

Isolation of Keratin Degraders and Development of Feather Hydrolysate

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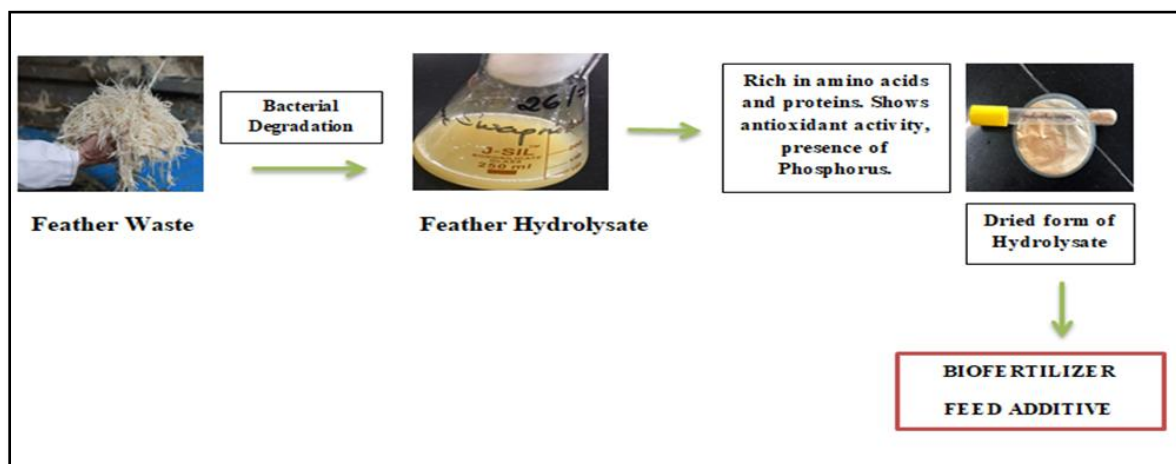
Abstract: Large amount of feather waste is generated every year by poultry processing plant. A small portion of feather waste is steamed, incinerated or chemically treated. Keratin from feathers can be biodegraded by some keratinolytic bacteria which serves as an alternate method. In this study keratin degrading bacteria were isolated from mangrove soil near Thane creek, Mumbai, (India). The production of feather hydrolysate was investigated for its protein content, Nitrogen content, antioxidant activity, invitro digestibility and phosphorus content. Isolate was identified to be *Bacillus cereus* CC-1 which degraded 90% of feather in 4 days. The crude enzyme showed good protease activity (50 µg/ml/min). *Bacillus cereus* CC-1 showed ability to degrade raw feather under optimal temperature of 37°C and pH 8. Also, Feather hydrolysate showed high protein content (1.9mg/ml), phosphorus content (5.5µg/ml) and good invitro digestibility. The antioxidant activity of feather hydrolysate was found to be 0.45mg/ml. Finally, the hydrolysate was checked as a biofertilizer and significant results were achieved.

Keywords: Keratinolytic bacteria, Feather hydrolysate, Antioxidant, Invitro digestibility, Mangroves.

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Graphical Abstract



I. Introduction

Being a structural protein that is fibrous in nature, keratin is rendered with low solubility in water and numerous inorganic solvents. Keratin is majorly present in vertebrate exoskeleton such as hair, feathers, nails, horns, claws, hide, skin, wool, scales. It makes the outer coat of human and animal organs thus preventing the loss of body fluids. About 5 – 7% (w/w) of total weight of mature chickens represents feathers and are generated in large amounts as a waste product thus reaching millions of tons per year worldwide [Manczinger et al. 2003]. The waste feathers generated are generally incinerated, buried or used as land fillings, which causes problem for storage, foul odor and ash disposal. Various human ailments including Chlorosis (RBC), Mycoplasmosis and Foul cholera (Avians) have resulted due to discarded feathers [Williams et al. 1990]. Keratin consists of extensive disulphide bonds and cross linkages which makes degradation difficult. [Kaluzewska et al. 1991]. Cysteine bridges which are cross linked confers high mechanical stability and resistance to proteolytic degradation by papain, trypsin and pepsin. However keratin can be degraded by a class of serine protease [Williams et al. 1990]. Two enzymes are involved in degradation – Disulfide reductase and Serine protease. These enzymes work in co-operative manner. First, Disulfide reductase reduces the disulfide bonds between keratin and later Serine protease (keratinase), an inducible enzyme breaks down keratin and release free amino acids [Yamamura et al. 2002] like cystine, lysine, proline and serine. Keratinolytic enzymes are widely used in various biotechnological process which includes dehairing of hides in leather industries, detergent additive for removal of stains. After hydrolysis of feather keratin, the hydrolysate can be used as feedstuffs or converted to glues, films and is also a source of rare amino acids such as cystine, proline and serine [Gupta et al. 2006].

