

Preparation of Feather Digests as Fertilizer with *Bacillus pumilis* KHS-1

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Abstract The present study was undertaken to assess the capacity of *Bacillus pumilis* KHS-1 to grow on chicken flour and to prepare feather digest as fertilizer. To increase keratinolytic activity, the addition of cysteine (5.0 mM) showed the highest keratinolytic activity (245 unit) among the reducing agents tested. The production of soluble protein (feather digests) paralleled the tendency to the production of keratinolytic protease. In the growth curve of *B. pumilis* KHS-1 at 30°C in the feather medium with 5 mM cysteine, the maximum keratinolytic activity of *B. pumilis* was about 161 units/ml after 84 h of incubation. The maximum enzyme activities were observed at the late logarithmic growth phase, and remained thereafter with little changes. Using 27-day plant growth assays on carrot and Chinese cabbage, feather digests and reference fertilizer were compared. In terms of the length and the weight of the above-ground vegetations, feather digests showed the same effect as that of the fertilizer. Therefore, our investigation shows that the feather digests can be used in agriculture.

Key words: *Bacillus pumilis* KHS-1, feather digests, keratinolytic protease, fertilizer

Animal wastes have been used as a nutrient source for crop production. During the last three decades, many researches have been conducted to improve the agronomic utilization of animal waste, including poultry waste. The large increase in the size of individual production facilities and the total poultry industry has resulted in an enormous increase in waste, particularly litter, to be managed. Feather waste, generated in large quantities as a byproduct of industrial poultry processing, is nearly pure keratin protein [11]. Considering that millions of tons of feathers are produced annually worldwide and that they are made up primarily of

keratin (90% or more), this byproduct represents a potential alternative to more expensive dietary ingredients, for example, for poultry production [19].

Currently, feather waste is utilized on a limited basis as a dietary protein supplement for animal feedstuffs. Prior to being used, the feather is steam pressure-cooked or chemically treated to make it more digestible. These treatment processes require significant energy and destroy certain amino acids [12]. Consequently, biodegradation by microorganisms, possessing keratinolytic activity, represents an alternative method to improve the nutritional value of feather waste. However, only limited information on such microbial treatment of feathers is currently available [6].

We isolated feather-degrading bacteria, *Bacillus subtilis*, *Bacillus pumilis*, and *Bacillus cereus*, from poultry waste. The production of keratinolytic proteases by *B. pumilis* and *B. cereus* was increased with feathers, but the presence of casein greatly increased the production of the enzyme in *B. subtilis*. Among those isolates, *B. subtilis* and *B. pumilis* showed similar keratinolytic activities, but the enzyme stability of *B. pumilis* was greater than that of *B. subtilis* [6]. In this study, we examined the capacity of *Bacillus pumilis* KHS-1 to grow on chicken flour and attempted to prepare feather digest as a fertilizer.

MATERIALS AND METHODS

Media and Preparation of Feather Digests

Bacillus pumilis KHS-1 isolated from poultry waste [6] was used in this study. The experiments on cultivation conditions were carried out in 500-ml Erlenmeyer flasks containing 100 ml of basal medium (0.05 g of NH₄Cl, 0.05 g of NaCl, 0.03 g of K₂HPO₄, 0.04 g of KH₂PO₄, and 0.01 g of MgCl·6H₂O) with 0.5 g of hammer-milled chicken feather. The culture was incubated at 30°C at 110 rpm for five days and the fermentation broth was

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sampled for analysis of bacterial growth, pH, and keratinase activity.

The feather medium used for the preparation of feather fertilizer contained, per liter, the following: 5 g of peptone, 0.5 g of NH_4Cl , 0.5 g of NaCl , 0.3 g of K_2HPO_4 , 0.4 g of KH_2PO_4 , 0.1 g of $\text{MgCl}\cdot 6\text{H}_2\text{O}$, 0.6 g of cysteine, and 5 g of chicken feathers. The pH was adjusted to 6.0. Feathers were washed, dried, and hammer-milled prior to being added to the medium. The medium was autoclaved and the incubation was carried out in a 30-l fermenter containing 20 l of medium, with a stirring rate of 300 rpm and an aeration rate of 1 vvm at 40°C for 3 days. After centrifugation (2,800 \times g for 15 min), the supernatant was concentrated with a rotary evaporator at 60°C (5 times concentration) and the concentrates were used for fertilizer. Cell growth was determined by the plate count method on nutrient agar.

