks of wave numbers representing the presence of Amide A, I, II, and III bands is shown in **Fig. 4.** The hydrolysate

exhibited a peak at 3404.39 cm^{-1}, which showed the presence of amide A with a wave number close to 3500-3200 cm^{-1}. N-H stretching vibration is associated with the absorption characteristic of amide A [33]. Amide I exhibit the wavenumber of 1700-1600 cm^{-1}, which is due to the stretching vibration of C=O bonds, whereas Amide II exhibits the wave number 1580-1480 cm^{-1}. The occurrence of amide II is derived from N-H and C-H stretching vibrations. Amide I possess the strongest transmission band and is very sensitive to secondary based on different hydrogen-bonding environments for α-helix, β-sheet, turn, and unordered conformation [34]. The protein hydrolysate exhibited the wavenumbers of 1634.87 cm^{-1} and 1585.55 cm^{-1}, therefore, confirmed the presence of Amide I and amide II. Also, the presence of a band at 1634.87 reflects that the protein is comprised up of strong beta sheets. The absorption peak of protein hydrolysates at 1300-1200 cm^{-1} stands for amide III, which signifies the stretching of C-N and deformation of N-H bonds. The keratin hydrolysate exhibited the wavenumber of 1247.28 $cm^{-1,}$ which showed the presence of amide III. The presence of bands between 1034-1078.28 cm^{-1} represents the presence of cysteine, formed as the result of disulfide bond broken down during the dissolution of keratin. The feather keratin hydrolysate exhibited band at wave number of 1077.28 cm^{-1} conforming the same. Similar results were reported by Colembergue *et al.* (35) with chicken feather protein.

Fig. 4 here

3.3.5 Amino acid composition

The study of amino acid composition is significant as the biological and functional activities of protein hydrolysates depend on the type and composition of amino acids within the protein sequence. The amino acid composition of feather protein hydrolysates was determined, as shown in **table 4.** The amino acid -glutamic acid, leucine, proline, valine, and aspartic acid were found to be present in the highest amount. Similarly, the protein hydrolysates showed

high levels of hydrophobic amino acids (alanine, cystine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine, and valine) contributing to 55.01% and good quantities of aromatic amino acids (tryptophan, phenylalanine, and tyrosine) contributing to 14.29 % of the total amino acids respectively. These amino acids are known to possess antioxidant activities, which help to justify the high free radical scavenging abilities obtained from the fermented protein hydrolysates. The protein hydrolysates also demonstrated good amounts of essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) in various food and cosmetic applications. Zhao *et al.* (36) evaluated the composition of essential amino acids in chicken feather as histidine (0.5 mg/ 100 g protein), isoleucine (3.51 mg/ 100 g protein), leucine (6.16 mg/ 100 g protein), lysine (1.12 mg/ 100 g protein), methionine (0.41 mg/ 100 g protein), phenylalanine (3.18 mg/ 100 g protein), Threonine (4.04 mg/ 100 g protein) and cysteine (5.07 mg/ 100 g protein). In our study, as illustrated in **table 4**, all essential amino acids increased with the most significant increment seen in lysine (3.78 mg/ 100 g protein) and Methionine (0.99 (mg/ 100 g protein). Improved amino acid profile can potentially enhance the growth and meat weight of the chicken applicable to chicken feed industry.

Table 4 here

3.3.6 In vitro antioxidant properties of feather protein hydrolysates

As illustrated in **Fig. 5**, the antioxidant abilities of feather protein hydrolysates were studied with DPPH radical scavenging activity, which showed that the inhibition activity increases with the hydrolysate concentration. DPPH being a free radical, when protonated, is scavenged, which reduces the absorbance at 517 nm, which is the measure of radical scavenging activity. The IC_{50} value of the feather keratin hydrolysate was found to be 0.7 mg/ mL. The IC_{50} DPPH radical scavenging activity of this hydrolysate was found to be

lower (meaning higher antioxidant abilities) than the two chemically extracted keratin hydrolysate A and hydrolysate C by Alahyaribeik and Ullah (37) with IC_{50} of 8.21 ± 0.231 mg/ mL and 2.23 ± 0.316 mg/ mL respectively. Fakhfakh *et al.* (23) observed an IC_{50} value of 0.3 mg/ mL from the chicken feather hydrolysate using *B. pumilus* A1 while *Chryseobacterium sediminis* RCM-SSR-7 isolated and identified from feather dumping sites in India exhibited 0.102 mg/ mL radical scavenging activity in its hydrolysates [38]. The free radical scavenging abilities of the feather keratin hydrolysate can be positively correlated with its amino acid composition. As can be observed from the amino acid analysis, the protein hydrolysates showed high levels of hydrophobic amino acids (alanine, cystine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, tyrosine, and valine). Cysteine is produced as a product during the breakdown of the disulfide bond present in the feather by microbial keratinase, which acts as a potent antioxidant. Also, Cysteine-SH present in feather peptide is a strong hydrogen donor to free radicals. Furthermore, sulfenic acid (–SOH) is produced when the chicken feather is reduced under alkaline conditions. This acid is yet another prime antioxidant in keratin hydrolysate [12]. This confirms the presence of electron-donating hydrolysates and peptides in feather keratin hydrolysate, which could be used as primary antioxidants that are applicable to many food, pharmaceutical, and cosmetic industrial products.

Fig. 5 here

4. Conclusion

Chicken feather degrading bacteria *Bacillus amyloliquefaciens* KB1 was isolated and identified from chicken farm bed and the same isolated bacteria with maximum total soluble protein (250.33 mg/ mL) and the highest feather degradation (86.17 %) obtained after fermentation was utilized successfully to degrade chicken feather. The fermentation process of a chicken feather by isolated bacteria KB1 was optimized using feather concentration, initial pH, and incubation temperature. The feather protein hydrolysates were characterized using FTIR spectroscopy and amino acid analysis. Similarly, these hydrolysates enhanced functional properties like antioxidant abilities and *in-vitro* digestibility, which can be associated with the breakdown of protein (keratin) during the fermentation process. Thus, the application of green technology-based fermentation by newly isolated and identified bacteria was highly effective in valorizing feather waste in producing feather keratin hydrolysate. Such hydrolysates of chicken feathers from chicken feather waste hold tremendous potential for various feed, pharmaceutical, and cosmetic industries.

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Conflict of interest

There are no conflict of interest among the author (s).

Availability of data and material

Available (if necessary)

Code availability

Not applicable

Authors' contributions

All authors contributed to the study conception and design. **Conceptualization**: Saugat Prajapati, Sushil Koirala, **Methodology:** Saugat Prajapati, Anil Kumar Anal, **Formal analysis and investigation:** Saugat Prajapati, Anil Kumar Anal, **Writing - original draft preparation:** Saugat Prajapati, **Writing - review and editing**: Sushil Koirala, Anil Kumar Anal, **Supervision:** Anil Kumar Anal. All authors read and approved the final manuscript.