The present study is aimed at isolating keratin degraders from mangrove soil thinking that the microflora of mangrove lives in stressful conditions and are resistant to various parameters and thus the degradation rate as compared to other bacteria would be higher. The study also focuses on development of feather hydrolysate using chicken feathers as source of carbon and nitrogen. Further the hydrolysate was characterized for its protein content, Nitrogen, Carbon, Hydrogen, phosphorus and for its antioxidant potential and invitro digestibility. The study also aims at using the developed feather hydrolysate as a biofertilizer.

II. Materials and Methods

2.1 Collection of waste feathers

Waste Feathers were collected from nearby Poultry shop. Feathers were washed with tap water to remove the dirt, blood stains and intact feathers were dried in Hot air oven at 55° C for 24 hours and then were stored at room temperature prior to microbial treatment.

2.2 Isolation of keratin degrading bacteria

250g of Soil sample was collected from Mangrove area near Thane creek, Mumbai in a clean ziplock bag. 0.5g of soil was suspended in sterile peptone broth (20ml) for 2 days. The suspension was then reinoculated in sterile Feather meal media containing NaCl, K₂HPO₄, KH₂PO₄, MgCl₂, Feathers 1%, pH – 7. The flask was incubated at room temperature (30⁰C) on rotary shaker (120 rpm) for 7 days and the growth of bacteria in the form of turbidity was observed.

2.3 Characterization and molecular identification of bacteria

Bergey's manual of systemic bacteriology was used for preliminary characterization of isolated strain and 16S rRNA analysis for authentication of strain. DNA sequencing was done in highly automated gene sequencer. Sequencing was carried out with 8F primer using BDT v3.1 cycle

sequencing kit on ABI 3730xl Genetic analyzer. Further the sequence was used to carry out BLAST with the database of NCBI genebank databases.

2.4 Production of feather hydrolysate

The Optical Density of Isolated colony was adjusted to 0.5 at 530nm and then was inoculated in sterile feather meal media and incubated for 4 days at Room temperature (30°C) under shaker condition (120 rpm). The broth was then centrifuged at 10000 rpm for 10 minutes to remove the cell debris and cell free extract was used as feather hydrolysate for determination of protein content, keratinolytic activity, antioxidant potential, protease activity and phosphorus content.

2.5 Estimation of protein content and detection of amino acids

The amount of soluble protein in the culture filtrate was quantified by method explained by Lowry et al. (1951). The amino acids present in feather hydrolysate were determined using paper chromatography.

2.6 Antioxidant assay

Quantitative determination of antioxidant potential was done by Total Antioxidant Capacity Assay which is a spectroscopic technique. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of green phosphate molybdate complex at acidic pH. Ascorbic acid standards were prepared containing concentration varying from 4 – 20 µg into series of test tubes and made up to 2ml with distilled water. The samples were then combined with 2ml of phosphomolybdenum reagent containing 6M H₂SO₄, 28Mm sodium phosphate and 4mM ammonium molybdate. The tubes were capped and incubated in boiling water bath for 90mins. Absorbance was measured at 695nm against blank. A typical blank solution had 2ml of reagent solution and 4ml of water. For samples of unknown composition, antioxidant assay was performed in the same way and the values were determined with the aid of standard graph.

2.7 Protease assay

2.7 A Qualitative method

The colony obtained from enrichment was isolated on milk agar plate to see protease activity in the form of clearance around the colonies.

2.7 B Quantitative Method

Protease activity was carried out by modified method of Tsuchida et al (1986) by using Casein as a substrate. The color developed by addition of ninhydrin reagent was recorded by taking absorbance at 530nm.

