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# Isolation and characterization of a feather-degrading bacterium from the poultry processing industry

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**A *Flavobacterium* sp. producing a high keratinolytic activity was isolated from a poultry industry after growth on selective feather meal agar. This bacterium grew on feather meal broth, producing keratinase, and was also capable of complete degradation of raw feathers. The proteolytic activity was assessed in the presence of specific protease inhibitors. The crude enzyme showed mainly metalloprotease character. This novel isolate would have potential biotechnological use in processes involving keratin hydrolysis.**
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## Introduction

Feathers, which are almost pure keratin protein, are produced in large amounts as a waste byproduct of poultry processing. At present, feathers are converted to feather meal, a digestible dietary protein for animals, using physical and chemical treatments. Processing of feather meal destroys certain amino acids influencing protein quality and digestibility [21]. Feather meal, consequently, is a poorly digestible feed ingredient.

The use of microbial or enzymatic treatment for nutritional upgrading of feather meal has been described. Feather meal fermented with *Streptomyces fradie* and methionine resulted in a comparable growth rate of broilers with those fed isolated soybean protein [4]. *Bacillus licheniformis* feather lysate with amino acid supplementation produced growth of chickens similar to that achieved with soybean meal [23]. The use of crude keratinase prepared from *B. licheniformis* significantly increased the total amino acid digestibility of raw feathers and commercial feather meal [9]. This enzyme increased digestibility of a commercial feather meal up to 82% and could replace up to 7% of the dietary protein for growing chickens [19].

Keratinolytic microorganisms and their enzymes may have important applications in biotechnological and industrial processing involving keratin-containing wastes from poultry and leather industries through the development of nonpolluting processes [13,18]. These enzymes are produced by some species of *Bacillus* [1,22], actinomycetes [3,24] and fungi [8,17].

Keratinolytic activity has been observed in microorganisms isolated from the poultry industry [16,22]. Recently, feather-degrading activity was associated with a *Vibrio* sp. strain isolated from decomposing feathers [16]. In this report, we describe the isolation and characterization of a novel proteolytic bacterium able to completely degrade raw feathers.

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## Materials and methods

### Reagents

Azocasein, phenylmethylsulphonyl fluoride (PMSF), *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and pepstatin were from Sigma (St. Louis, MO, USA). Other reagents were from Merck (Darmstadt, Germany). Azokeratin was synthesized as described elsewhere [20].

### Isolation and selection of microorganisms

Samples were collected at a poultry processing plant from several sites where feathers were deposited and incubated in 5 g $l^{-1}$ peptone solution at 30°C for 24 h. Bacterial suspensions were streaked on feather meal agar plates (10 g $l^{-1}$ feather meal, 0.5 g $l^{-1}$ NaCl, 0.3 g $l^{-1}$ $K_2HPO_4$, 0.4 g $l^{-1}$ $KH_2PO_4$ and 15 g $l^{-1}$ agar), which were incubated at 30°C for 3 days. Single colonies were isolated and screened for their ability to grow on feather meal agar. Feather-degrading capacity was determined on raw feather medium (10 g $l^{-1}$ native feather, 0.5 g $l^{-1}$ NaCl, 0.3 g $l^{-1}$ $K_2HPO_4$, 0.4 g $l^{-1}$ $KH_2PO_4$).

### Enzyme production

The organisms were cultivated in feather meal broth (10 g $l^{-1}$ feather meal, 0.5 g $l^{-1}$ NaCl, 0.3 g $l^{-1}$ $K_2HPO_4$, 0.4 g $l^{-1}$ $KH_2PO_4$) for up to 8 days at 30°C. Enzyme was obtained by centrifugation at 10,000×*g* for 5 min, and culture supernatants were used as crude enzyme preparations.

### Enzyme assay

Keratinase activity was assayed with azokeratin as a substrate [15]. The reaction mixture contained 100 $\mu$l of enzyme preparation and 500 $\mu$l of 20 g $l^{-1}$ azokeratin in 50 mM Tris buffer, pH 8. The mixture was incubated at 50°C for 15 min and the reaction was then stopped by the addition of trichloroacetic acid (final concentration, 100 g $l^{-1}$). After centrifugation at 10,000×*g* for 5 min, the absorbance of the supernatant fluid was determined at 440 nm. One unit of enzyme activity was the amount of enzyme that caused a change of absorbance of 0.01 at 440 nm in 15 min at 50°C. A