Ethics approval

Not Applicable (NA)

Consent to participate

Not Applicable (NA)

Consent for publication

Consent approved

References

1. Łaba, W., Żarowska, B., Chorążyk, D., Pudło, A., Piegza, M., Kancelista, A., & Kopeć, W. (2018) New keratinolytic bacteria in valorization of chicken feather waste. *AMB Express,* 8**,** 9.
2. Tesfaye, T., Sithole, B., Ramjugernath, D., & Chunilall, V. (2017) Valorisation of chicken feathers: Characterisation of chemical properties. *Waste Management,* 68**,** 626-635.
3. Lasekan, A., Bakar, F. A., & Hashim, D. (2013) Potential of chicken by-products as sources of useful biological resources. *Waste Management,* 33**,** 552-565.
4. Haddar, H. O., Zaghloul, T. I., & Saeed, H. M. (2009) Biodegradation of native feather keratin by Bacillus subtilis recombinant strains. *Biodegradation,* 20**,** 687.
5. Gupta, R., Rajput, R., Sharma, R., & Gupta, N. (2013) Biotechnological applications and prospective market of microbial keratinases. *Applied Microbiology and Biotechnology,* 97**,** 9931-9940.
6. Brandelli, A., Daroit, D. J., & Riffel, A. (2010) Biochemical features of microbial keratinases and their production and applications. *Applied Microbiology and Biotechnology,* 85**,** 1735-1750.
7. Taskin, M., & Kurbanoglu, E. (2011) Evaluation of waste chicken feathers as peptone source for bacterial growth. *Journal of Applied Microbiology,* 111**,** 826-834.
8. Lange, L., Huang, Y., & Busk, P. K. (2016) Microbial decomposition of keratin in nature—a new hypothesis of industrial relevance. *Applied Microbiology and Biotechnology,* 100**,** 2083-2096.
9. Anbu, P., Gopinath, S. C., Hilda, A., Mathivanan, N., & Annadurai, G. (2006) Secretion of keratinolytic enzymes and keratinolysis by Scopulariopsis brevicaulis and Trichophyton mentagrophytes: regression analysis. *Canadian Journal of Microbiology,* 52**,** 1060-1069.
10. Syed, D. G., Lee, J. C., Li, W.-J., Kim, C.-J., & Agasar, D. (2009) Production, characterization and application of keratinase from Streptomyces gulbargensis. *Bioresource Technology,* 100**,** 1868-1871.
11. Kumar, R., Balaji, S., Uma, T., Mandal, A., & Sehgal, P. (2010) Optimization of influential parameters for extracellular keratinase production by Bacillus subtilis (MTCC9102) in solid state fermentation using horn meal—a biowaste management. *Applied Biochemistry and Biotechnology,* 160**,** 30.

12. Wan, M.-Y., Dong, G., Yang, B.-Q., & Feng, H. (2016) Identification and characterization of a novel antioxidant peptide from feather keratin hydrolysate. *Biotechnology Letters,* 38**,** 643-649.

13. Forgács, G., Lundin, M., Taherzadeh, M. J., & Horváth, I. S. (2013) Pretreatment of chicken feather waste for improved biogas production. *Applied Biochemistry and Biotechnology,* 169**,** 2016-2028.

14. Veenayohini, K., & Sangeetha, D. (2016) Isolation and identification of keratinolytic bacteria from poultry waste and assessment of its keratinase activity on chicken feathers. *International Journal of Applied Research,* 2**,** 396-402.

15. Dada, M., & Wakil, S. (2019) Screening and Characterisation of Keratin-Degrading Bacillus sp. from Feather Waste. *Biotechnology Journal International***,** 1-12.

16. Sahoo, D. K., Das, A., Thatoi, H., Mondal, K. C., & Mohapatra, P. K. D. (2012) Keratinase production and biodegradation of whole chicken feather keratin by a newly isolated bacterium under submerged fermentation. *Applied Biochemistry and Biotechnology,* 167**,** 1040-1051.

17. Cheong, C. W., Lee, Y. S., Ahmad, S. A., Ooi, P. T., & Phang, L. Y. (2018) Chicken feather valorization by thermal alkaline pretreatment followed by enzymatic hydrolysis for protein-rich hydrolysate production. *Waste Management,* 79**,** 658-666.

18. AOAC, G. (2016) Official methods of analysis of AOAC International. Rockville, MD: AOAC International, ISBN: 978-0-935584-87-5.

19. Daroit, D. J., Corrêa, A. P. F., & Brandelli, A. (2011) Production of keratinolytic proteases through bioconversion of feather meal by the Amazonian bacterium Bacillus sp. P45. *International Biodeterioration & Biodegradation,* 65**,** 45-51.

20. Bernal, C., Diaz, I., & Coello, N. (2006) Response surface methodology for the optimization of keratinase production in culture medium containing feathers produced by Kocuria rosea. *Canadian Journal of Microbiology,* 52**,** 445-450.

21. Kruger, N. J. (2009), in The protein protocols handbook, Springer, pp. 17-24.

22. Jain, S., & Anal, A. K. (2016) Optimization of extraction of functional protein hydrolysates from chicken egg shell membrane (ESM) by ultrasonic assisted extraction (UAE) and enzymatic hydrolysis. *LWT-Food Science and Technology,* 69**,** 295-302.

23. Fakhfakh, N., Ktari, N., Haddar, A., Mnif, I. H., Dahmen, I., & Nasri, M. (2011) Total solubilisation of the chicken feathers by fermentation with a keratinolytic bacterium,

Bacillus pumilus A1, and the production of protein hydrolysate with high antioxidative activity. *Process Biochemistry,* 46**,** 1731-1737.

24. Raungrusmee, S., Shrestha, S., Sadiq, M. B., & Anal, A. K. (2020) Influence of resistant starch, xanthan gum, inulin and defatted rice bran on the physicochemical, functional and sensory properties of low glycemic gluten-free noodles. *LWT-Food Science and Technology***,** 109279.

25. Raungrusmee, S., & Anal, A. K. (2019) Effects of Lintnerization, Autoclaving, and Freeze-Thaw Treatments on Resistant Starch Formation and Functional Properties of Pathumthani 80 Rice Starch. *Foods,* 8**,** 558.

26. Vidal, A. R., Duarte, L. P., Schmidt, M. M., Cansian, R. L., Fernandes, I. A., de Oliveira Mello, R., Demiate, I. M., & Dornelles, R. C. P. (2020) Extraction and characterization of collagen from sheep slaughter by-products. *Waste Management,* 102**,** 838-846.

27. Dhakal, D., Koomsap, P., Lamichhane, A., Sadiq, M. B., & Anal, A. K. (2018) Optimization of collagen extraction from chicken feet by papain hydrolysis and synthesis of chicken feet collagen based biopolymeric fibres. *Food Bioscience,* 23**,** 23-30.

28. Garrido, T., Gizdavic-Nikolaidis, M., Leceta, I., Urdanpilleta, M., Guerrero, P., de la Caba, K., & Kilmartin, P. A. (2019) Optimizing the extraction process of natural antioxidants from chardonnay grape marc using microwave-assisted extraction. *Waste Management,* 88**,** 110-117.