2.8 CHN (carbon, hydrogen, nitrogen) analysis

CHN analysis was done using CHN Analyzer based on the principle of Dumas Method. This technique involves complete oxidation of sample by flash combustion. The combustion products are separated using chromatographic column further the concentration of individual component is detected by thermal conductivity detector.

2.9 Effect of pH and temperature on the rate of degradation

Optimum pH and Temperature plays an important role in degradation of feathers. Two sets of feather meal media were made one for pH and other for temperature. *Bacillus cereus* CC-1 OD was adjusted to (0.5) at 530nm was inoculated in Sterile Feather meal media with varying pH from 5 to 8

and other set was held at different temperature - 4°C, Room temperature (30°C), 37°C, 55°C and 100°C. The amount of amino acid released in supernatant was measured using Ninhydrin Test. The concentration was determined using methionine as a standard.

2.10 Phosphorus estimation

Phosphorus in the sample can be quantified colorimetrically. Soluble Phosphorus is converted to hetero complex in the presence of molybdate ions, which is reduced in presence of stannous chloride to give blue color, which is measured at 660nm. Potassium dihydro phosphate (KH₂PO₄) standards were prepared containing concentration varying from 20 – 100 µg/ml into series of test tube and made up to 10ml with distilled water. The sample was then combined with 4ml of Ammonium molybdate and SnCl₂. The tubes were shaken and incubated at Room temperature for 15 mins. Absorbance were measured at 660nm using a Blank. For samples of unknown composition, phosphorus estimation was performed in the same way and the values were determined with the aid of standard graph.

2.11 In vitro digestibility of feather hydrolysate.

In vitro digestibility of the sample was done adding pepsin and pancreatin to the sample. 2mg/ml of pepsin was added to 1g of sample and was incubated for 2 hrs in 2M HCl. After incubation the pH was adjusted to 8 using Sodium Taurochlorate and 2M NaHCO₃. 2mg/ml of Pancreatin was added and the reaction mixture was incubated at 37 for 16 hours. The amount of soluble protein released from the hydrolysate and intact feathers (control) were compared.

2.12 Use of Feather hydrolysate as a biofertilizer

In this experiment three groups were made in triplicates. 1 – Control (C) where seeds were sown in garden soil, 2 – Test (T) containing feather hydrolysate as biofertilizer and 3 - Positive control, Chemical fertilizer (CF) where urea was applied to the soil. 1g of feather hydrolysate was applied to 200g of soil similarly 1g of urea was applied to 200g of soil. Various parameters were studied which include root height, shoot height, Number of leaves and plant weight and the three groups were compared. Four Ground nut seeds were planted in each pot.

2.13 Statistical Analysis

Statistical analysis was carried out using one way analysis of Variance (ANOVA) with Tukey HSD post hoc test. GraphPad Prism 7.03 software was used for all statistical analysis.

III. Results and Discussions

3.1 Isolation, Screening and identification of keratin degraders

0.5g of soil sample was suspended in sterile peptone broth (20ml) for 2 days. The suspension was then reinoculated in sterile Feather meal media. Eight colonies were obtained when plated on media. All eight colonies were then separately inoculated in feather media and rate of degradation was checked. Colony 4 was found to be best feather degrader and it degraded feather in 4 days (Fig 1). After performing biochemical tests and 16 S rRNA sequencing the isolate was found to be *Bacillus cereus* CC-1. *Bacillus* strain had been widely utilized for enzyme production, including the keratinases which degrade feathers [Lal et al. 1999; Gessesse et al. 2003]. Other *Bacillus* spp include *B. licheniformis*, *B. pumilus* and *B. halodurans* [Williams et. al, 1990; Kim et. al, 2001 and Gessesse et. al, 2003] had also been reported for their keratinolytic activity against chicken feathers. Among all these strain *Bacillus cereus* CC-1 isolated from mangrove soil in the present study degraded feathers

much faster than any other reported, except for *B. licheniformis* RG1 which degraded feathers completely in 24 hours [Ramnani et. al, 2004].

