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Isolation and Characterization of a Feather-Degrading Bacterium for Recycling of Keratinous Protein Waste

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The aim of this study was to isolate chicken feather-degrading bacteria with high keratinolytic activity and to investigate cultural conditions affecting keratinolytic enzyme production by a selected isolate. A chicken feather-degrading bacterial strain CH3 was isolated from poultry wastes. Isolate CH3 degraded whole chicken feather completely within 3 days. On the basis of phenotypical and 16S rDNA studies, isolate CH3 was identified as *Bacillus thuringiensis* CH3. This strain is the first *B. thuringiensis* described as a feather degrader. The bacterium grew with an optimum at pH 8.0 and 37℃, where maximum keratinolytic activity was also observed. The composition of optimal medium for keratinolytic enzyme production was feather 0.1%, sucrose 0.7%, casein 0.3%, K_2HPO_4 0.03%, KH_2PO_4 0.04%, $MgCl_2$ 0.01% and NaCl 0.05%, respectively. The keratinolytic enzyme had a pH and temperature optima 9.0 and 45℃, respectively. The keratinolytic activity was inhibited ethylenediaminetetraacetic acid, phenylmethylsulfonyl fluoride, and metal ions like Hg^{2+}, Cu^{2+} and Zn^{2+}. The enzyme activated by Fe^{2+}, dithiothreitol and 2-mercaptoethanol.

Key Words : Feather, Keratin, Keratinolytic enzyme, Waste

1. Introduction

Feathers, which are almost pure keratin protein, are produced in large amounts as a waste by-product at poultry-processing plants. Millions of tons of feathers are produced annually worldwide and represent a potential alternative to more expensive dietary ingredients for animal feed. However, the current processes to obtain feather meal are expensive and also destroy certain amino acids, yielding a product with poor digestibility and variable nutrient quality[1].

Keratin is the insoluble structural protein of feathers and wool and is known for its high stability[2]. The composition and molecular configurations of its constituent amino acids warrant structural rigidity. The keratin chain is tightly packed in the α-helix (α-keratin) or β-sheet (β-keratin) into a supercoiled polypeptide chain, resulting in mechanical stability and resistance to common proteolytic enzymes such as pepsin, trypsin and papain[3]. In addition, cross-linking of protein chains by cysteine bridges confers high mechanical stability and resistance to proteolytic degradation of keratin. Nevertheless, feathers do not accumulate in nature, since structural keratin can be degraded by some microorganisms[4].

Keratinolytic enzymes may have important uses in biotechnological processes involving keratin-containing wastes from poultry and leather industries, through the development of non-polluting processes[4]. After hydrolysis, the feathers can be converted to feedstuffs, fertilizers, glues, and films or used for the production of the rare amino acids serine, cysteine, and proline[5]. It is reported that keratinolytic enzymes are mainly produced by mesophilic fungi and actinomycetes, but some thermophilic species of *Bacillus* produce feather-degrading enzymes[5].

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The aim of this study is to isolate mesophilic feather-degrading bacteria from industrial poultry waste and to identify bacteria showing high feather-degrading ability, with potential application in biotechnological processes. In addition, we investigated the production and characteristic of keratinolytic enzyme by feather-degrading isolate from poultry waste.

2. Materials and Methods

2.1. Isolation and identification of feather-degrading microorganism

Soil and feather wastes were collected from several sites at a local poultry farm. The samples were flooded in phosphate-buffered saline and the suspension were streaked on skim milk agar plate (1.0% tryptone, 0.5% yeast extract, 5.0% skim milk, 0.5% NaCl, 2.0% agar, pH 8.0). After a 3 day incubation at 37℃, the plates were examined for the presence of colonies developing clear haloes. Colonies with clear haloes resulting from proteolysis were selected for further investigation. The purity of the isolate was ascertained through repeated streaking. The pure isolates were subsequently grown in a feather medium (0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.01% $MgCl_2$, 0.05% NaCl, 0.05% urea, 0.1% chicken feather, pH 8.0). Cultures were grown at 37℃ and 200 rpm for 5 days. Isolates that completely broke down feathers in the medium were selected. The individual isolates were then examined for their keratinolytic activities. One bacterium having the highest keratinolytic activity was selected for further study and characterized to the species level using API 50CHB and API 20E kit. The 16S rRNA gene was also sequenced after genomic DNA extraction and polymerase chain reaction amplification as described elsewhere[6,7]. Databases in GenBank were searched for sequences similar to the 16S rDNA sequence.