29. Sella, S. R., Vandenberghe, L. P., & Soccol, C. R. (2014) Life cycle and spore resistance of spore-forming Bacillus atrophaeus. *Microbiological Research,* 169**,** 931-939.

30. Fan, B., Blom, J., Klenk, H.-P., & Borriss, R. (2017) Bacillus amyloliquefaciens, Bacillus velezensis, and Bacillus siamensis form an "operational group B. amyloliquefaciens" within the B. subtilis species complex. *Frontiers in Microbiology,* 8**,** 22.

31. Parrado, J., Rodriguez-Morgado, B., Tejada, M., Hernandez, T., & Garcia, C. (2014) Proteomic analysis of enzyme production by Bacillus licheniformis using different feather wastes as the sole fermentation media. *Enzyme and Microbial Technology,* 57**,** 1-7.

32. Zou, Y., Wang, L., Cai, P., Li, P., Zhang, M., Sun, Z., Sun, C., Xu, W., & Wang, D. (2017) Effect of ultrasound assisted extraction on the physicochemical and functional

properties of collagen from soft-shelled turtle calipash. *International Journal of Biological Macromolecules,* 105**,** 1602-1610.

33. Zhou, C., Li, Y., Yu, X., Yang, H., Ma, H., Yagoub, A. E. A., Cheng, Y., Hu, J., & Otu, P. N. Y. (2016) Extraction and characterization of chicken feet soluble collagen. *LWT-Food Science and Technology,* 74**,** 145-153.

34. Miller, L. M., Bourassa, M. W., & Smith, R. J. (2013) FTIR spectroscopic imaging of protein aggregation in living cells. *Biochimica et Biophysica Aacta (BBA)-Biomembranes,* 1828**,** 2339-2346.

35. Colembergue, J. P., Rios, D. G., & Prentice, C. (2016) Evaluation and characterization of protein isolates from poultry processing industries by-product. *International Journal of Applied Science and Technology,* 6**,** 79-87.

36. Zhao, W., Yang, R., Zhang, Y., & Wu, L. (2012) Sustainable and practical utilization of feather keratin by an innovative physicochemical pretreatment: high density steam flash-explosion. *Green Chemistry,* 14**,** 3352-3360.

37. Alahyaribeik, S., & Ullah, A. (2020) Methods of keratin extraction from poultry feathers and their effects on antioxidant activity of extracted keratin. *International Journal of Biological Macromolecules,* 148**,** 449-456.

38. Kshetri, P., Roy, S. S., Sharma, S. K., Singh, T. S., Ansari, M. A., Prakash, N., & Ngachan, S. (2019) Transforming chicken feather waste into feather protein hydrolysate using a newly isolated multifaceted keratinolytic bacterium Chryseobacterium sediminis RCM-SSR-7. *Waste and Biomass Valorization,* 10**,** 1-11.

Figure captions

Fig. 1 Chicken feather degradation by bacterial isolate KB1 (a) control (without bacteria) and (b) solubilized feather by bacterial isolate KB1 in a minimal basal media

Fig. 2 Phylogenetic relationship of the 16S rDNA sequence of keratinolytic bacteria KB1 with 16S rDNA of closest *Bacillu*s species

Fig. 3 (a) 3-D response surface plot showing the interactive effect of initial pH, initial feather concentration, and temperature on the production of total soluble protein

Fig. 3 (b) 3-D response surface plot showing interactive effect of initial pH, initial feather concentration and temperature on the degree of feather degradation

Fig. 4 Fourier-transform infrared (FTIR) spectrum of feather protein hydrolysates

Fig. 5 *In vitro* % inhibition of DPPH antioxidant activity of feather protein hydrolysates

