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Characterization of a Novel Feather-Degrading *Bacillus* sp. Strain

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Abstract

Feather waste is generated in large amounts as a byproduct of commercial poultry processing. This residue is almost pure keratin, which is not easily degradable by common proteolytic enzymes. A feather-degrading bacterium was isolated from poultry feathers in decomposition. The strain identified as kr16 showed important feather-degrading activity when grown on basal medium containing 10 g/L of native feather as the source of energy, carbon, and nitrogen. The isolate was characterized according to the phenotypical characteristics and biochemical profiling that belong to the *Bacillus* genus. Keratinolytic activity of this isolate was monitored during cultivation of the bacterium on raw feathers at different temperatures. Maximum growth and feather-degrading activity were observed at 30–37°C. The keratinolytic enzyme had a pH optimum ranging from 8.0 to 11.0 and a temperature optimum of 45–65°C. The keratinase was strongly inhibited by EDTA and the metal ions Hg^{2+} and Sn^{2+}.

Index Entries: *Bacillus*; feather digestion; keratinase; poultry waste; protease.

Introduction

Animal wastes have been used as a nutrient source in crop and animal production. The large increase in the proportion of the poultry industry resulted in an enormous increase in waste requiring management. Feather waste, generated in large quantities as a byproduct of industrial poultry processing, is nearly pure keratin protein *(1)*. Native keratin protein is not degradable by common proteolytic enzymes such as trypsin, pepsin, and

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papain. However, this protein is recycled in the environment, and keratinolytic activity has been reported for species of *Streptomyces (1,2)*; *Bacillus (3,4)*; some fungi *(5,6)*; and, more recently, mesophilic Gram-negative bacteria *(7,8)*.

Feather waste is currently converted into feather meal, which 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 also destroy certain amino acids, resulting in a product with poor digestibility and variable nutrient quality *(9,10)*.

Enzymatic hydrolysis by keratinolytic microorganisms represents an alternative method to improve the nutritional value of feather waste. It has already been demonstrated that feather-lysate obtained by *Bacillus licheniformis* PWD-1 has similar nutritional features to soybean protein for feed use *(11)*. In addition, the use of crude keratinase increased the amino acid digestibility of raw feathers and commercial feather meal *(12)*. Therefore, the development of bioprocesses using keratinolytic microorganisms addresses three important needs: ecologic, because the degradation of feathers in the environment leads to production of sulfur compounds; economic, because the current processes are expensive; and nutritional, resulting in a product with better digestibility and nutritional value.

In this article, we describe the isolation and characterization of a novel keratinolytic strain able to accomplish the degradation of chicken feathers.

Materials and Methods

Medium and Culture Conditions

The basal medium used for isolation, maintenance, growth, and fermentation analysis of the feather-degrading microorganism contained 0.5 g/L of NaCl, 0.3 g/L of Na_2HPO_4, 0.4 g/L of NaH_2PO_4 (pH 7.0) supplemented with 10 g/L of raw feathers, or 10 g/L of feather meal and 15 g/L of agar for plates. Feathers were washed in distilled water prior to being sterilized by autoclaving (121°C at 105 kPa for 15 min). For growth determination, the microorganisms were cultured in 500-mL Erlenmeyer flasks containing 100 mL of raw feather medium. Growth was followed at 25, 30, 37, and 42°C with aeration by rotation of the flasks at 150 cycles/min. Aliquots of 0.5 mL were removed at different times, and cell counts were determined by plate count on nutrient agar (Difco BRL, São Paulo, Brazil) following standard procedures *(13)*. Samples were prepared for plating as described previously *(3)*. All experiments were done in triplicate.

Isolation of Microorganisms

Feathers were collected from several sites at a local poultry company (Avipal, Porto Alegre, Brazil). Feathers were flooded in phosphate-buff-

ered saline, and suspension was used to streak feather-meal agar plates, which were allowed to grow at 30°C for 1–5 d. Single colonies were screened for their ability to hydrolyze keratin in feather meal agar plates. Colonies producing clearing zones in this medium were selected for further work. The purity of isolates was checked by repeated streaking.

Characterization of Feather-Degrading Isolate

A feather-degrading isolate was identified according to morphologic examinations and several biochemical tests *(14)*, and using an API 50CH kit with automated interpretation by APILAB Plus software (BioMérieux, Marcy-l'Etoile, France).