2.2. Enzyme preparation

The organism was cultivated for 3 days in a feather medium, from a 10^6 CFU/ml culture. The culture medium was centrifuged at 14,000 rpm for 15 min, and the supernatant was used as a crude enzyme preparation to study culture conditions.

The crude enzyme was precipitated from the supernatant by the gradual addition of ammonium sulfate to 80% saturation, allowed stand for 2 h and centrifuged at 10,000 rpm for 30 min. The pellet was dissolved in Tris-HCl buffer (25 mM, pH 7.8), dialyzed against 10 mM of the same buffer for 24 h and concentrated. The concentrate was further concentrated in an ultrafiltration cell, using an ultrafilter that removes substances smaller than about 10 kDa. To study characteristics of keratinolytic enzyme, this concentrate was used. All operations were performed at 4℃.

2.3. Dimethyl sulfoxide soluble feather keratin preparation

Soluble keratin was prepared from chicken feathers by the method of Wawrzkiewicz et al.[8]. Native chicken feathers (10 g) in 500 ml of dimethyl sulfoxide were solubilized by heat treatment in a reflux condenser at 100℃ for 1 h. Soluble keratin was then precipitated by addition of cold acetone (1 l) at -70℃ for 2 h followed by centrifugation at 9,000 rpm for 10 min. The precipitate was lyophilized and grinded, and then suspended in 28 mM Tris/HCl buffer (pH 7.8). This keratin suspension was used as a substrate for keratinolytic activity determination.

2.4. Keratinolytic activity assay

Keratinolytic activity was assayed with soluble keratin as a substrate according to the method of Fridrich et al.[9]. A crude enzyme solution (1 ml) was mixed with 4 ml of 28 mM Tris/HCl buffer (pH 7.8) containing 4 mg of keratin powder and incubated for 1 h at 45℃. The reaction was stopped by adding 2 ml of 10% trichloroacetic acid and centrifuged at 14,000 rpm for 15 min at 4℃. The absorbance of the supernatant was measured at 280 nm. One unit (U) of keratinolytic activity was defined as the amount of enzyme that resulted in an increase in absorbance at 280 nm of 0.01 under the above conditions. The protein concentration was determined by the Bradford method[10] with bovine serum albumin as the standard.

3. Results and Discussion

3.1. Isolation of feather-degrading microorganism

About thirty different strains of bacteria were isolated from soil and feather waste. Isolated strains producing large clear zone on the skim milk agar plate were selected primarily. Among them, isolate CH3 showed the highest feather-degrading activity in a feather medium. The degradation of whole chicken