Figure 1

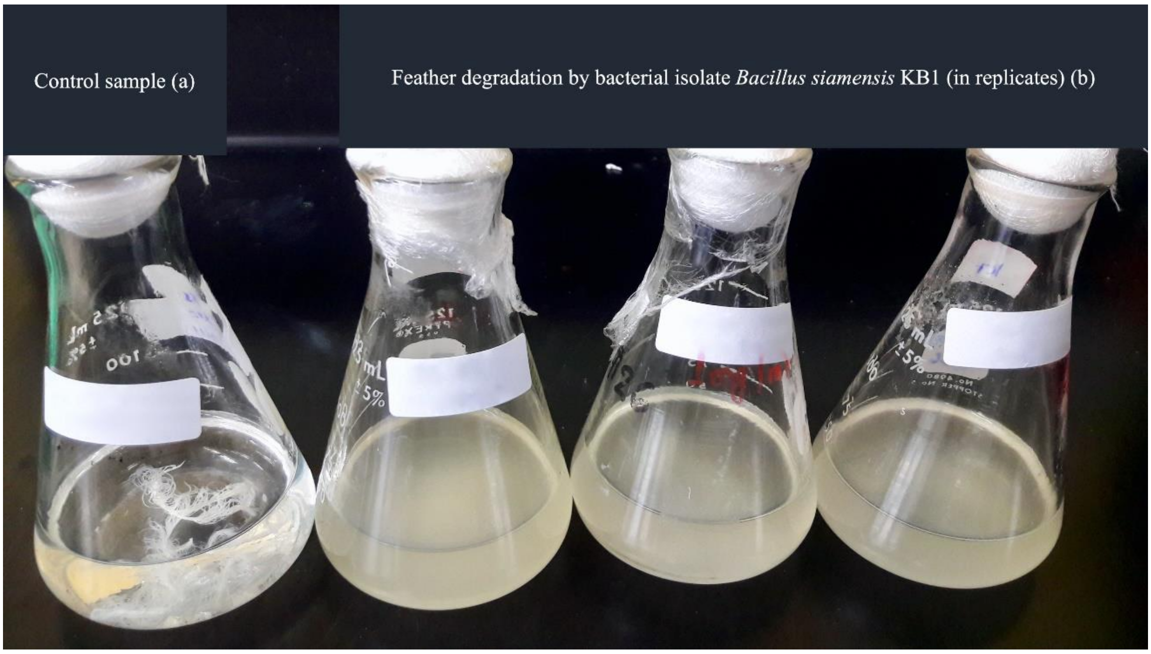


Figure 2

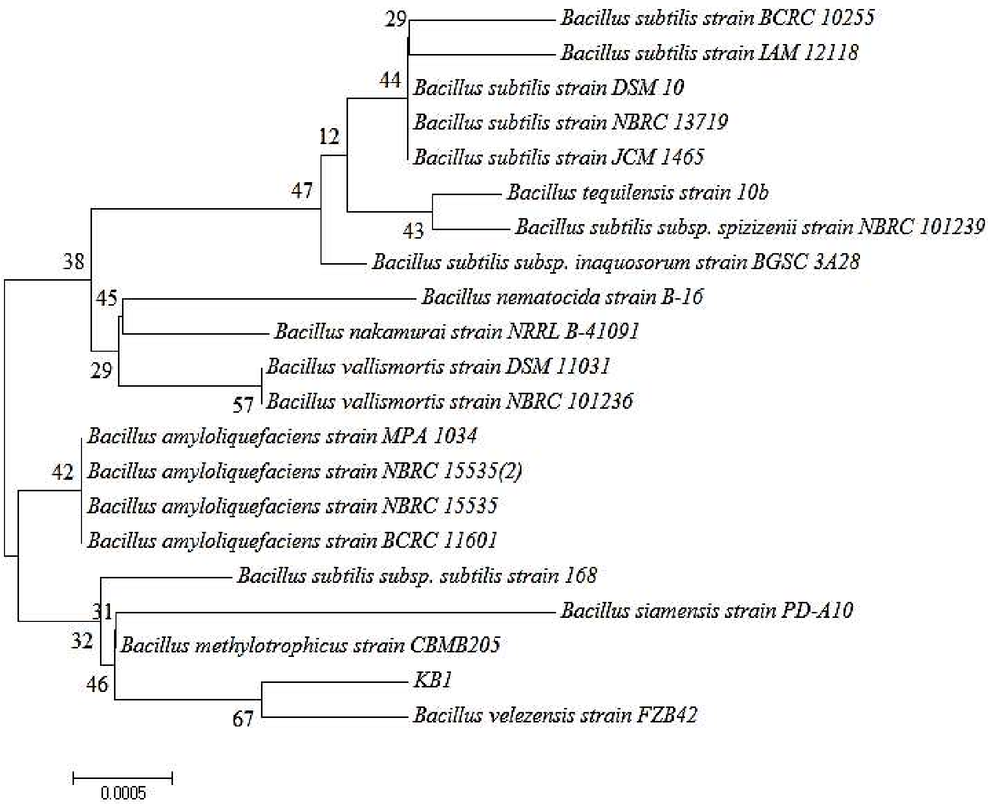


Figure 3 (a)

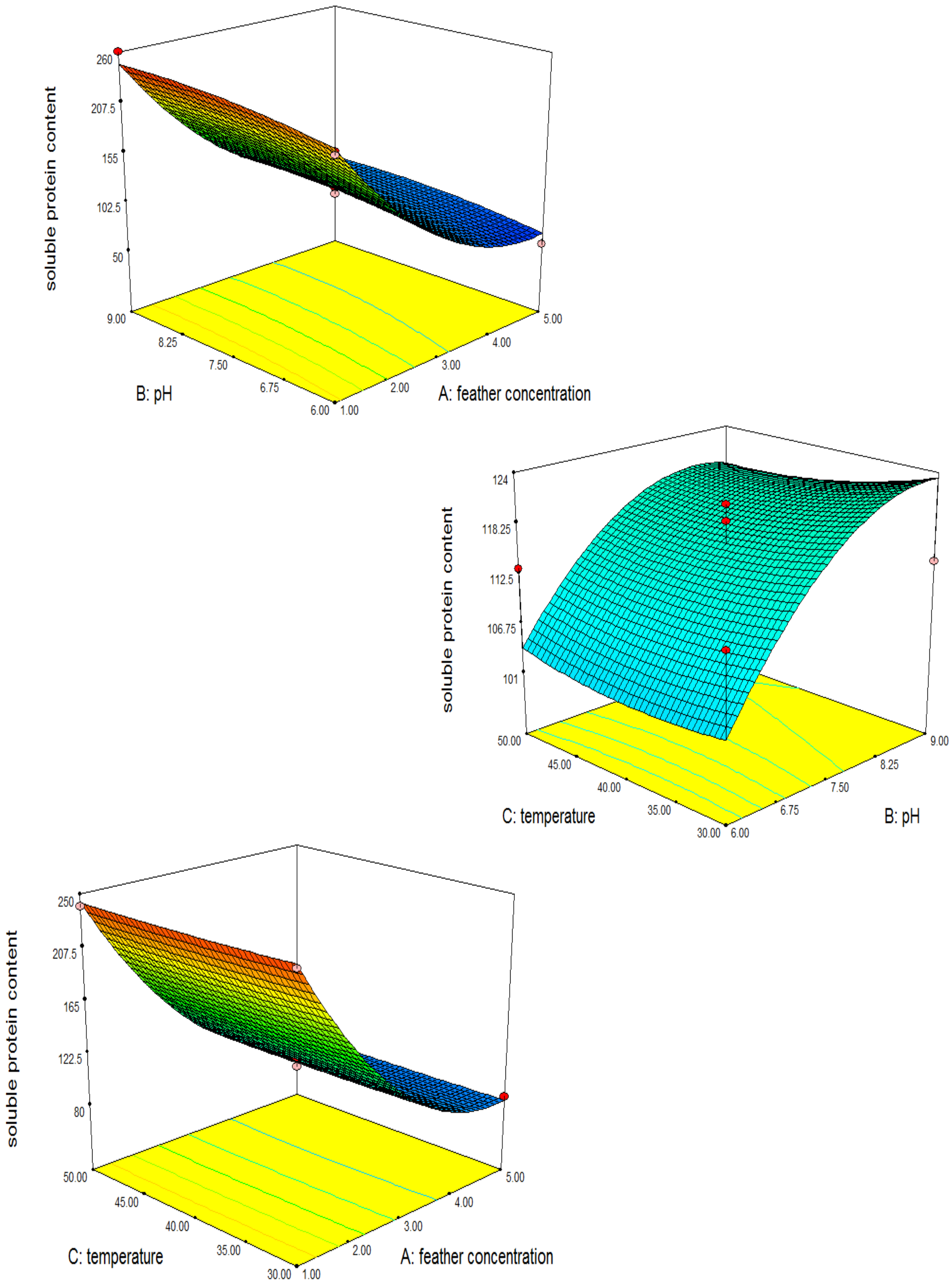
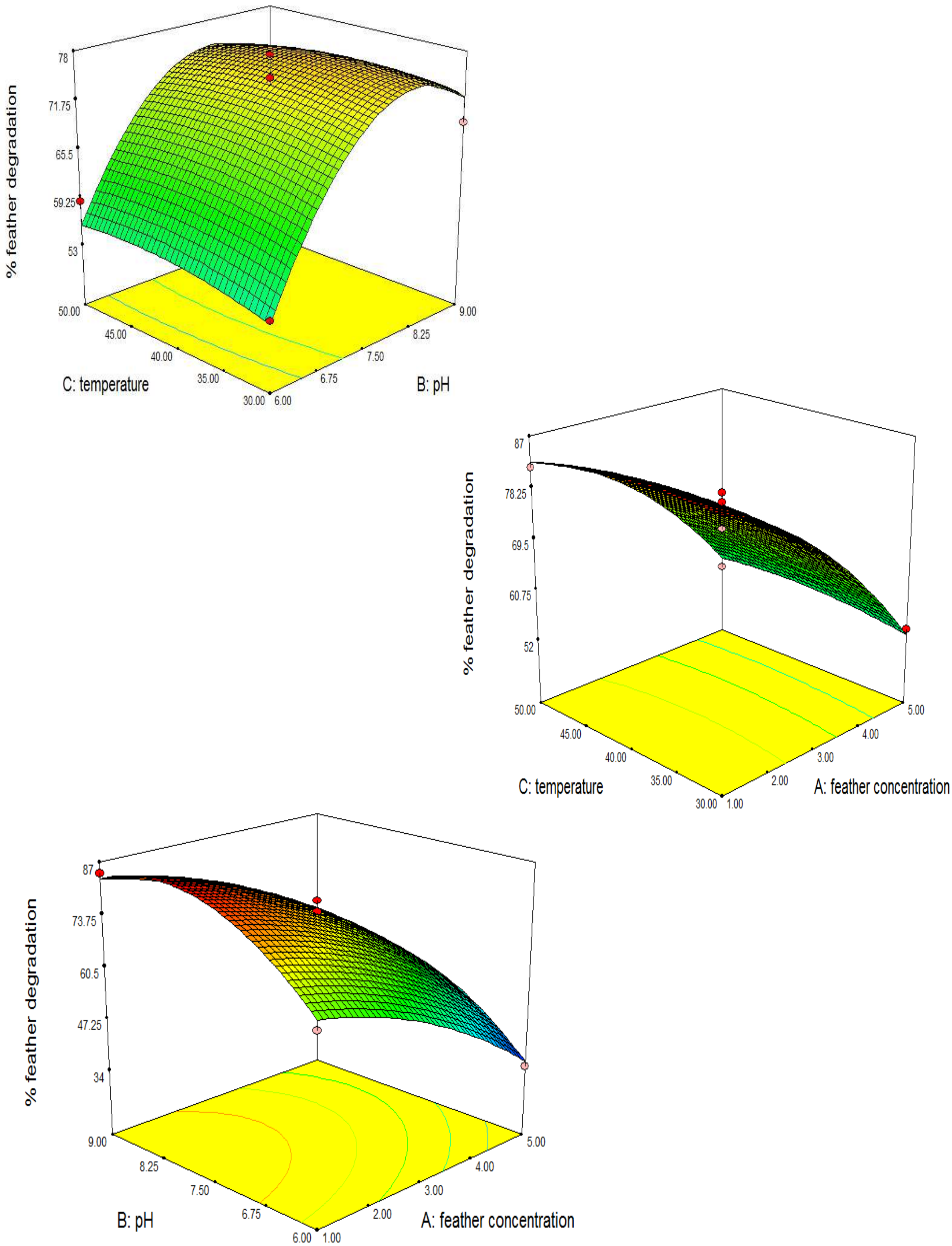