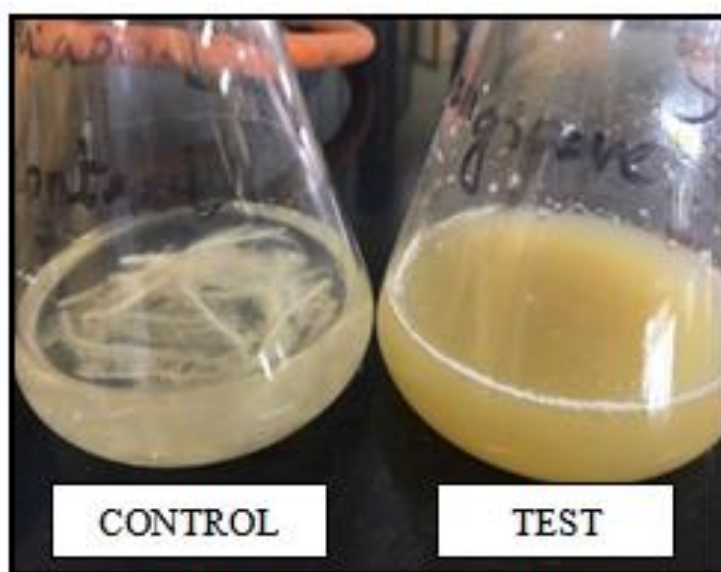


Fig No 1. – Degradation of Chicken feathers by *Bacillus cereus* CC-1

3.2 Determination of Protein content and Identification of amino acids in hydrolysate

Total Soluble Protein content of feather hydrolysate was determined using Folin Lowry method. Bovine Serum Albumin was used as standard for quantification of unknown sample and Amino acids in the hydrolysate were determined using Paper chromatography. Protein content was found to be 1.9 mg/ml (Fig 2) and amino acids were identified as cysteine, methionine, tyrosine, serine, and phenylalanine. Baker et al, 1981 reported that feathers contain large amount of cysteine, glycine, arginine and phenylalanine. The present study also shows the presence of tyrosine and serine.

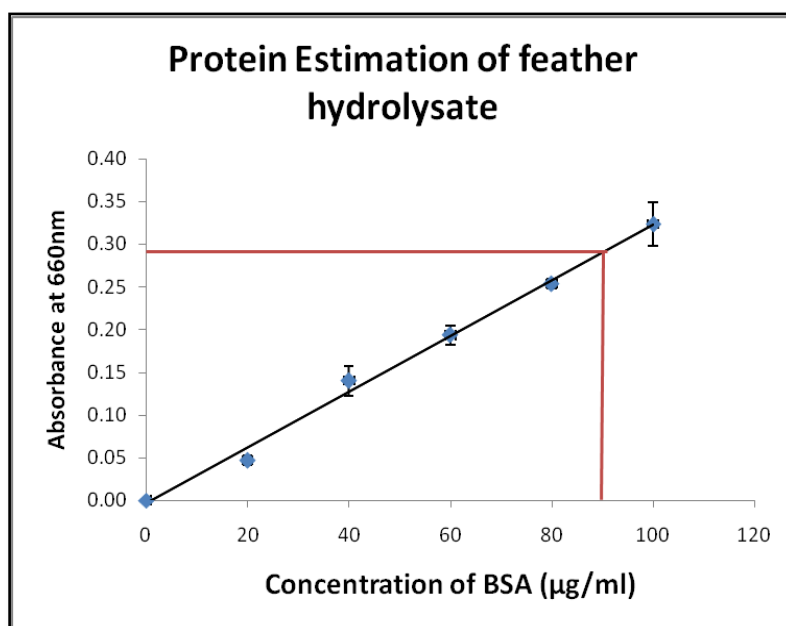


Fig No 2. - Protein concentration of feather hydrolysate

3.3 Antioxidant potential of feather hydrolysate.

Antioxidant in hydrolysate was determined using Phosphomolybdenum assay with Ascorbic acid as the standard. The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and subsequent formation of green phosphate Molybdate complex at acidic pH. The Antioxidant potential of feather hydrolysate was found to be 0.45 mg/ml equivalent of Ascorbic acid (Fig 3). Fakhfakh et al, 2011 found the antioxidant potential by DPPH, which is stable free radical that shows maximum absorbance at 517nm. In presence of proton donating substrate (antioxidant) the radical would scavenge thus reducing the absorbance. This decrease in the absorbance is taken as radical scavenging activity. They reported radical scavenging activity with an IC₅₀ value of 0.3mg/ml. However the values obtained in the present study were found to be greater than the values reported by Fakhfakh et al, 2011.