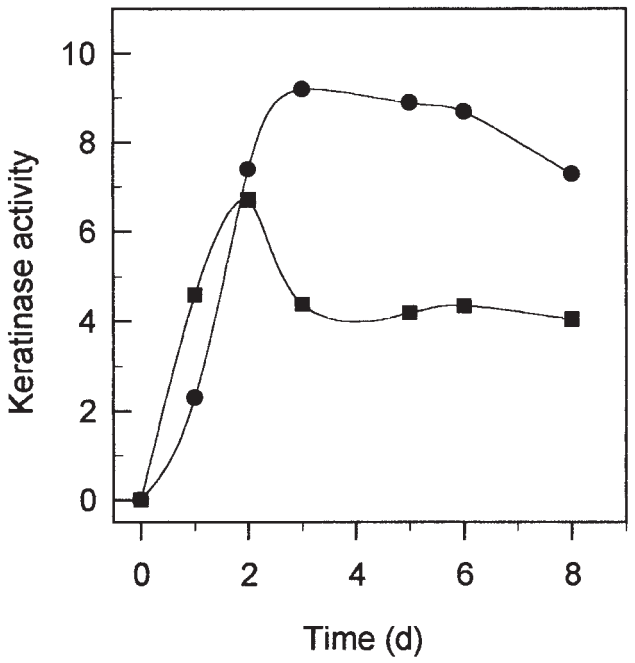


**Figure 1** Keratinase activity during growth of keratinolytic bacterium kr6 in feather meal broth. Enzyme activity was measured using azokeratin as substrate. Each point represents the mean of three independent experiments. (●) Volumetric activity (units $ml^{-1}$); (■) specific activity (units mg $protein^{-1}$).

similar protocol was used to determine enzyme activity on azocasein.

### *Enzyme inhibition*

The effects of protease inhibitors on azokeratin hydrolysis were measured. Culture supernatants of the selected isolates were used as enzyme sources. The protease inhibitors PMSF, ethylenediaminetetraacetic acid (EDTA), E-64 and pepsatin were used to inhibit serine, metallo-, cysteine and aspartic protease activities, respectively. The inhibitor working concentrations were 1 mM PMSF, 5 mM EDTA, 5 $\mu$M E-64 and 1 $\mu$M pepstatin [2]. The control consisted of azokeratin activity without inhibitors. The activity of each type of protease was calculated as follows: percentage of specific protease$=(A-A_i/A)\times 100$, where $A$ is the total activity without inhibitors and $A_i$ is the activity with specific inhibitor [11].

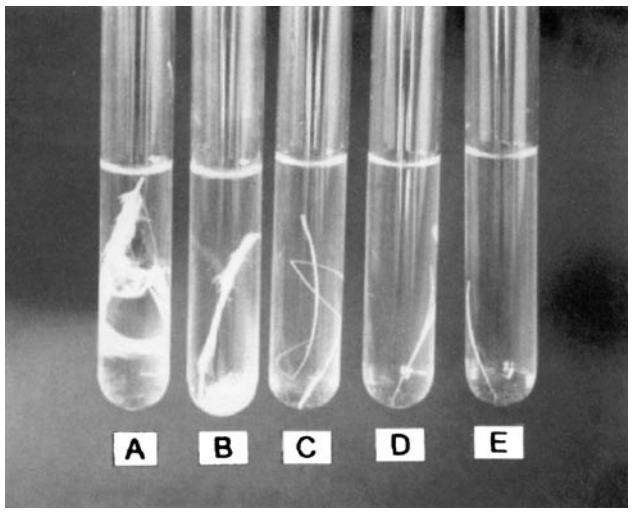


**Figure 2** Feather degradation by microorganism kr6 after (A) 1, (B) 24, (C) 48, (D) 72 and (E) 96 h.

**Table 1** Extracellular protease profile of isolate kr6[a]

| Enzyme | Activity (%) |
|---|---|
| Serine protease | 16.7 |
| Metalloprotease | 66.7 |
| Cysteine protease | 14.3 |
| Aspartic protease | 23.8 |

[a]Total activity was calculated by azokeratin hydrolysis at 50°C without inhibitors; percentages of specific activities were calculated as described in Materials and methods section.

### *Taxonomic studies*

Bacterial identification was conducted based on morphology and biochemical tests [12], and using an API20 kit (Bio-Mérieux, Marcy-l Étoile, France). The morphological, cultural and physiological characteristics of the isolated bacteria were compared with data from *Bergey's Manual of Determinative Bacteriology* [6].

## Results

### *Isolation of keratinase-producing strains*

Eight distinct colony morphologies were observed on feather meal agar plates. These isolates were isolated for the purpose of characterization of their keratin-degrading properties. They were grown on feather meal agar plates and transferred at frequent intervals to feather meal broth for adaptation to keratin degradation. The isolate, kr6, presented the highest feather-degrading activity among the isolates.

Growth and proteolytic activity were screened on milk agar plates. Strain kr6 grew at 22–46°C and produced its highest proteolytic activity at 30–37°C.