Nutrient broth and nutrient agar were purchased from BBL Microbiology Systems (Cockeysville, U.S.A.), and other media for the identification and characterization of microorganisms were purchased from Difco Laboratories (Detroit, U.S.A.).

Keratinolytic Activity

Bacillus pumilis KHS-1 was incubated for 3 days in feather medium, starting from a 10^6 CFU/ml culture. The culture medium was centrifuged at 2,800 \times g for 15 min, and the supernatant was used as enzyme preparation.

Azokeratin was prepared by reacting ball-milled feather powder with sulfanilic acid and NaNO_2 by the method similar to that described by Tomarelli *et al.* [21] for albumin. For a standard assay, 5 mg of azokeratin were added to a 1.5-ml centrifuge tube along with 0.8 ml of 50 mM potassium phosphate buffer (pH 7.5). This mixture was agitated until the azokeratin was completely suspended. A 0.2 ml aliquot of an appropriately diluted enzyme solution was mixed with azokeratin, and the mixture was incubated in a water bath at 30°C for 1 h. The reaction was terminated by the addition of 0.2 ml of 10% trichloroacetic acid (TCA), and the mixture was filtered. A_{450} of the filtrate was measured with a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). A control was prepared by adding TCA to a reaction mixture before adding the enzyme solution. One unit of keratinolytic activity was defined as an increase of A_{450} by 0.001. Protein concentration was measured by absorbance at 280 nm or by the method of Bradford [2], using bovine serum albumin as a standard.

Digested Material as Fertilizer (27-Day Plant Growth Assay)

The standard practice of the American Society for Testing and Materials [1] was applied to compare the feather digests and the reference (a commercial fertilizer) to fertilize the soil for the growth of carrot (*Daucus carota*) and Chinese cabbage (*Brassica campestris* var. *chinensis*).

The soil substrate contained sieved and mixed sphagnum peat (Kekkilä, Finland), the pH of which was raised to 7.0 by limestone ($\text{CaCO}_3 + \text{MgCO}_3 + \text{CaCO}_3$; Ca 30%, Mg 2%, Kekkilä, Finland). The seeds were then planted in the substrate in polyethylene plant pots (6 l), 19 seeds per pot to test. Feather digests (188 ml of feather digests/kg substrate) and the fertilizer (700 mg of fertilizer/kg substrate) were diluted with tap water and poured onto the top of the substrate to obtain the desired concentration of soluble nitrogen (110 mg of N/kg substrate). The pots were placed in an environmental chamber at 20°C for 27 days, and tap water was added daily to replace the evaporation loss.

RESULTS AND DISCUSSION

Keratinase Production

Keratinolytic cultures were screened on feather meal agar. The rod-shaped and Gram-positive bacterium demonstrated a clearing zone and a high feather-degrading activity. The isolate was selected for the purpose of identification and further study. The bacterium grew aerobically and had a strong catalase activity. Since preliminary morphological and biochemical characteristics of this bacterium coincided with those of *B. pumilis* [6], we designated it as *B. pumilis* KHS-1.

B. pumilis KHS-1 grew and produced keratinase, using carbon sources such as feather, glucose, galactose, lactose, sucrose, and molasses. The feather as the carbon source showed the highest level of keratinase production (143.3 unit) (data not shown). The strain grew and produced keratinase using chicken feather as the sole source of nitrogen and carbon. Complete solubilization of feather was observed

Table 1. Keratinase production by *B. pumilis* KHS-1 grown on protein and feather media in the presence of different supplements.