soluble protein content
260
207.5
155
102.5
50
9.00
8.25
7.50
6.75
6.00
B: pH
1.00
2.00
3.00
4.00
5.00
A: feather concentration
soluble protein content
124
118.25
112.5
106.75
101
50.00
45.00
40.00
35.00
30.00
C: temperature
6.00
6.75
7.50
8.25
9.00
B: pH
soluble protein content
250
207.5
165
122.5
80
50.00
45.00
40.00
35.00
30.00
C: temperature
1.00
2.00
3.00
4.00
5.00
A: feather concentration

Figure 3 (b)



% feather degradation
78
71.75
65.5
59.25
53
50.00
45.00
40.00
35.00
30.00
C: temperature
6.00
6.75
7.50
8.25
9.00
B: pH
% feather degradation
87
78.25
69.5
60.75
52
50.00
45.00
40.00
35.00
30.00
C: temperature
1.00
2.00
3.00
4.00
5.00
A: feather concentration
% feather degradation
87
73.75
60.5
47.25
34
9.00
8.25
7.50
6.75
6.00
B: pH
1.00
2.00
3.00
4.00
5.00
A: feather concentration

Figure 4

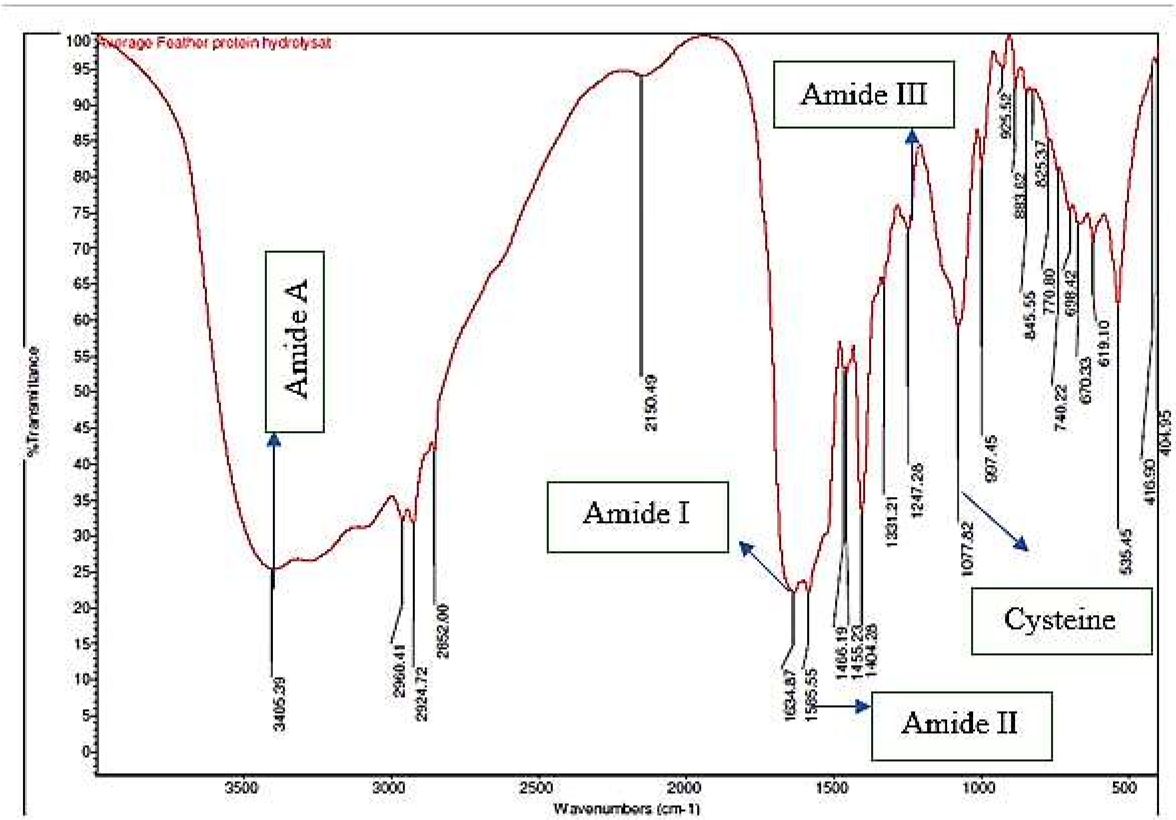
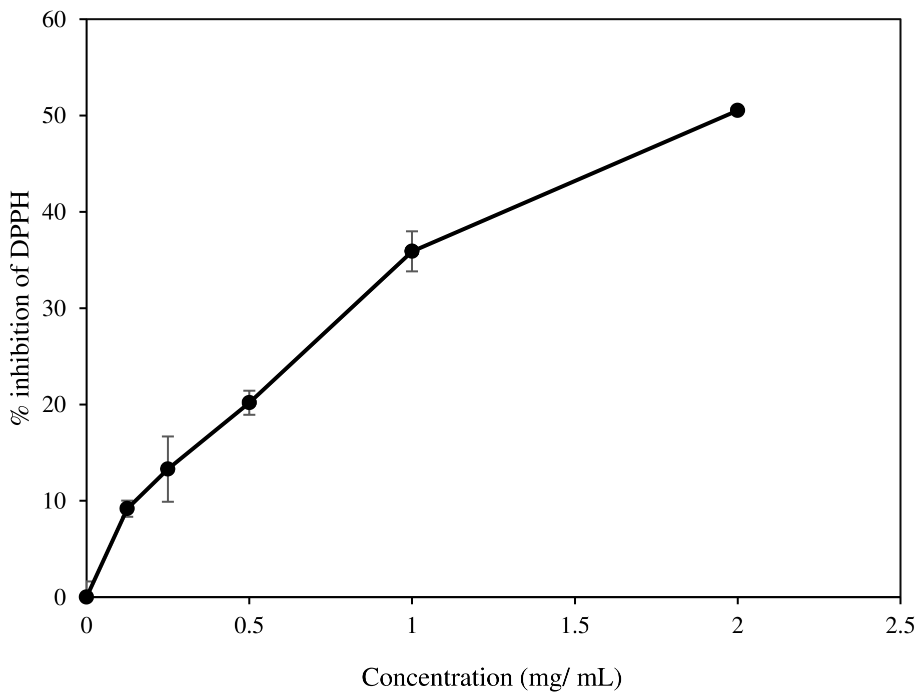