Preparation of Enzyme

The isolate was cultivated for 48 h in whole-feather medium, pH 7.0, at 30°C from a 10^6 CFU/mL culture. The culture medium was centrifuged at 10,000*g* for 10 min, and the supernatant was used as enzyme preparation. The protein concentration of this enzyme preparation was 6.1 mg/mL, determined with Folin phenol reagent *(15)*.

Proteolytic Activity

Proteolytic activity was determined as described previously *(7)* using azokeratin as substrate. The reaction was initiated by pipetting 150 µL of enzyme into 250 µL of azokeratin (2% in 50 m*M* Tris, pH 8.0). The reaction mixture was incubated for 30 min at 50°C, and then 1.2 mL of 10% trichloroacetic acid was added. The contents were mixed and allowed to stand for 15 min to ensure complete protein precipitation. Samples were centrifuged at 10,000*g* for 5 min, 1.2 mL of the supernatant was transferred to a clean tube, and absorbance at 440 nm was determined. One unit of enzyme is the amount of enzyme that causes a change in absorbance of 0.01 at 440 nm/30 min at 50°C. Azokeratin was synthesized as described elsewhere *(16)*.

Analysis of Soluble Protein

Cell-free supernatant of each feather culture was used for determination of soluble protein by the Folin phenol reagent method *(15)*.

Effect of Chemicals on Keratinase Activity

Chemicals were added to the enzyme preparations and incubated for 15 min at 25°C before testing for proteolytic activity. The substances were used at the working concentrations provided in Table 1. Phenylmethylsulfonyl fluoride (PMSF) and *p*-chloromercuric benzoate were from Sigma (St. Louis, MO). EDTA, 1,10-phenanthroline, dimethylsulfoxide (DMSO), and 2-mercaptoethanol were from Merck (Darmstadt, Germany).

Table 1
Effect of Chemicals on kr16 Keratinase

Compound (working concentration)	Relative activity (%)[a]
None	100
PMSF (2 m*M*)	70
EDTA (5 m*M*)	25
1,10-Phenanthroline (5 m*M*)	60
DMSO (5 m*M*)	98
p-Chloromercuric benzoate (5 m*M*)	123
2-Mercaptoethanol (2.5 m*M*)	287
$CaCl_2$ (5 m*M*)	111
$MgCl_2$ (5 m*M*)	67
$ZnCl_2$ (5 m*M*)	75
$MnCl_2$ (5 m*M*)	103
$CoCl_2$ (5 m*M*)	133
$CuCl_2$ (5 m*M*)	44
KCl (5 m*M*)	56
$SnCl_2$ (5 m*M*)	0
$HgCl_2$ (5 m*M*)	0

[a]Relative activity was calculated considering the enzyme activity with no additions as 100%.

Results and Discussion

Isolation and Adaptation of Feather-Degrading Microorganism

Keratinolytic cultures were screened on feather meal agar. One rod-shaped bacterium demonstrated complete feather degradation in liquid medium and pronounced clearing zones on agar medium. The isolate was selected for the purpose of identification and to further characterize its feather-degrading properties. Cells of the isolate were grown on whole feathers and transferred at frequent intervals to the basal medium, containing whole raw feathers. We propose kr16 as the strain designation for this keratinolytic strain.

Characterization of Feather-Degrading Isolate

Identification of this bacterial isolate was based on cell morphology, colony morphology, growth characteristics, and several biochemical tests. Microscopic observation of the isolate showed a straight rod with endospores. The bacterium grew aerobically, was Gram-positive, and was strongly catalase positive. Collectively, the biochemical characteristics indicated that the isolate was a *Bacillus* sp. strain. Additionally, analysis by APILAB Plus indicated a very good identification; the isolate shared 99.2% identity with *Bacillus cereus*.

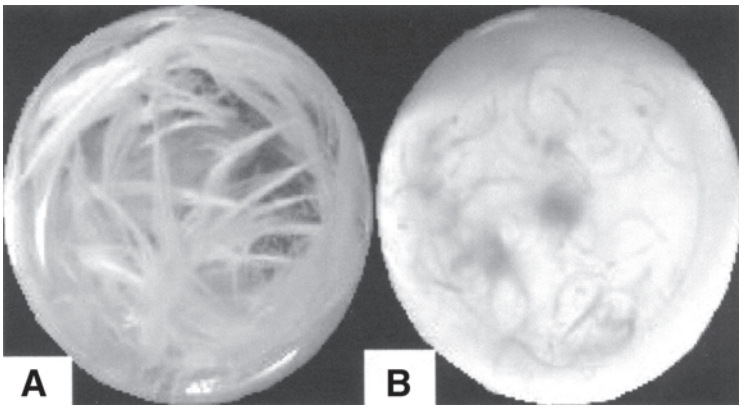

Fig. 1. Feather degradation by *Bacillus* sp. kr16: bottom of Erlenmeyer flask after 5 d of incubation with **(A)** control inoculum and **(B)** kr16 inoculum.

