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Rapid Conversion of Chicken Feather to Feather Meal Using Dimeric Keratinase from *Bacillus licheniformis* ER-15

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Abstract

Dimeric keratinase from *Bacillus licheniformis* ER-15 completely degraded 25g boiled native chicken feather to feather meal within 8h at pH 8, 50°C and 150rpm. Feather degradation was a linear function of enzyme concentration and 2.5g chicken feather was degraded in presence of 1200U keratinase. Process for feather meal production comprised soaking of 25g feather in 250ml water followed by boiling for 10min-20min before enzyme addition. Feather meal thus produced was dried at 80°C and ground to obtain feather meal powder. Feather meal contained 14% nitrogen, 44% carbon with all essential amino acids and showed 73% *in-vitro* digestibility.

Keywords: *Bacillus licheniformis*; Feather Degradation; Feather Meal, *in-vitro* Digestibility, Keratinase

Introduction

Feather is protein rich waste product of poultry processing industries which are being generated in billion of tons every year [1-4]. These feathers are generally land filled or burnt which cause environmental problems [4]. Feather are also degraded to feather meal which is used as animal feed, organic fertilizers, feed supplements because it is made up of >90% protein and rich in hydrophobic amino acids and important amino acids like cystine, arginine, threonine [5,6]. Most popular method of feather meal production is by hydrothermal process where feather are cooked under high pressure at high temperature. However, hydrothermal treatment, results in destruction of essential amino acids like methionine, lysine, tyrosine, tryptophan and has poor digestibility and low nutritional value [7,8]. In this respect, microbial degradation of feather into feather meal has gained importance and new microbes are being looked upon for efficient degradation of feather. Feather are degraded during fermentation process where consortium of thermophilic/mesophilic bacterial cultures such as *Bacillus, Streptomyces, Vibrio, Chryseobacterium* strains are used [4,6,9]. During fermentation not more than 0.5-2% w/v can be used and also essential amino acids are utilized by micro-organism which decreases the nutritional value of feather meal. To combat this, focus of the research is changing towards developing of enzymatic methods of feather degradation using special class of proteases, the keratinases. Till date not more than 10% feather degradation is reported in the presence of keratinases however a novel dimeric keratinase from *Bacillus licheniformis* ER-15 was observed to degrade feather completely into feather meal [10]. Here various process parameters for enzymatic degradation of feather to feather meal have been standardized. Amino acid analysis and *in-vitro* digestibility of the feather meal is also compared with existing reports.

Material and Methods

Chemicals used for buffer and medium were obtained from Sisco Ranbaxy Laboratory (SRL, India). Soy flour and chicken feather were collected from local market only.

Analytical methods

Keratinase assay: Keratinase activity was measured using 20mg feather, 1ml of properly diluted enzyme and 4ml, pH 10 buffer (50mM Glycine-NaOH buffer). Reaction mixture was incubated at 60°C for 1h and stopped with 4ml of 5% w/v trichloro acetic acid (TCA) followed by incubation at room temperature for 30min and centrifugation at 8000rpm for 10min. Absorbance of supernatant was measured at A280. Similarly, control reaction was set up with 1ml of 5% w/v TCA Enzyme unit was defined as amount of enzyme required to release protein equivalent to absorbance of 0.01 from feather keratin under standard assay condition [2].

Protein determination: Protein in supernatant was measured at A280 and 1 absorbance was considered as 1mg/ml protein using bovine serum albumin as standard.

Production and downstream processing of keratinase from Bacillus *licheniformis* ER-15

Enzyme production: Keratinase was produced in soyflour feather medium in 60h as reported earlier [10]. A loopful bacterial culture was inoculated in 50ml nutrient broth in 250ml Ehrlenmeyer flask, grown at 37°C, 200rpm for 16h and used as seed culture. This seed culture was again grown in nutrient broth for 16h in similar conditions and used as inoculum for production medium. Four hundred ml of production medium in 2L flask (0.4%w/v soyflour, 3%w/v glucose, 0.3w/v KH2PO4, 0.9%w/v K2HPO4, 0.5%w/v feather) was inoculated with 4% v/v inoculum and incubated at 250rpm, 37°C for 60h.

Sedimentation and microfiltration: After 60h of production, fermentation broth was collected in a beaker and kept at room temperature in static condition for 24h. Most of the bacterial cells were settled along with feather meal at the bottom of beaker, supernatant was decanted and micro filtered through 0.2µ filters (MDI, India) using vacuum pump. This micro filtered supernatant was concentrated and used as enzyme.