Figure 5



Tables

Table 1. Degree of feather degradation (DFD) and total soluble proteins (TSP) of bacterial isolates from chicken farm bed.

Bacterial isolates	DFD (%)	TSP (mg/g)
KB1	74.78 ± 2.94^{a}	205 ± 0.03^{a}
KB2	11.1 ± 1.23^{f}	39 ± 0.06^{e}
KB3	16.06 ± 0.66^{def}	49 ± 0.04^{cde}
KB4	57.48 ± 5.32^{b}	150.72 ± 0.04^{b}
KB5	22.32 ± 1.88^{d}	61 ± 0.03^{c}
KB6	30.46 ± 0.98^{c}	50 ± 0.06^{edc}
KB7	20.4 ± 1.92^{de}	54 ± 0.01^{dc}
KB8	15.43 ± 2.15^{ef}	45 ± 0.03^{ed}

Values are the means of three replications of a sample ± SD. Different superscripts alphabet (a-f) used in the same column represent significant difference ($p < 0.05$)

Table 2. Box-Behnken experimental design with experimental and predicted values for soluble protein concentration and degree of feather degradation.

Run order	Independent variables			Response variables	
	X1	X2	X3	TSP (mg/g)	% DFD
1	1	9	40	260.0	86.16
2	3	7.5	40	120.43	77.51
3	1	6	40	220.30	63.60
4	5	9	40	90.46	55.45
5	5	6	40	56.20	34.91
6	3	9	30	114.03	69.07
7	3	9	50	110.00	69.72
8	3	7.5	40	118.50	74.60
9	1	7.5	30	242.10	84.25
10	5	3	30	86.42	53.73
11	3	6	50	113.16	58.76
12	1	7.5	50	240.10	81.80
13	5	7.5	50	85.96	53.0
14	3	6	30	112.16	53.0
15	3	7.5	40	111.53	71.39

Where X1, X2 and X3 are initial feather concentration (% w/v), pH and temperature (^{o}C) respectively.

Table 3. Physico-chemical composition of raw feathers and feather protein hydrolysates.

Composition (%)	Raw feathers	Feather protein hydrolysate
Protein	80.73 ± 1.53	78.45 ± 0.38
Fat	2.30 ± 0.05	0.034 ± 0.06
Moisture	10.06 ± 0.66	3.54 ± 0.04
Ash	0.83 ± 0.07	10.72 ± 0.04
In vitro digestibility	1.75 ± 0.5	82.36 ± 0.62

Table 4. Amino acid composition of feather protein hydrolysates.

Amino Acid	Composition (mg/ 100 g protein in feather protein hydrolysates (FPH))
Alanine	2.57
Arginine	2.61
Aspartic acid	5.51
Cystine	4.20
Glutamic acid	7.73
Glycine	4.93
Histidine*	0.86
Hydroxylysine	ND
Hydrosyproline	ND
Isoleucine*	3.54
Leucine*	6.25
Lysine*	3.78
Methionine*	0.99
Phenylalanine*	5.13
Proline	5.84
Serine	4.50
Threonine*	2.17
Tryptophan*	0.81
Tyrosine	4.25
Valine*	5.66

(*) denotes the essential amino acids

ND= Not Detected

Figures

Figure 1

Chicken feather degradation by bacterial isolate KB1 (a) control (without bacteria) and (b) solubilized feather by bacterial isolate KB1 in a minimal basal media