Basal medium plus	Supplement	Keratinase activity (unit)
Nitrogen source	None	38.2 \pm 2.3
	Feather	159.2 \pm 19.3
	Casein	139.2 \pm 12.8
	Peptone	129.3 \pm 13.5
	Yeast extract	122.7 \pm 32.7
	Tryptone	118.3 \pm 13.2
Feather	None	159.2 \pm 19.3
	Peptone	189.5 \pm 13.1
	Yeast extract	162.3 \pm 20.1
Casein	None	139.2 \pm 12.8
	Peptone	135.8 \pm 9.3
	Yeast extract	125.3 \pm 15.4

The basal medium was composed of (g/l): 0.5 g of NH_4Cl , 0.5 g of NaCl , 0.3 g of K_2HPO_4 , 0.4 g of KH_2PO_4 , and 0.1 g of $\text{MgCl}\cdot 6\text{H}_2\text{O}$. All the other supplements were included at a final concentration of 0.5%.

after 5 days and *B. pumilis* KS-1 produced a high level of keratinase activity in the feather medium (Table 1). The strain produced keratinase activity, when grown on either feather or casein as nitrogen and carbon sources; however, the level of keratinase production was higher when grown on feather than on casein. Increased level of keratinase production by the strain was also observed on addition of peptone.

The strain grew and produced an appreciable level of keratinase, using feather as the sole source of nitrogen. Compared to currently used methods to prepare feather meal, using high temperature and pressure treatments, the use of microbiological methods to hydrolyze feather is an attractive alternative [4, 17]. The ability of the strain to grow and produce an appreciable level of keratinase, using feather as a substrate, could offer tremendous potential for the development of biotechnological methods for the hydrolysis of feather. In particular, the high level of keratinase production by the strain, which is able to efficiently hydrolyze feather even in the presence of reducing agents, makes it extremely interesting.

Addition Effect of Reducing Agents and Culture Conditions

Because of the characteristically high cysteine content of feather, there exists a possibility of sulfitolysis. Understandably, the preponderance of cysteine in feather naturally confers a rigid biomolecular configuration through the formation of cysteine disulfide bridges. Therefore, reducing agents (thiol reagents), such as cysteine, mercaptoethanol, glutathione, thioglycolate, dithiothreitol (DTT), and sodium sulfite, are expected to affect the disulfide bridges of feather. As shown in Fig. 1, the reducing agents at various concentrations (0.5, 1.0, and 5.0 mM) were added to the medium, and the keratinolytic activities were measured. The addition

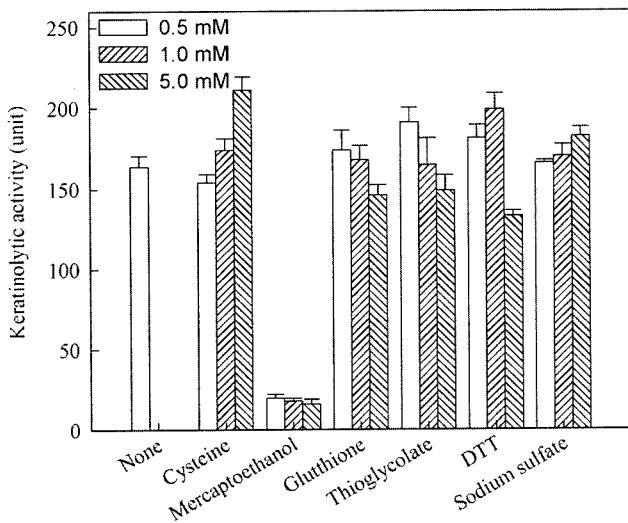
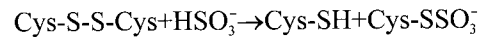


Fig. 1. Effect of reducing agents on the keratinase activity. Reducing agent was added to the basal medium with feather and peptone.

of cysteine (5.0 mM) showed higher keratinolytic activity (245 unit), however, the addition of mercaptoethanol resulted in the decrease of the activity. Sulfitolysis during keratin degradation was carefully examined by Kunert [7] and Malviya *et al.* [9]. According to Kunert [7], dermatophytes and non-dermatophytes metabolized free or combined cysteine as a source of sulfur and nitrogen [9], and the products of cysteine metabolism by fungi were inorganic sulfur and other intermediates. Kunert [7] indicated that the excess sulfur was excreted back to the medium in the oxidized form as sulfate and sulfite, and the sulfite reacts with cystine at neutral to alkaline pH, cleaving it to cysteine and S-sulfocystein, by the equation shown below [7]:



According to Kunert [7], this reaction also takes place with cystine in proteins, including keratin. Hence, keratin is denatured prior to the attack by protease (keratinolytic protease) by excretion of sulfite, which causes the sulfitolysis of the disulfide bonds.