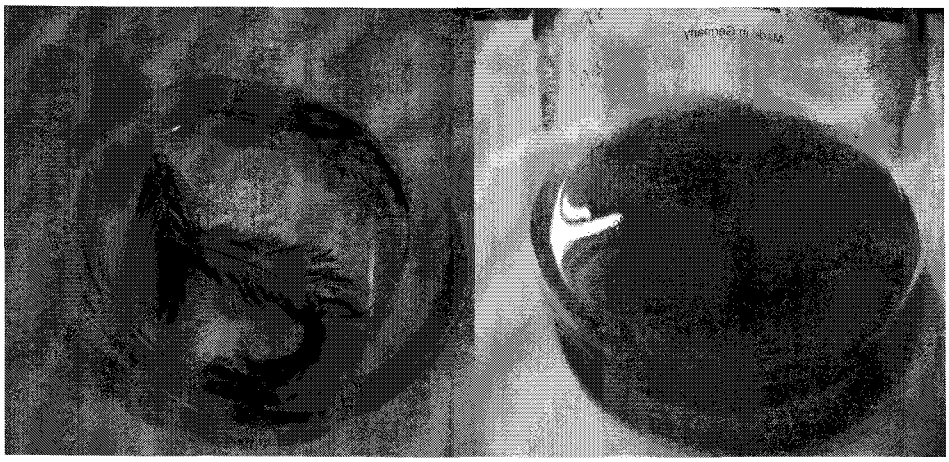

Fig. 1. Feather degradation by isolate CH3 after 0 (left) and 3 (right) days of incubation at 37℃.

feather by isolate CH3 is shown in Fig. 1. Complete degradation was observed after incubation at 37℃ for 3 days. It is notable result that all feather barbule and almost all feather rachis were degraded after 2 days. Williams et al.[11] demonstrated that *Bacillus licheniformis* PWD-1 degraded chicken feather completely at 50℃ in 10 days. Böckle et al.[12] reported that *Streptomyces pactum* DSM40530 partially degraded native chicken feather at 50℃. It is evident from the present study that isolate CH3 very efficiently degraded chicken feather in a short period at 37℃. This suggests that isolate CH3 could be useful in the biodegradation of poultry and abattoir wastes.

3.2. Identification of isolated strain

Isolate CH3 was a Gram-positive, endospore-forming, rod-shaped bacilli. API 50CHB and 20E kits which were used to differentiate *Bacillus* species, were selected for biochemical identification of isolate CH3. The data of API 50CHB/20E kit was read in the database (APILAB). Isolate CH3 was very close to *Bacillus thuringiensis* (data not shown). Determination of 16S rDNA sequence of isolate CH3 was also carried out. The 1.5 kbp PCR product amplified by two universal primers was confirmed (Fig. 2) and sequenced (Fig. 3). BLAST search showed that isolate CH3 showed high homology (99.9%) with *B. thuringiensis*. Therefore, isolate CH3 was tentatively named as *B. megaterium* CH3. So far as we know, isolate CH3 is the first *B. thuringiensis* described as a feather degrader.

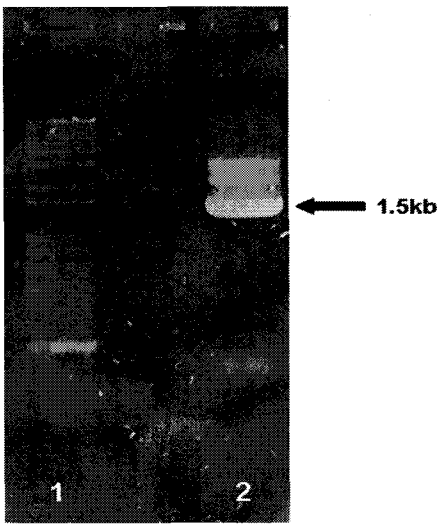

Fig. 2. Amplified PCR product using universal primer for 16s rDNA sequencing.
Lane 1, 1kb ladder; Lane 2, PCR product of isolate CH3.

3.3. Optimum conditions for keratinolytic enzyme production by *Bacillus thuringiensis* CH3

B. thuringiensis CH3 was cultured in 0.1% whole feather medium at various temperatures and initial pHs. The growth and keratinolytic activity of *B. thuringiensis* CH3 was the highest at 37℃ and pH 8.0, respectively (data not shown). Other previously described keratinolytic bacteria generally have optimum growth and keratinolytic activity at high temperature[4]. Williams and Shih[13] reported that maximal growth and keratinolytic activity of *B. licheniformis* PWD-1