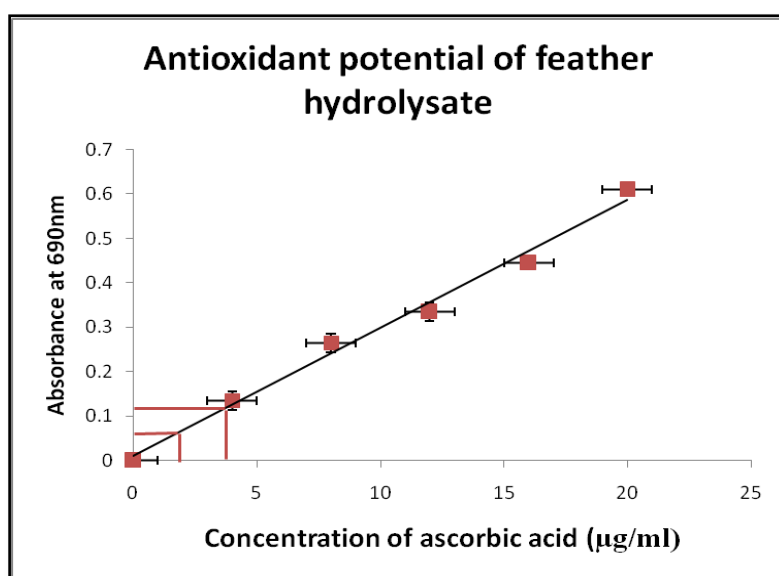


Fig No 3. - Antioxidant potential of Feather hydrolysate

3.4 Protease Activity and Enzyme activity.

Qualitative protease activity of organism was checked by plating it on milk agar plate. Organism was isolated on milk agar plate the plate was incubated at Room temperature for 24 hours. Zone of clearance was observed around the colonies after incubation indicating protease activity (Fig 4). In quantitative assay, Casein was used as substrate which was allowed to react with Crude enzyme for 30mins under buffer condition. The reaction was terminated by adding chilled TCA. Supernatant was reacted with Ninhydrin reagent. The Enzyme activity of crude enzyme was found to be 50µg/ml/min and specific activity was found to be 26.31 U/ml.



Fig No 4. Protease activity of Isolated *B.cereuson* Milk agar plate.

3.5 CHN analysis and Phosphorus estimation of hydrolysate.

CHN analysis was done using CHN Analyzer based on the principle of Dumas Method which involves the complete and instantaneous oxidation of sample by flash combustion and Phosphorus in feather hydrolysate was measured using colorimetric assay. The assay is based on conversion of soluble phosphorus into hetero complex in the presence of molybdate ions, which is reduced in the presence of stannous chloride to give blue color that is measured at 660nm. The carbon, Nitrogen and Hydrogen content of feather hydrolysate was found to be 27.4%, 10.2%, 5.5% respectively (Fig 5) and the concentration of Phosphorus in hydrolysate was found to be 5.5µg/ml (Fig 6).