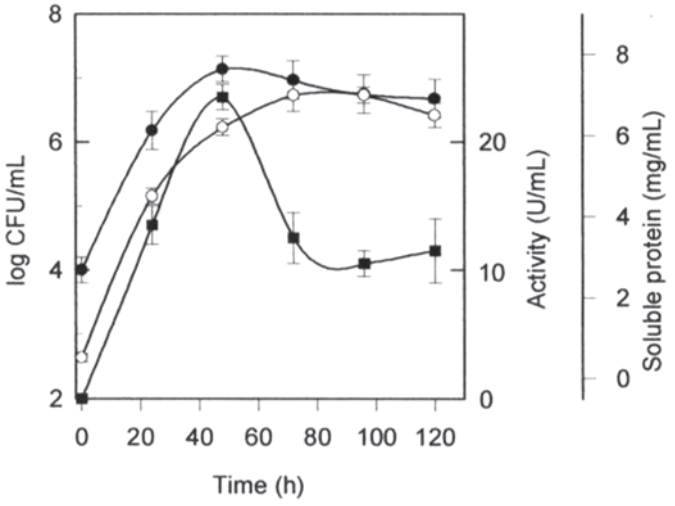

Fig. 2. Growth of *Bacillus* sp. kr16 on raw feathers at 30°C. The microorganism was grown on medium containing 10 g/L of native chicken feathers and monitored for viable counts (●), keratinolytic activity (■), and soluble protein concentration (○). Each point represents the mean ± SEM of three independent experiments.

Degradation of Feathers by Isolate

It was observed that aerobic growth of the isolate on raw feathers, with the feathers as its primary source of carbon, energy, nitrogen, and sulfur, resulted in degradation of the keratin protein after 5 d of incubation at 30°C (Fig. 1).

The microorganism grew well in a range of 25–42°C but did not at 55°C. Maximum growth and feather-degrading activity were observed at 30–37°C. The strain kr16 reached the end of exponential growth phase at 48 h during cultivation in raw feathers (Fig. 2). Keratinolytic micro-

organisms often have higher optimum temperatures of growth and feather degradation *(3,4,6)*. Keratinolytic activity of mesophilic organisms may have a relevant impact in natural systems *(8)* and may be an interesting property for biotechnological use because these microorganisms will be less energy consuming than the thermophilic ones.

The production of soluble protein was monitored. The maximum protein concentration was 6.8 g/L, observed from 72 h of growth (Fig. 2).

The production of keratinase by the organism was determined during feather biodegradation based on the hydrolysis of azokeratin. Generally, keratinolytic activity increased with cultivation time (Fig. 2), the maximum activity coinciding with the start of stationary phase. This was similar to results observed for *Streptomyces fradiae* growing on wool *(17)*, and *Vibrio* sp. kr2 growing on feathers *(7)*.

Feather waste biodegradation using microbial sources represents an alternative way to improve the nutritional value of feather, since feather waste is currently utilized on a limited basis as a dietary protein supplement for animal feedstuffs. The amino acid composition of keratin is characterized by a low content of lysine, which is the main limiting essential amino acid in food. Protein concentrates from keratin sources show a deficit of lysine, which can be enhanced by the addition of lysine-rich protein or by plastein reaction *(18)*. Indeed, the use of microbial keratinases to upgrade the nutritional value of raw feathers and feather meal has been described *(9,10)*.

Keratinase Properties

The effect of pH and temperature on keratinolytic activity was investigated. Maximum activity was observed between pH 8.0 and 11.0 at 50°C, with an optimum within this range (Fig. 3). The enzyme was active between 30 and 85°C, with an optimum at 45–65°C (Fig. 4). This optimum temperature for activity was in good agreement with that for other keratinolytic enzymes from *S. fradiae (19)*, and *Bacillus* spp. *(20,21)*. Data also suggest a highly alkali-stable proteinase, a favorable property for hair removal processes *(22)*.