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21   ACATGCAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCG   70
71   GAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGA  120
121  CGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGA  170
171  ACCGCATGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGG  220
221  ACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACG  270
271  ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG  320
321  GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA  370
371  AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAA  420
421  AACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTT  470
471  GACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGG  520
521  TAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGC  570
571  GCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAG  620
621  GGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCC  670
671  ATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAG  720
721  GCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCA  770
771  AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAG  820
821  TGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC  870
871  CGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGG  920
921  CCCGCACAAGCGGTGGAGCATGT-GGTTTAATTTCGAAGCAACGCGAAGA  970
971  ACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGGGCTTCTC 1020
1021 CTTCGGGAGCAGAGTGACAGGTGGNGCATGGAATCCGCCGACCGAGGTTT 1070
1071 TTCCAATGGGGTGGCTGAAGCCCACAATGTTTGAGAGCACCACACTGCCC 1120
1121 GCCACACATGTTCCGGGCCCTTGCATAAGTGGCGCCGTACGACTAGGCGC 1170
1171 TAATGATCGCTAAGGTCGAAGTACATCCGCTCAACGTCGGATGTTAGGCT 1220
1221 TGACTCTTGCCAAAATACTCTAATCGAGGTGGAGCGCCAGAACGTCGAGA 1270
1271 AACATGGCAGGTAACATCGTGCACACATCGGGTCCAGTATTCCCCGTACT 1320
1321 ACTAAACTGCAGTAGGGGTGGAAGGAGGCCAAACAGTGGCCGTCAGTGGA 1370
1371 ATCTCACGGGTTGAATTACTACCGTTGATTCTAGTTCCCAACGCGAGCAA 1420
1421 CGCCCTGAATTGGGTTGTAGAGTGCTGTGCTCGACTGCTGTTGG       1464
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Fig. 3. Nucleotide sequence of 16S rDNA from isolate CH3.

was observed at 50℃, respectively. *B. thuringiensis* CH3 was mesophile. Therefore, our strain will be less energy-consuming than the thermophilic strains usually used in feather processing.

The influence of the addition of various carbon sources to the feather medium is shown in Table 1. Keratinolytic activity was increased by glucose, dextrin, sucrose, maltose, glycerol, mannitol and soluble starch. The maximum carbon source was glycerol that presents 47.3 U/ml, but sucrose as a optimal carbon source was selected because sucrose can be used easier than glycerol. The maximum keratinolytic activity was found in the medium containing 0.7% sucrose (data not shown). Santos et al.[14] reported that the presence of glucose in the medium decreased the keratinolytic activity of *Aspergillus fumigatus*. It has been also reported that the presence of glucose decreases the keratinase production of *Streptomyces fradiae*[15].

The effect of nitrogen sources on keratinolytic enzyme production is shown in Table 2. Peptone, beef extract, yeast extract, soybean flour, casein, and skim milk had a positive influence on keratinolytic activity,

Table 1. Effect of carbon source on keratinolytic activity of *B. thuringiensis* CH3

Carbon source (0.1%)	Keratinolytic activity (U/ml)	Total protein (μg/ml)
None	23.9	12.5
Glucose	29.9	10.3
Fructose	23.3	9.8
Rhamnose	17.1	9.8
Xylose	19.6	9.7
Arabinose	19.0	10.7
Dextrin	27.2	11.3
Sucrose	42.2	11.2
Maltose	26.1	10.6
Lactose	18.0	10.1
Galactose	17.9	10.8
Glycerol	47.3	11.0
Sorbitol	21.5	9.7
Mannitol	24.2	10.0
Soluble starch	30.7	10.0

Table 2. Effect of nitrogen source on keratinolytic activity of *B. thuringiensis* CH3

Nitrogen source (0.1%)	Keratinolytic activity (U/ml)	Total protein (μg/ml)
None	25.9	11.0
Peptone	58.1	9.5
Beef extract	37.3	11.4
Malt extract	18.9	7.1
Yeast extract	52.7	10.3
Soybean flour	46.8	11.2
Casein	89.1	11.3
Skim milk	78.5	10.7
Trypton	27.9	10.0
$NaNO_3$	31.1	3.9
$(NH_4)_2HPO_4$	72.2	10.0
NH_4NO_3	8.2	5.3
NH_4Cl	15.8	9.7
$NH_4H_2PO_4$	7.4	4.9
KNO_3	23.1	8.1
$(NH_4)_2SO_4$	11.7	7.0
$NaNO_2$	17.6	3.7

resulting in 124, 44, 103, 81, 244, and 203% increases over the control. $NaNO_3$ and $(NH_4)_2HPO_4$ among the inorganic nitrogen sources increased keratinolytic activity. The optimal nitrogen source for keratinolytic activity was casein, and the maximum casein concentration was 0.3% (data not shown).