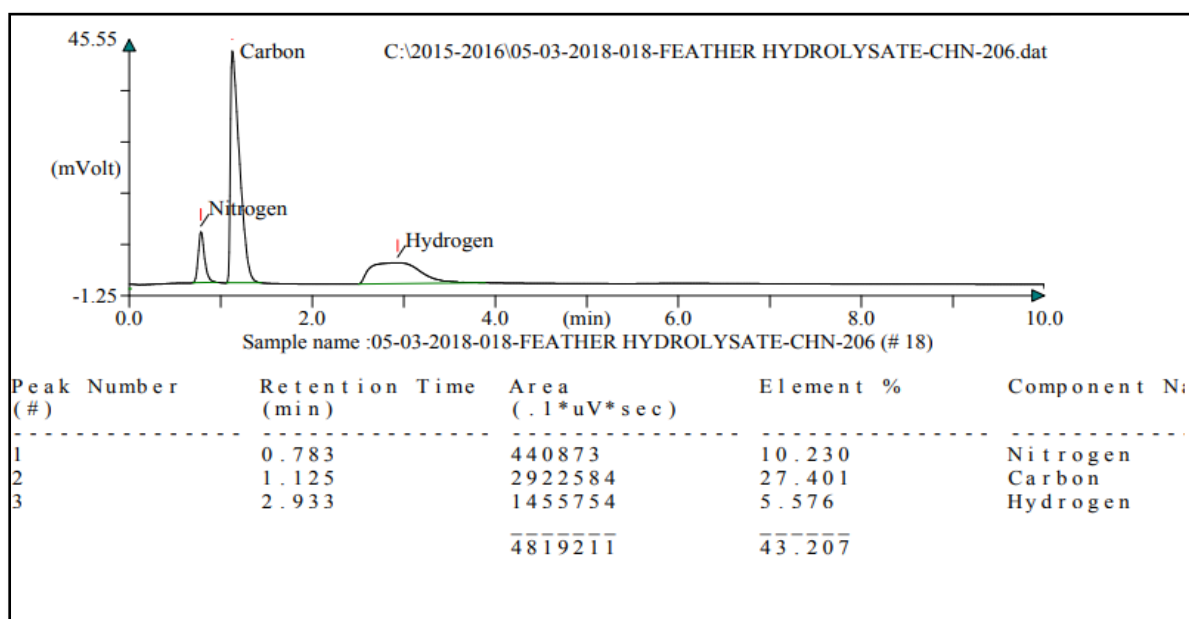


Fig No 5. CHN Content of Feather hydrolysate

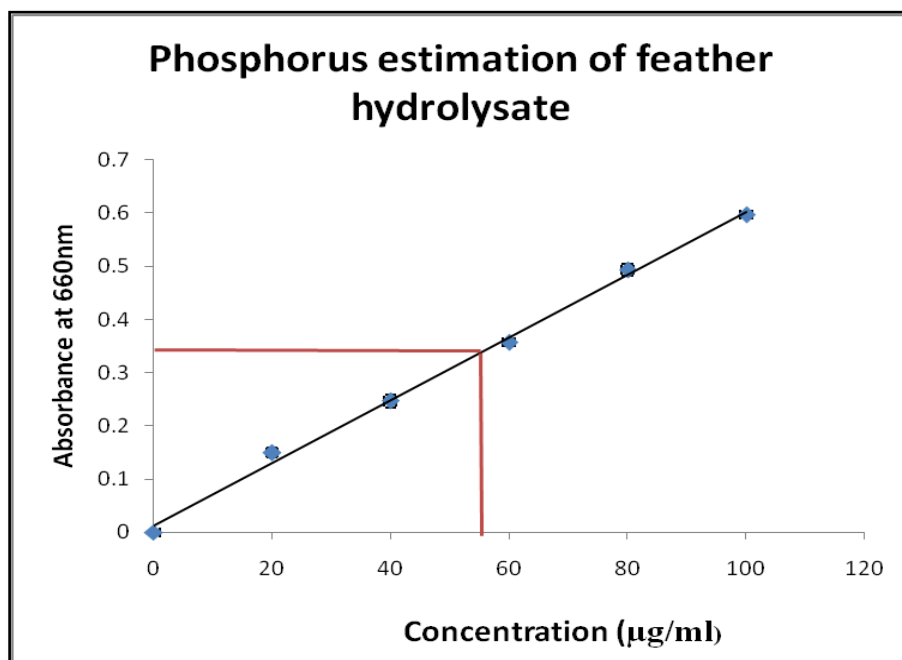


Fig No 6. Phosphorus estimation of Feather hydrolysate

3.6 Effect of pH and Temperature on rate of degradation.

Feather meal media with different pH was inoculated with *Bacillus cereus* CC-1 and incubated at Room temperature for 4 days. In other set the pH was kept constant and media with inoculated *Bacillus cereus* CC-1 was incubated at different temperature. It was found that pH 8 is optimum pH for production of hydrolysate (Fig 7). The results are in line with those published by Suntornsuk et al, 2003 which shows at pH 9, complete degradation of feather by *Bacillus sp* FK46 was achieved. Optimum temperature for the growth of organism and enzyme production was found to be 37°C (Fig 8).