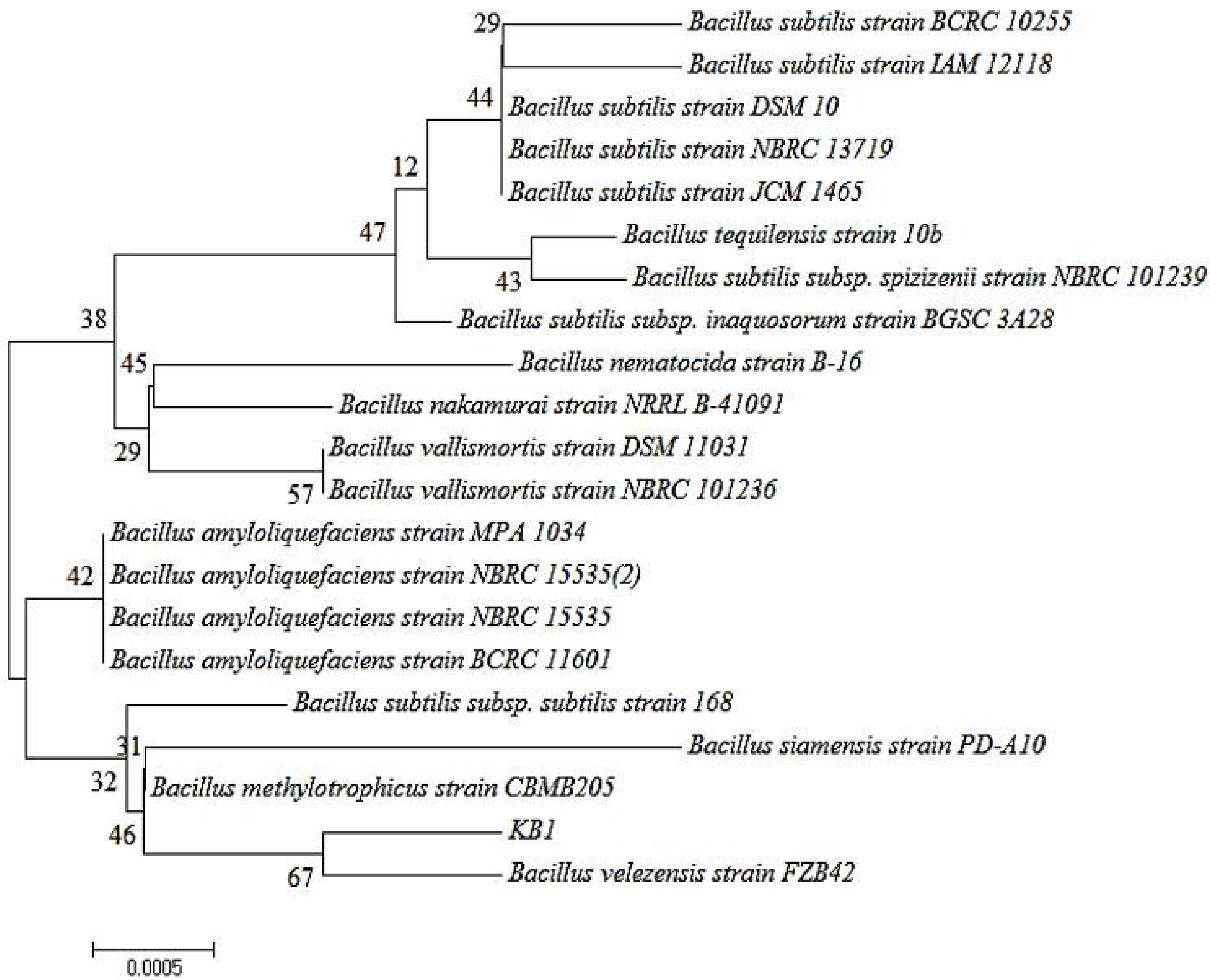


Figure 2

Phylogenetic relationship of the 16S rDNA sequence of keratinolytic bacteria KB1 with 16S rDNA of closest Bacillus species

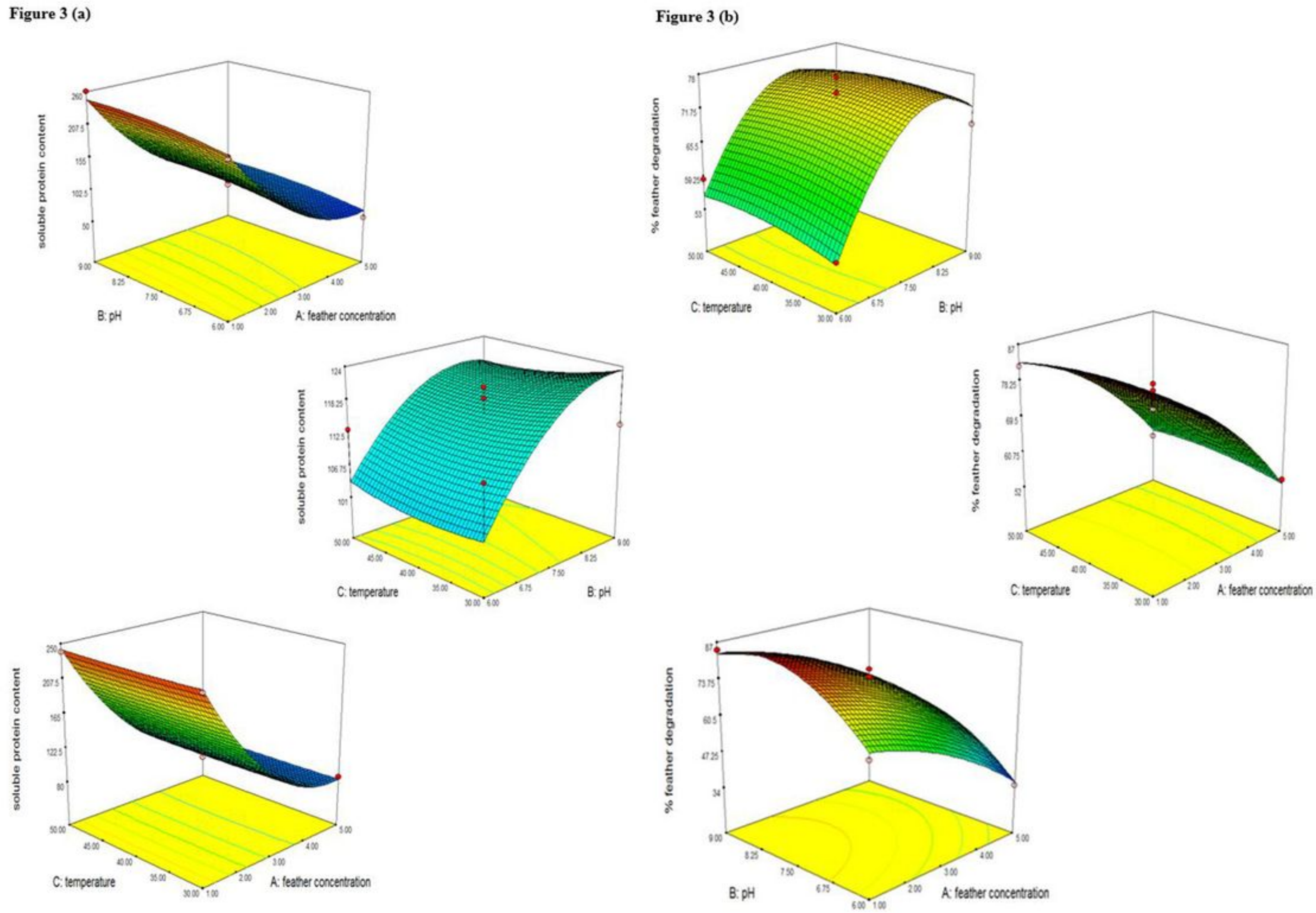


Figure 3

(a) 3-D response surface plot showing the interactive effect of initial pH, initial feather concentration, and temperature on the production of total soluble protein (b) 3-D response surface plot showing interactive effect of initial pH, initial feather concentration and temperature on the degree of feather degradation