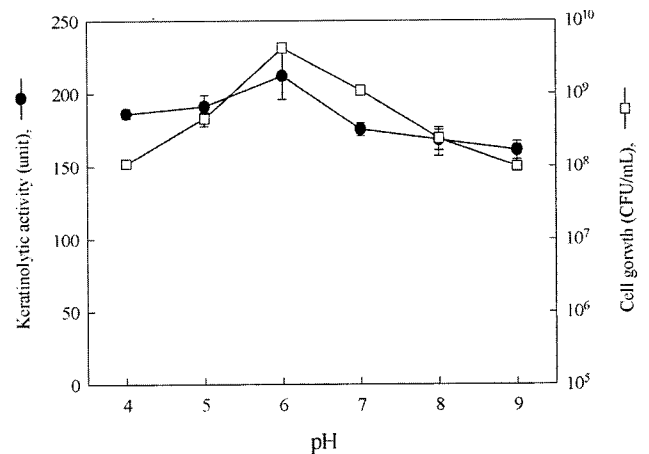
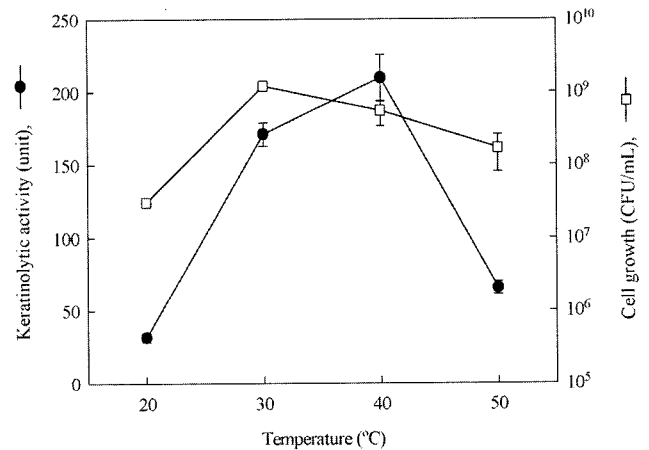


Fig. 2. Effect of temperature and initial pH on the keratinase production. Cultivation was carried out in a basal medium, containing feather, peptone, and cysteine, for 5 days.

The growth and keratinase production of *B. pumilis* KHS-1 were optimal at an incubation temperature of 40°C (Fig. 2). *Bacillus* species typically are mesophilic and grow well within a temperature range of 30–40°C [18], except for *B. licheniformis*, which had maximum growth and keratinase production at 45–50°C [3, 23]. Keratinase production by *B. pumilis* KHS-1 was relatively low at 50°C.

The initial pH of the medium also greatly affected bacterial growth and keratinase production. As seen in Fig. 2, the optimum pH for bacterial growth and keratinase production was 6. These results are in line with previous report indicating that keratinase was a neutral protease and most active under neutral or basic conditions [3, 6].

Keratinolytic Activity and Soluble Protein Production

It was observed that the aerobic growth of the isolates in the media, containing the feathers as primary sources of carbon, energy, nitrogen, and sulfur, resulted in degradation of the keratin protein after 4 days of incubation (Fig. 3).

Figure 3 shows the growth curves of *B. pumilis* KHS-1 at 40°C in the feather medium with 5 mM cysteine. The maximum keratinolytic activity of *B. pumilis* was 161 units/ml after 84 h of incubation, and remained thereafter with little changes. Lin *et al.* [8] reported that *B. licheniformis* PDW-1, like *B. pumilis* KHS-1, secreted the enzyme at the late logarithmic growth phase. Sangali and Brandelli [15] also reported that the keratinolytic activity increased as the incubation time increased, and that the maximum activity was observed at the beginning of the stationary phase, which were in agreement with the results on *Streptomyces fradiae* [16].

As shown in Fig. 3, the production of soluble protein (feather digests) by *B. pumilis* KHS-1 strain was monitored. The production of soluble protein had the same tendency as that of keratinolytic protease, and the maximum level of the protein production was observed during 72 and 84 h of incubation.