On the basis of the above results, the optimum medium contained 0.1% feather, 0.7% sucrose, 0.3% casein, 0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.01% $MgCl_2$,

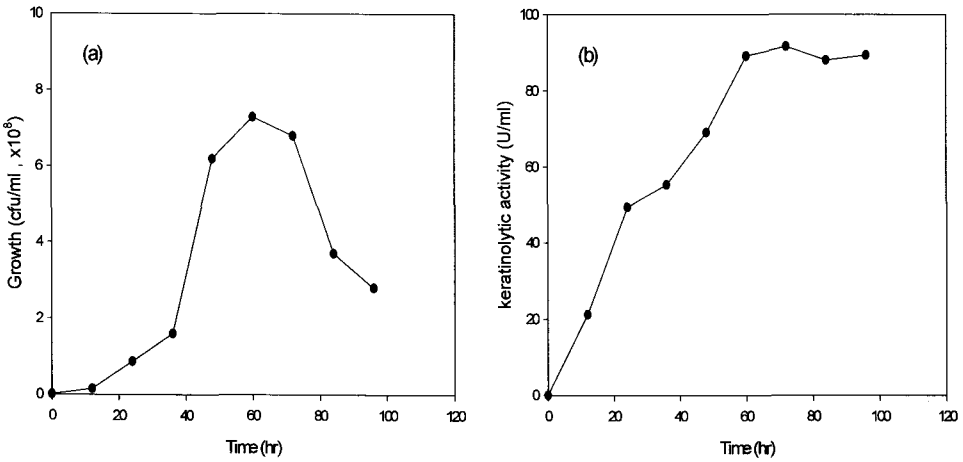

Fig. 4. Cell growth and keratinolytic activity of *Bacillus thuringiensis* CH3.
(a), cell growth ; (b), keratinolytic activity.

0.05% NaCl with an initial pH 8.0. Fig. 4 shows the time course of growth and keratinolytic activity of *B. thuringiensis* CH3 during its growth in the optimal medium at 37℃. Cells growing in medium containing feather reached the stationary phase after 60 h of incubation. The maximum keratinolytic activity of *B. thuringiensis* CH3 was about 91.7 U/ml after 72 h of cultivation.

3.4. Characteristics of keratinolytic enzyme

The effect of temperature on keratinolytic activity was investigated by having a constant pH 7.8 with varying temperatures. The enzyme was active between 37℃ and 50℃, with an optimum temperature at 45℃ (Fig. 5). For thermal stability, residual activity was measured after 20 min of preincubation in Tris/HCl buffer (pH 7.8) at different temperature levels. The keratinolytic activity was stable up to 50℃ (data not shown).

The effect of pH on keratinolytic activity was investigated by measurements at 45℃ in buffers of various pH (28 mM). The optimum pH was found at pH 9.0 (Fig. 6). For pH stability, residual activity was measured after 20 min of preincubation at 45℃ in buffers of the same pH. The enzyme was stable over the pH range between 7.0 and 10.0, retaining >80% activity in the lower limit (data not shown).