Keratinase produced by kr16 was inhibited by EDTA and 1,10-phenanthroline and partially inhibited by PMSF (Table 1), suggesting that the supernatant fluid could contain several enzymes affecting keratinolysis. Similar results were observed for species of *Streptomyces (23)*. The stimulatory effect of 2-mercaptoethanol may be associated with a reduction of disulfide bridges of azokeratin, resulting in a substrate more susceptible to enzyme attack. Among the metal ions tested, Hg^{2+} and Sn^{2+} strongly inhibited the enzyme, whereas Ca^{2+} and Co^{2+} increased proteolytic activity. It has been suggested that the inhibition by Hg^{2+} ions is not just related to binding of the thiol groups but may be a result of an interaction with tryptophan residues or with the carbonyl group of amino acids in the enzyme *(24)*. Partial inhibition by KCl was observed. Monovalent cations such as Na^{+}

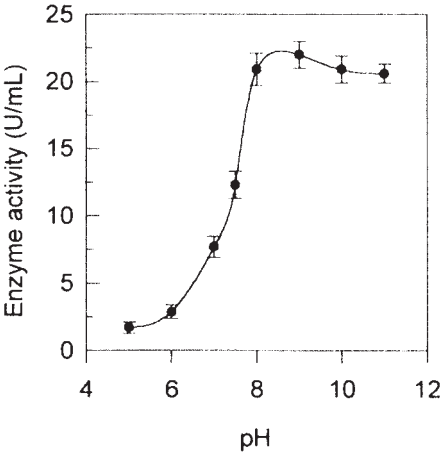

Fig. 3. Effect of pH on activity of kr16 keratinase. Activity was measured at 50°C against azokeratin in 50 m*M* citrate-phosphate buffer (pH 3.0–7.0) or 50 m*M* Tris (pH 8.0–11.0). Each point represents the mean ± SEM. of three independent experiments.

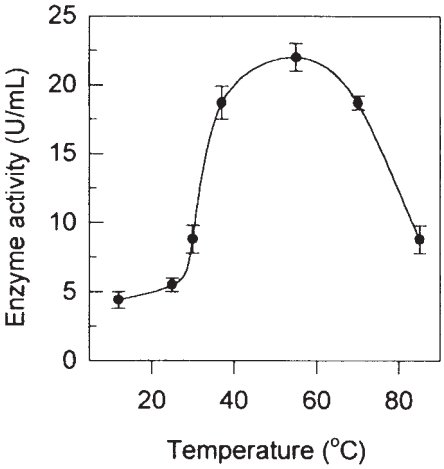

Fig. 4. Effect of temperature on activity of kr16 keratinase. Activity was measured at different temperatures against azokeratin in 50 m*M* Tris, pH 8.0. Each point represents the mean ± SEM of three independent experiments.

and K^+ can compete for divalent cation-binding sites, resulting some unexpected kinetics properties of enzymes *(25)*. The ions Zn^{2+} and Mg^{2+} showed a biphasic effect (Fig. 5), in agreement with the fact that some metallopeptidases are inhibited by excess metal ions, particularly at neutral to alkaline pH *(26)*. These data resemble some of the characteristics of keratinase of *Chryseobacterium* sp. *(16)*. The data also suggest that a metalloprotease and a serine protease may be involved in keratin degradation by *Bacillus* sp. kr16.

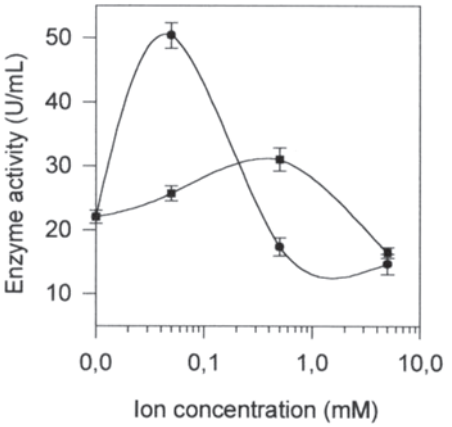

Fig. 5. Effect of magnesium (●) and zinc (■) concentration on activity of kr16 keratinase. The enzyme was preincubated for 15 min at 25°C with different concentrations of either $MgCl_2$ and $ZnCl_2$. A control with no additions was set as 100%. Each point represents the mean ± SEM of three independent experiments.

The strain kr16 shows potential for biotechnological use because it produces a keratinase that allows complete degradation of chicken feathers. Its activity at alkaline pH is also a desirable property for use in the leather industry.

Acknowledgment

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