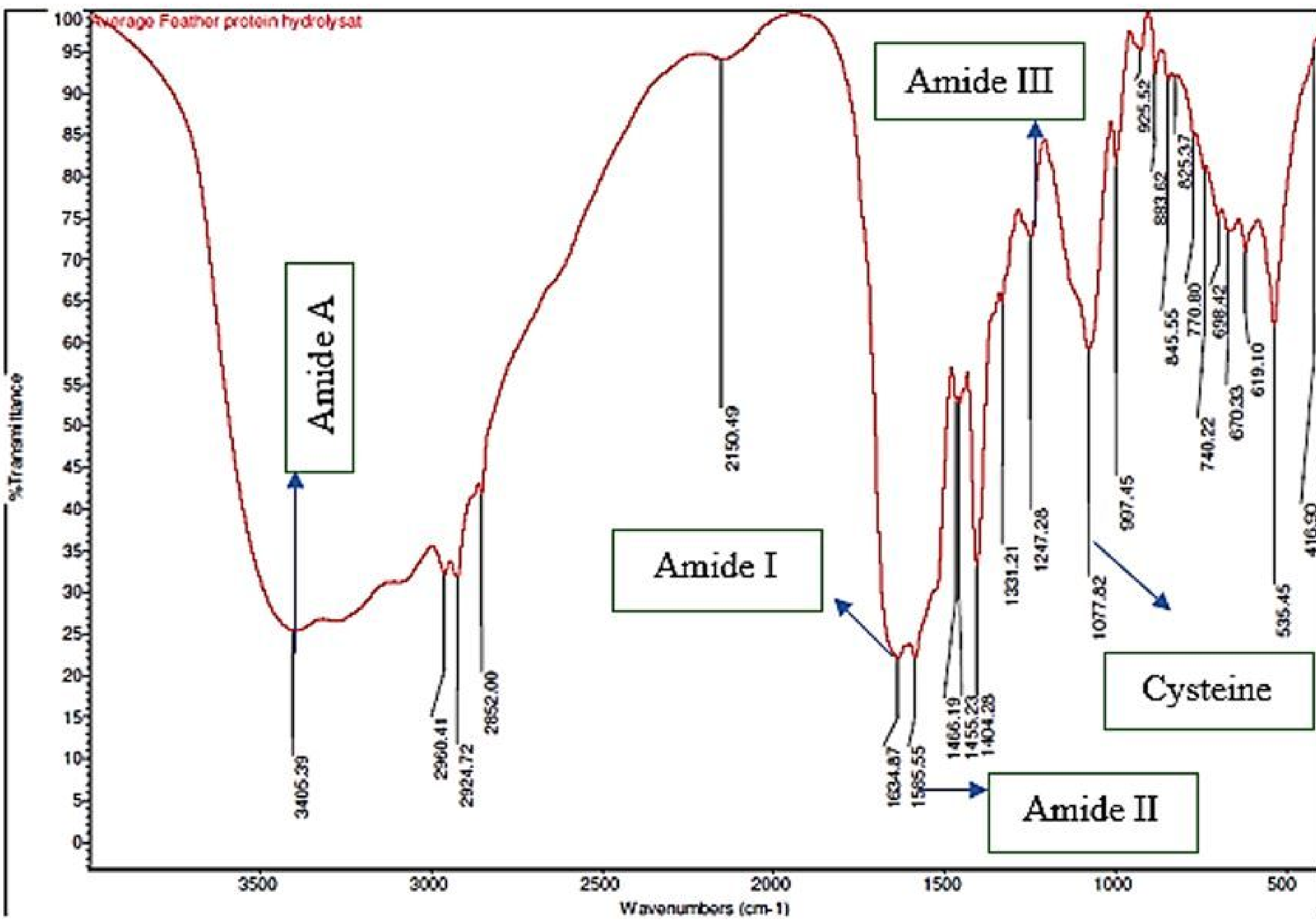


Figure 4

Fourier-transform infrared (FTIR) spectrum of feather protein hydrolysates

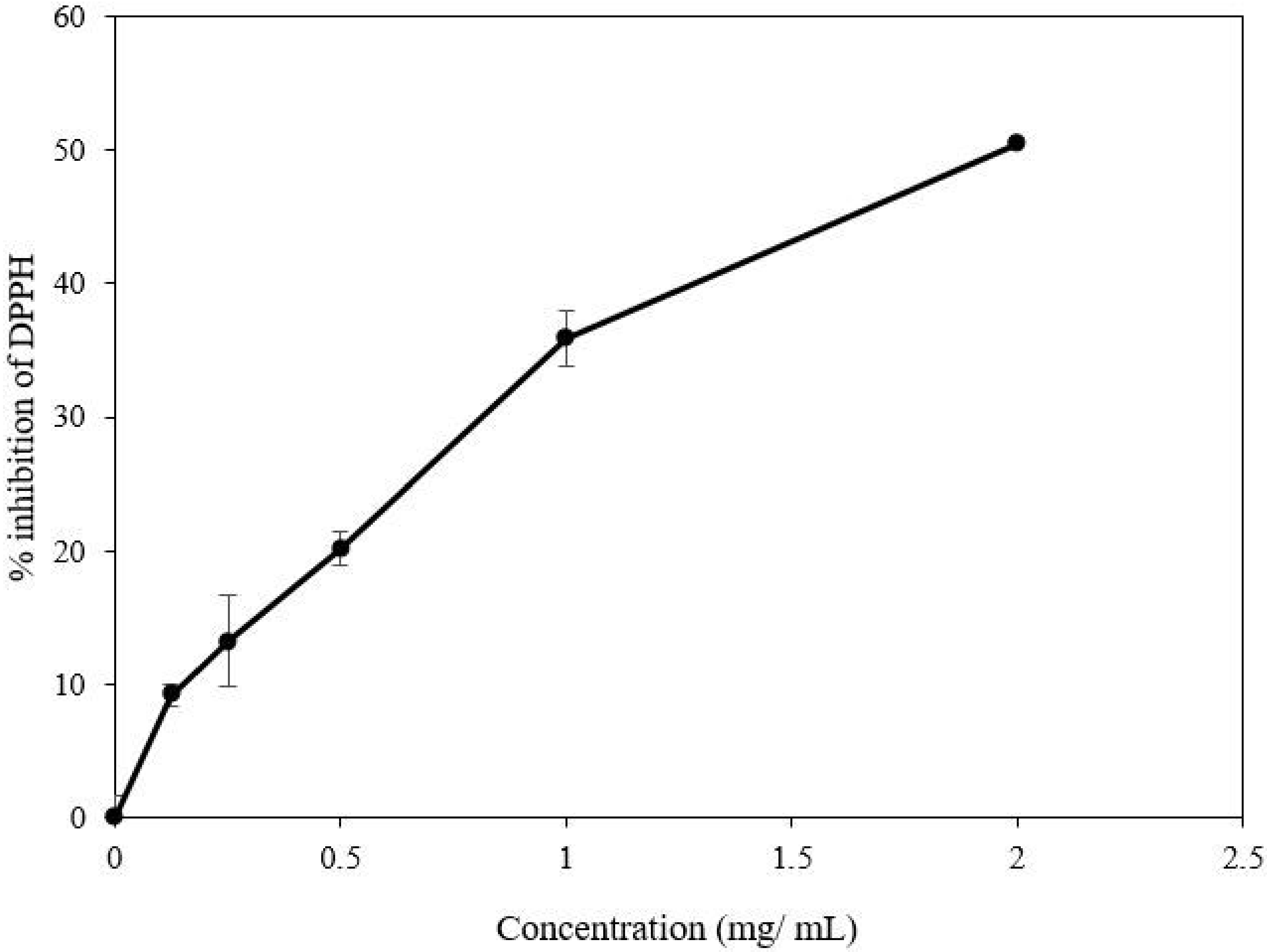


Figure 5

In vitro % inhibition of DPPH antioxidant activity of feather protein hydrolysates

Supplementary Files

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