Enzyme concentration and shelf life: Micro filtered supernatant was concentrated using 85% saturation of ammonium sulphate. Precipitated enzyme was collected after centrifugation, dissolved in pH 7

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phosphate buffer and stored at 4°C. concentrated enzyme was used for feather meal production after required dilution. Enzyme was checked for shelf life for a period of 1 year at room temperature.

Feather processing and feather meal production

Procurement of feather and feather processing: Chicken feather were procured from the local market. Feathers were washed with detergent and detergent was removed by several washing with tap water followed by distilled water. Washed feather were dried at 80°C for 6h and were used for subsequent experimentation.

Standard protocol for feather degradation: Feather (2.5g) was autoclaved at 15psi for 15min in a 250ml flask containing 25ml, 25mM pH 8 phosphate buffers. Volume was made upto 50ml with properly diluted enzyme. Flasks were kept at 150rpm, 50°C for 12h or till specified time. After degradation, feather meal was filtered through 2mm sieve and residual feather were dried at 80°C till constant weight. Percent degradation was calculated on the basis of dry weight. Experiments were set up in triplicate and repeated twice. Data is presented as mean (± SD).

Process Parameters

Effect of enzyme concentration

Effect of enzyme concentration on feather degradation was checked using 150-1500U keratinase on 2.5g feather in 50ml volume, for a period of 12h and percent degradation was studied using dry weight method.

Effect of temperature and time

Feather degradation was studied as a function of time for the period of 2h-12h or till complete degradation at 37°C and 50°C using 2.5g from in 50ml volume and 1200U enzyme under standard conditions. Percent feather degradation, protein release and residual keratinase activity was determined after every 2h. Structural changes in feather were also analyzed by scanning electron microscopy (SEM). Feather were washed with 50mM phosphate buffer and dried at room temperature for scanning studies. Feather were coated with gold particle and observed using scanning electron microscopy (LEO 435VP SEM, Carl Zeiss NTS, GmbH, Germany) at Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India, Department of Science and Technology supported service centre.

Scale-up

Keratinase degradation was further scaled up for degradation of 5, 10, 15 20 and 25g feather in a 2L flask at pH 8 and 50°C under optimized condition (5%w/v feather, 150rpm and 1200U enzyme/2.5g feather).

Pre-soaking and boiling of feather

Pre-soaking and boiling method was substituted for autoclaving. Twenty five gram feather was soaked in 250ml water for 2h and boiled for 10-20 min with intermittent mixing instead of autoclaving in standard protocol. It was cooled to room temperature and 12000U enzyme along with remaining 50% moisture i.e. 250ml pH 8 buffer was added and mixed properly. It was kept at 50°C and 150rpm till complete degradation of feather. Feather meal was dried at 80°C and was ground to form homogenous powder.

Amino acid and CHN analysis of feather meal

One g of feather meal powder was hydrolyzed with 10ml of 6N HCl at 60°C for 12h, filtered. Filtrate was concentrated by speed vac and analyzed for amino acid profiling by HPLC (Agilent 1100 HP-HPLC) after derivatization with orthopthalaldehyde. Sample was run into the Aminex column using mobile phase (A (20mM sodium acetate + 0.018% triethylamine) and B (20% of 100mM sodium acetate + 40% methanol + 40% acetonitrile) with the flow rate of 0.5ml/ min at 40°C and detected at 338nm using VW detector. Amino acids were quantified using HPLC standards at Shanker Nethrayala, Chennai, India.

Carbon, nitrogen, sulfur and hydrogen contents of feather meal were analyzed using 1g feather meal powder at USIC facility, Delhi University, North campus, Delhi, India Using CHNS analyzer (Elementar, Vario El, Germany).

In-vitro digestibility of feather meal

For *in-vitro* digestibility, 1g feather meal was resuspended in 10ml, 2N HCl, 2mg/ml pepsin was added and mixture was incubated 37°C for 2h. Further, pH of the mixture was adjusted to pH 8 by adding 2M sodium bicarbonate and 2mg/ml trypsin was added and incubated at 37°C for 16h [11]. After pepsin and trypsin treatment peptide release was measured at 280nm. Percent digestibility was calculated by total protein released/ total protein of 1g feather.