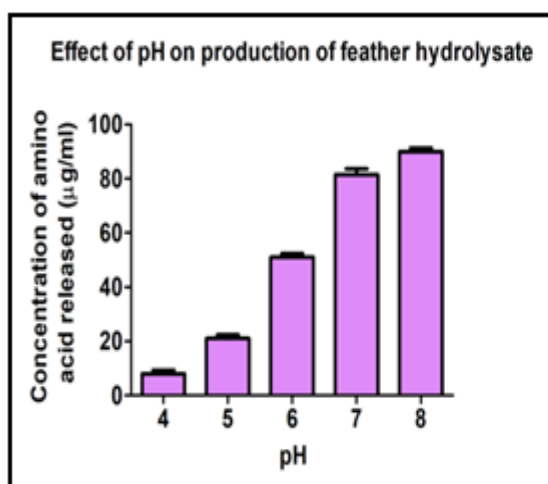


Fig No 7. Amount of amino acid released at different pH after incubation at room temperature for 4 days

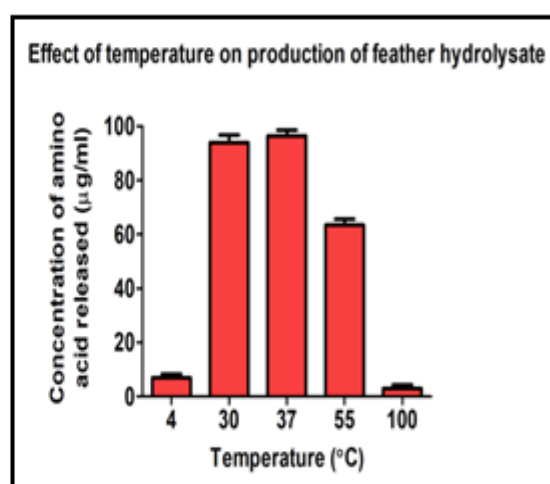


Fig No 8. Amount of amino acid released at different temperature with pH 7 and incubation time of 4 days

3.7 Invitro digestibility test

Invitro digestion test suggests that only 15 µg/ml of protein was released from intact feather whereas 60 µg/ml (Fig 9) of protein was released from Feather hydrolysate indicating good digestive capacity of hydrolysate by stomach and intestinal enzymes. The results obtained are same as those reported by Fakhfakh et al, 2011 who used strain *Bacillus pumilus*A1 and recorded that feather hydrolysate is digested much faster than intact feathers in their study.

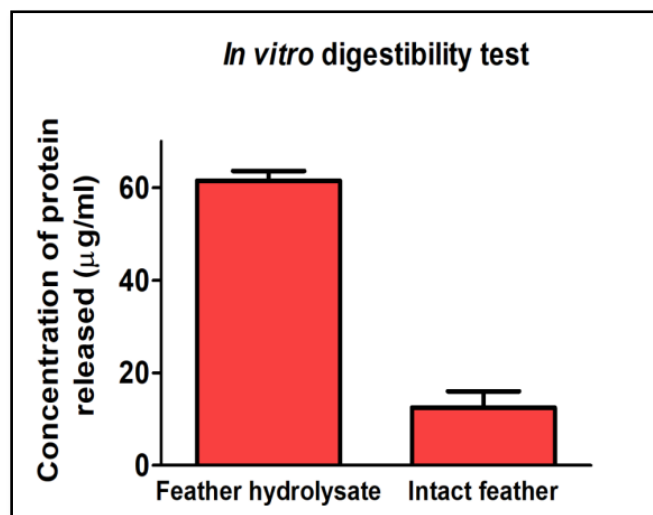


Fig No 9. Amount of protein released after invitro digestion of (A)Feather Hydrolysateand (B)Intact feather

3.8 Feather hydrolysate as a Biofertilizer

Growth pattern of groundnut plant were studied for 21 days. It was seen that plants with Chemical fertilizer (urea) and Organic Fertilizer (Feather hydrolysate) used was almost same. The root length, shoot length and number of leaves were almost same for both but the plant wet weight was higher in case of plants grown with organic fertilizer. It was seen that the organic fertilizer showed a significant influence on the root height, wet weight as compared to the control plant with $p < 0.05$. The organic fertilizer also showed a significant influence on the shoot height and number of leaves as compared to the control with $p < 0.01$. However there was no significant difference between organic and chemical fertilizer for both root and shoot height (Fig 10, 11, 12).