The enzyme was strongly inhibited by ethylenediaminetetraacetic acid (EDTA) although other protease inhibitor such as phenylmethylsulfonyl fluoride (PMSF) also had some effects, suggesting that the

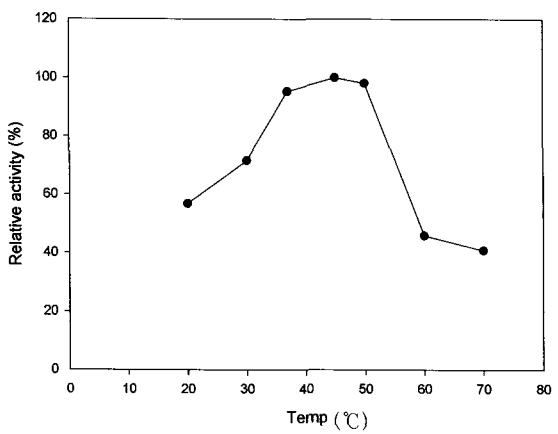

Fig. 5. Effect of temperature on keratinolytic enzyme activity.

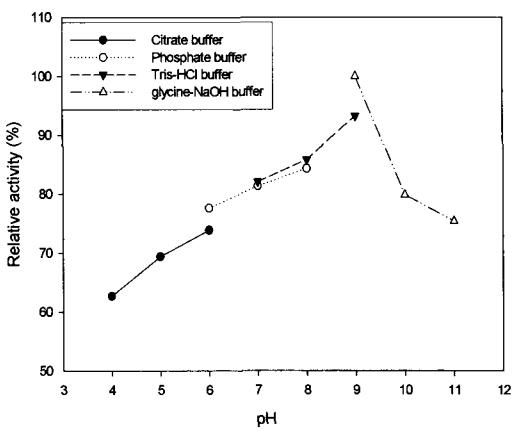

Fig. 6. Effect of pH on keratinolytic activity.

Table 3. Effect of metal ion and chemical on the activity of keratinolytic enzyme from *Bacillus thuringiensis* CH3

Compound	Relative activity (%)
None	100
FeSO4 (10 mM)	105
CuCl2 (10 mM)	44
ZnCl2 (10 mM)	35
HgSO4 (10 mM)	27
PMSF (1 mM)	88
PMSF (10 mM)	67
EDTA (1 mM)	40
EDTA (10 mM)	36
DTT (1 mM)	179
DTT (10 mM)	225
2-Mercaptoethanol (0.1%)	125
2-Mercaptoethanol (1%)	132

culture supernatant could contain several enzymes affecting keratinolysis. The use of reducing agents such as dithiothreitol and 2-mercaptoethanol caused increases in keratinolytic activity (Table 3). The use of reducing agents to enhance keratin degradation by keratinolytic enzymes has been described[4]. The stimulatory effect of reducing agent on keratinolytic activity of strain CH3 may be explained by the reduction of disulfide bridges, allowing a more accessible substrate.

Among the metal ions tested, Hg^{2+}, Cu^{2+} and Zn^{2+} strongly inhibited the enzyme, whereas Fe^{2+} slightly increased keratinolytic activity. The effect of Hg^{2+} inhibiting the enzyme suggests that a free cysteine is present at or near the active site[16]. In this regard, a novel trypsin-like thiol protease produced by a keratinolytic *B. licheniformis* strain was recently described[16]. Thus, our results suggest that serine protease, cysteine protease and metalloprotease may be involved in feather degradation by *B. thuringiensis* CH3. Similar result was observed for species of *Streptomyces*[17]. It has been also reported that *Flavobacterium* sp. kr6 exhibited predominantly metalloprotease activity, but demonstrated some serine, cysteine and aspartic protease activities[18].

4. Conclusions

Feathers are generated in large amounts as a waste byproduct at commercial poultry processing plants and they are made up primarily of keratin. Generally they become feather meal used as animal feed after undergoing physical and chemical treatments. These processes require significant energy and also cause environmental pollutions. Therefore, biodegradation of feather by microorganisms represents an alternative method to prevent environment contamination. The results presented here clearly indicate the strong keratinolytic nature of *B. thuringiensis* CH3 toward chicken feather as the keratin source. The keratinolytic protease of *B. thuringiensis* CH3 offers the possibility for bioconversion of poultry feathers from a pollutant to a nutritionally upgraded protein-rich feedstuff for livestock.

Acknowledgements

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