Results and Discussion

Keratinase from *Bacillus licheniformis* ER-15 was produced in soy flour feather medium for 60h in a 2L flask as reported earlier [10]. Fermentation broth was kept at room temperature for 24h which allowed the settling of >90% bacterial biomass onto feather meal. Feather are made up hydrophobic amino acids which may have facilitated settling of microbial cells on the degraded feather specially the *Bacillus* sp. which is known to produce biosurfactant making the cell surface hydrophobic [1,12]. Sedimentation step can easily substitute centrifugation in downstream processing of fermentation broth of biosurfactant producing microbes.

The keratinase was concentrated with 85% ammonium sulphate saturation which resulted in > 80% enzyme recovery. Concentrated enzyme was stored at room temperature with shelf life of upto a year with almost no loss in activity (data not shown).

Standardization of feather degradation

Keratinase from *B. licheniformis* ER-15 exhibited maximum activity at pH 11 and 70°C [10]. Although, alkaline pH and high temperature would facilitate rapid feather degradation by reducing disulfide bonds [2] but is not often recommended for the direct use of feather meal in feed due to loss of some essential amino acids [3,13]. Therefore, feather degradation was studied at pH 8 and 50°C where present keratinase exhibited >60% activity [10].

Effect of enzyme concentration

Degradation of feather was observed to be a linear function of enzyme concentration with >600U (Figure 1). No visible degradation was observed upto 600U even after prolonged treatment. By increasing enzyme concentration from 600 to 900U, 60% feather degradation was achieved which increased to >90% with dissolution of shaft as concentration was increased to 1200-1500U/2.5g feather. Feather degradation using keratinase have been reported in presence of reducing agents like hypochlorite, dithiothretol, glutathione or in presence of live cells which provides reducing environment [1,14]. Majority of the feather meal production involves fermentation using keratinolytic microbes and subsequent fermentation broth was regarded as feather meal

[2,6,11,15]. During fermentation, feather degradation is supposed to be achieved by co-operative action of protease and cell redox [16-18]. In this context, present process is better than the existing ones since no fermentation is required for feather meal production.

Effect temperature on feather degradation

Feather degradation was studied at 37°C and 50°C and >90% degradation was observed after 24h at 37°C and 8h at 50°C. Further dissolution of shaft was observed only at 50°C. complete degradation at 50°C may be a result of faster breakdown of disulfide bonds at higher temperature which may have resulted in dissolution of shaft [1]. Further, the present enzyme has optimal activity at 70°C with >90% activity at 50°C and 67% at 37°C [10]. Thus, the faster degradation at 50°C may be result of both enzyme concentration and temperature.

Protein release and structural changes during feather degradation

Protein release and structural changes in feather was studied at 50°C (Figure 2). From figure 2, it can be observed that maximum protein was released in the first 2h with only 20% degradation (Figure 2A) and not much visible changes in feather (Figure 2B). First visual observation of feather degradation was made with shedding of barbules after 4h of incubation accompanied by additional 20% loss in weight. However, no substantial release of protein in supernatant was observed as was after first 2h. Similar trend in protein release was observed on further incubation upto 8h where complete degradation of feather was obtained. This suggests that, smaller peptides are released mostly during first 2h of degradation and later on most of the protein remains in the feather meal. This is the first report where keratinase alone could degrade feather completely within 8h. Hydrothermal hydrolysis of degradation also required longer time (16h) and high temperature (120°C) for feather degradation [1]. Further microbial degradation of feather generally requires more than 24h which may extend upto 5 week with the microbe used [2].

During feather degradation enzyme stability was also studied and 80% enzyme activity was recovered at 50°C after 8h which is higher than earlier report where half life was 5h at 50°C. This observation suggests that thermo-stability of present keratinase was enhanced in presence of substrate. This is in confirmation with commonly observed phenomenon that substrate protects enzyme against thermal destabilization [19].

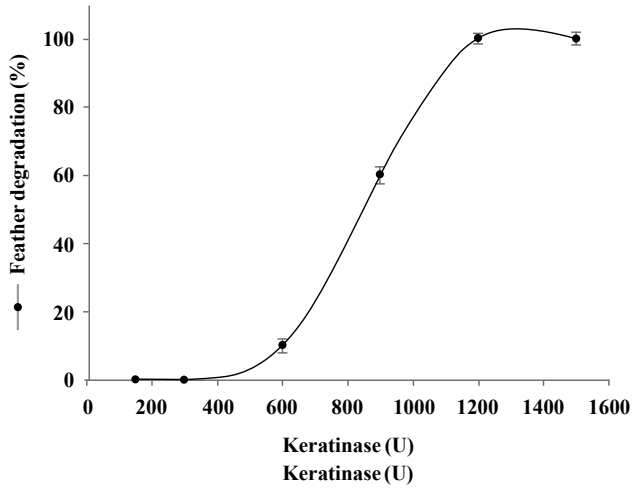

Figure 1: Feather degradation as a function of enzyme
Feather degradation was performed with 250mg feather in pH 8 buffer at 50˚C, 150 rpm for 12h. Percent degradation was calculated by measuring residual feather, dried at 80˚C for 12h.