In order to evaluate a biotechnological application of the keratinolytic protease of *B. pumilis* KHS-1, more detailed information on the factors that influence the activity of these enzymes on keratin substrates is needed, and more research on the characteristics of these interesting enzymes will be carried out.

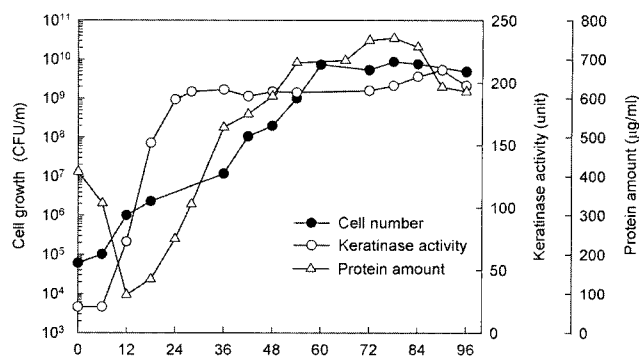


Fig. 3. Growth of *B. pumilis* KHS-1 and the production of soluble protein.

Feather Digests as Fertilizer

The degraded feather had certain slightly higher amino acids contents than untreated feather, possibly because of the microbial protein biomass added to the residue (data not shown). The results are similar to the report of Elamayergi and Smith [5], which found that feather meal fermented by *S. fradiae* had higher contents of methionine, lysine, tyrosine, and histidine than the meal without fermentation. However, low levels of amino acids such as aspartic acid, threonine, serine, glutamic acid, proline, valine, and isoleucine were found in the degraded feather. The difference in the results might have occurred due to differences in fermentation time, type of substrate and microorganism, and cultivation conditions.

Furthermore, additional proteins and amino acids were abundantly accumulated in fermented broth due to degradation of feathers by bacterial activities [20]. Therefore, the broth would be used as a nitrogen source for plants after concentration. The method could be easily applied for the biodegradation of feathers into digestible proteins as fermented feather and feather protein concentrate for use in plant or animal feed.

Using 27-day plant growth assays on carrot and Chinese cabbage and chemical and physical analysis, we investigated the effectiveness of the digested poultry waste as fertilizer in agriculture. The total nitrogen content of feather digests was 0.6 g/l. Compared to the reference fertilizer in terms of root length and the length and weight of the above-ground vegetation (Table 2), feather digests showed the same effect as that of the reference fertilizer on the growth of carrot and Chinese cabbage. Salminen *et al.* [14] reported

Table 2. Effect of feather digested material (54 g FDM-1/kg substrate) on the growth of carrot and Chinese cabbage as compared to the reference fertilizer (700 mg/kg) (110 mg N/kg substrate, N=30).

Material	Carrot		Chinese cabbage	
	Feather digests	Fertilizer	Feather digests	Fertilizer
Above-ground vegetation length (mm)	10.1±1.2	10.8±1.5	10.4±1.2	12.2±1.4
Root length (mm)	5.1±1.4	6.2±1.1	9.6±0.8	10.4±2.1
Above-ground vegetation dry weight (mg)	16.4±4.2	19.3±8.3	303.4±28.9	321.3±56.8
Root dry weight (mg)	1.6±0.7	1.5±0.5	19.4±1.2	22.3±9.2

that the anaerobically-digested material showed the fertilizer effect on carrot, but inhibited the growth of Chinese cabbage. Organic acids, intermediates of anaerobic degradation, are potentially phytotoxic, and since they are microbial substrates, they enhance the microbial growth, thereby depleting the soil oxygen and inhibiting the growth of Chinese cabbage [10]. Therefore, aerobic treatment could be used to ensure maturation of anaerobically-digested material and reduce the content of volatile inhibitors such as ammonia and organic acids [22]. This process, however, can also result in a significant loss of nitrogen through the volatilization of ammonia [13].

In conclusion, the growth effect of the feather digests on carrot and Chinese cabbage as nitrogen sources was found to be almost comparable to that of the fertilizer. Therefore, the feather digests, concentrated before use, can potentially be used as a fertilizer in agriculture. To evaluate the feather digests as a practical fertilizer in agriculture, more detailed plant assays are needed.

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