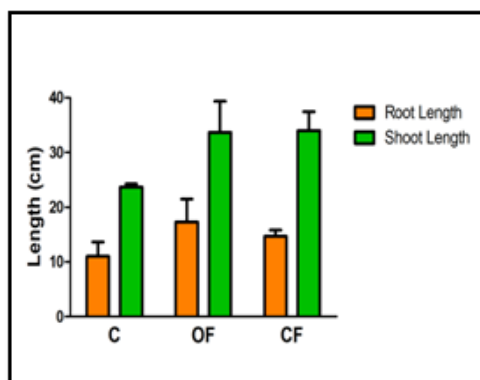


Fig No10. Root length and shoot length of Control(C) plants, plants grown with organic fertilizer (OF) and plants grown with chemical fertilizer (CF).

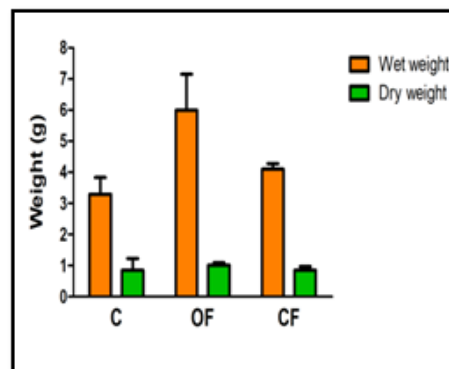


Fig No 11. Wet and Dry weight of Control plants, plants grown with organic fertilizer and plants grown with chemical fertilizer

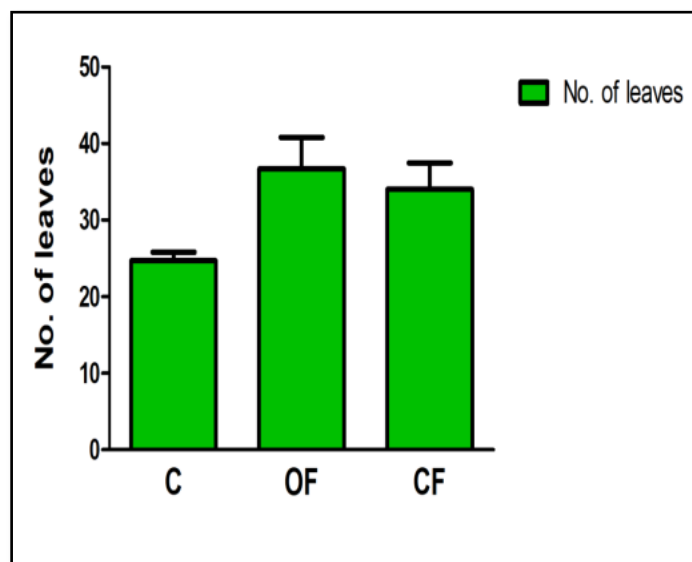


Fig No 12. Number of leaves of Control plants, plants grown with organic fertilizer and plants grown with chemical fertilizer

IV. Conclusion

Bacterial degradation of feathers can be a good choice to derive usable byproducts from feather waste. The present study revealed excellent finding of thermophilic and alkaliphile *Bacillus cereus* CC-1 with good protease (keratinase) activity. Poultry industry use many amino acids for feed formulation for better growth of poultry animals and in this present finding *Bacillus cereus* was able to degrade feathers efficiently and release a significant amount of amino acids which could be positively considered for feather meal production. Apart from high amino acids content feather hydrolysate also shows antioxidant activity which gives an added advantage to be used as feed additive. Chemical fertilizers have several harmful effects which include water pollution, chemical burn to the crops, increased air pollution, acidification of soil and mineral depletion. Therefore there is increasing demand for organic fertilizer. Organic fertilizers are pollution free and nontoxic. Feather hydrolysate contains nitrogen and phosphorus which helps it to be an excellent Biofertilizer.

Acknowledgement

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