Scale up

Feather degradation was scaled upto 5-25g feather in 2L flask in standardized condition and >90% degradation was achieved within 12h in >10g feather. Thus, complete feather degradation of feather in large volume was obtained in same condition and process was successfully scaled up.

Presoaking and boiling method

Since, for bulk degradation, 5% w/v feather involves large volume and would lead to cumbersome downstream process therefore pre-soaking and boiling was attempted with 25g feather. Feather was soaked in 250ml water for 2h and approximately 50% absorption of water. This pre-soaked feather was boiled for 10-20min and cooled till room temperature. Further 250ml enzyme (12000U) in pH 8 buffer was added to the boiled feather and >90% feather degradation was achieved at 50°C within 12h. This process formed a thick meal which was dried, ground and stored directly (Figure 3).

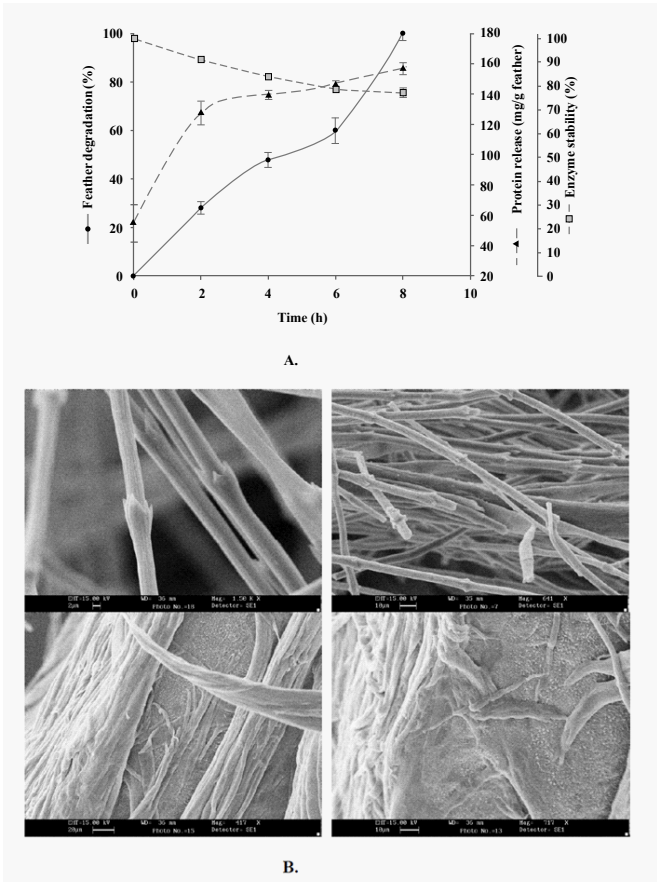

Figure 2: Time kinetics (A) and structural changes (B) of feather during degradation.
A. Feather degradation was performed at pH 8, 50°C and 150rpm till complete degradation.
B. Structure changes of feather at 0h (a), 2h (b), 4h (c) and 6h (d) after enzyme addition.