### *Keratinolytic activity*

Keratinolytic activity of the isolates was monitored during growth in feather meal broth. The keratinolytic activity of isolate kr6 reached maximum values after at 2–3 days of cultivation (Figure 1). An increase in pH was observed during growth on feather meal broth. Strain kr6 showed feather-degrading activity during growth in whole feather medium. Aerobic growth on minimal medium with raw feathers as its primary source of carbon,

**Table 2** Hydrolysis of azoproteins by proteases[a]

| Enzyme | Activity on azocasein (U/mg protein) | Activity on azokeratin (U/mg protein) | Keratin:casein ratio |
|---|---|---|---|
| Keratinase kr6 | 17.0 | 5.3 | 0.31 |
| Trypsin | 21.6 | 4.4 | 0.20 |
| Papain | 21.9 | 2.5 | 0.11 |
| Pronase | 26.5 | 6.7 | 0.25 |
| Alcalase | 36.9 | 11.4 | 0.30 |

[a]Commercial enzymes were dissolved at 1 mg $ml^{-1}$ in 50 mM Tris, pH 8.0, and then assayed for azoproteins as described in Materials and methods section.

energy, nitrogen and sulfur resulted in significant degradation of keratin after 4 days of incubation (Figure 2). All feather barbule and almost all feather rachis were degraded after 2 days. Based on these results, strain kr6 was selected for future characterization.

### *Characterization of strain kr6*

Identification of the feather-degrading bacterium, kr6, was based on cell morphology, colony morphology and several biochemical tests. Microscopic observation of the isolate, kr6, showed a Gram-negative rod without endospores. Strain kr6 produced yellow colonies. Together with the results of physiological tests, the characteristics indicate that kr6 belongs to the genus *Flavobacterium*. This strain was deposited in our culture collection under the identification LBM 9006.

### *Characterization of proteolytic activity*

Protease activity (azokeratin hydrolysis) was measured in the presence of protease inhibitors. Strain kr6 exhibited predominantly metalloprotease activity, but demonstrated some serine, cysteine and aspartic protease activities (Table 1).

The proteolytic activity of kr6 was compared with commercial enzymes by determining the hydrolysis of azokeratin and azocasein. Keratinase presented the highest azokeratin/azocasein ratio (Table 2). The enzyme preparation of kr6 was similar in hydrolysis of keratin substrates compared with commercially available microbial proteases such as pronase derived form *S. griseus* and alcalase from *B. licheniformis*.

## Discussion

A bacterium showing feather-degrading activity was isolated from a poultry processing plant. Strain kr6 was a Gram-negative rod with a broad temperature range of growth. Proteolytic activities were maximum in the range of 30–37°C, near the environmental temperature, whereas keratinolytic bacteria mostly show feather-degrading activity at elevated temperatures [1,22]. However, this strain behaves in a similar fashion to *Vibrio* strain kr2, previously isolated from decomposing feathers [16]. An increase in pH during cultivation was observed, which is indicative of the keratinolytic potential of microorganisms [7]. Preliminary identification tests indicate that kr6 is a *Flavobacterium* sp. This is the first report of keratin-degrading activity by *Flavobacterium* spp.

The strain showed mainly metalloprotease activity, but the effect of protease inhibitors on crude enzyme suggests that different proteolytic activities were present. Although raw feathers should not be attacked by any protease, many proteolytic activities could support the hydrolysis of resulting keratin peptides. Previously described keratinases are serine-type proteases [3,10,15]. *Bacillus* spp. isolated from canola meal compost also showed partial metalloproteolytic character, but these isolates exhibited weak keratinolytic activity when compared with the isolates where serine protease activity was predominant [11]. Metalloproteases are the most diverse of the types of proteases. They include enzymes from a variety of origins such as collagenases from higher organisms and microorganisms, and thermolysins from bacteria [2]. The keratinolytic activity produced by kr6 appears to belong to the group of alkaline metalloproteases since it was inhibited by chelating agents and was active in the pH range from 7 to 9, similar to that described for metalloproteases produced by *Pseudomonas aeruginosa* and *Serratia* spp. [14]. In addition, the proteolytic activity of *Flavobacterium meningosepticum* has been described, but it was related to a neutral metalloprotease with unique properties [5].

The azokeratin/azocasein hydrolysis ratio of kr6 was higher than for other proteases, suggesting higher specificity for the substrate keratin and consequently major utilization of keratin as nutrient source.

Considering that feather protein has been determined as an excellent source of metabolizable protein, and that microbial keratinases enhance the digestibility of feather keratin [9,19], *Flavobacterium* sp. kr6 could be used to produce feed protein. This novel isolate may have a potential biotechnological use in processes involving keratin hydrolysis.

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