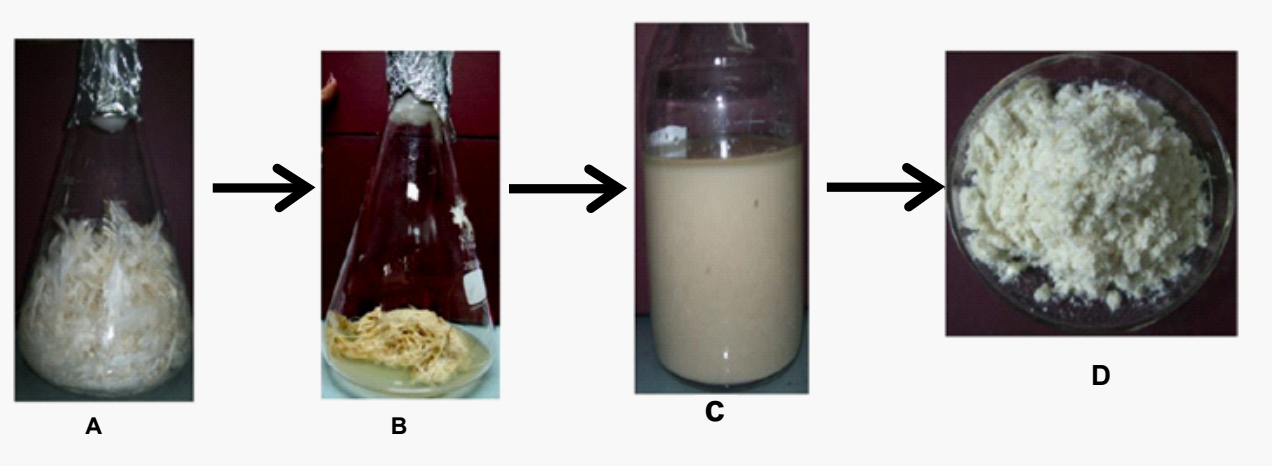

Figure 3: Various step of conversion of feather to feather meal. A. 25g feather before pre-soaking and boiling, B. Feather after pre-soaking and boiling, C. Feather meal after 12h with 12,000U enzyme treatment, D. Dried feather meal.

Amino acid	mg/g of feather (present work)	Steam hydrolyzed mg/g feather (Eggum, 1970)	acid hydrolyzed mg/g feather (Eggum, 1970)	mg amino acid/g CP (present work)	mg amino acid/g CP (Grazziotin et al., 2006)
Aspartic acid	51	-	-	55.85	57.8
Glutamic acid	56.1	-	-	61.45	92.2
Serine	73.8	-	-	80.85	108.3
Histidine	65.9	7.2	6.3	72.15	9.3
Glycine	51.05	-	-	55.9	59.6
Threonine	50.9	4.84	4.87	55.75	36.6
Alanine	46.3	-	-	50.7	54.2
Arginine	32.85	2.08	2.3	35.95	84.3
Tyrosine	37.85	2.8	3.11	41.45	32.9
Valine	33.15	7.25	7.73	36.3	85.6
Methionine	31.6	.72	0.76	34.6	17.0
Phenyl alanine	69.95	4.61	4.85	76.6	54.2
Isoleucine	51.85	4.82	5.55	56.8	62.8
Leucine	17.15	8.25	8.27	18.75	66.9
Lysine	14.85	2.08	2.23	16.25	24.1

Table 1: Amino acid composition of feather meal and comparison with reported feather meals.

Quality of feather meal

Quality of feather meal was checked by CHN analysis as well as amino acids profiling. The feather meal contained 14%w/w nitrogen, 44%w/w carbon, 3.2%w/w sulfur and 1.4%w/w hydrogen which suggests that feather meal is a protein rich meal with 87% protein by weight. Amino acid composition of feather meal is presented in Table 1 and compared with steam cooked, acid hydrolyzed, culture supernatant hydrolyzed (CSH) feather meal [11,20]. Enzymatic hydrolysis of feather meal was observed to be rich in essential amino acids in comparison to steam cooked/ acid hydrolyzed feather meal. Amino acid content of present feather meal was comparable to CSH produced by keratinolytic bacterium *Vibrio* kr6 [11] except for few quantitative differences. The present feather meal had higher content of essential amino acids histidine, phenylalanine, methionine and threonine while CSH revealed high content of glutamate, serine, arginine and leucine. These differences may be due to the different processes used for feather degradation. Since the CSH was a result of crude supernatant after feather degradation during fermentation by *Vibrio* kr 6 and present process was a cell free enzymatic degradation.

In-vitro digestibility of feather meal was determined by pepsin followed by trypsin treatment. Feather meal was found to be digestible by pepsin and trypsin by releasing 670 mg protein/g feather meal after 18h of digestion. *In- vitro* digestibility results showed that digestibility of the present feather meal is 0.734 i.e. 73.4% which is better than commercial feather meal (0.578) or milled feather (0.096) and comparable to whole cell hydrolysate (WCH) (0.834) feather meal produced by fermentation [11]. This suggests that present feather meal can be used as feed for chickens, cattle and fish as reported earlier for feather meal produced by fermentation [21].

Conclusion

The present process of bioconversion of feather into feather meal is completely an enzymatic process. To the best of our knowledge, this is the only process where no additional redox has been provided. The process is not only simple and time saving but at the same time economically viable as it does not require any bioreactor for feather degradation. Thus, bulk feather can be easily recycled into feather meal using keratinase from *Bacillus licheniformis* ER-15 within 12h.

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