A comparative study about decomposable ability of two thermophilic bacteria to keratin

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Abstract. Keratin is a refractory protein which widely exists as animal hairs, feathers and so on in nature. Because the structure of keratin becomes loose and easy to be degraded at high temperatures, thermophilic bacteria and keratinases have attracted extensive attention. Bacillus sp. WF146 and Thermoactinomyces sp. CDF are two thermophilic bacteria isolated from soil in our previous studies. They can both degrade keratin well. By changing the nitrogen source in the medium to cause nutritional stress, and monitoring protease degradation of feather substrate, we found that the expressed protease is the key to the degradation of keratin. The results showed that there were some differences in the feather degradation process of two strains, they may related to damaging disulphide bond of keratin with reducing power provided by the cells, and physics destroy the structure of feather. That is what led to their different feather-degrading characteristics.

1. Introduction
Keratin is a refractory protein which widely exists as animal hairs, feathers and so on in nature. Keratin wastes such as animal hairs and feathers can be transformed into available peptides and amino acids, which have significant application values in many fields such as fodder, leather treatment, foods and pharmaceuticals industry. Compared with traditional physical and chemical methods, bio-degradation of keratin wastes also has obvious environmental significance. Because the structure of keratin becomes loose and easy to be degraded at high temperatures, thermophilic bacteria and keratinases have attracted extensive attention.

Currently, known mesophilic keratin degradative bacteria mainly belong to Bacillus and Streptomyces [1]. Such as Streptomyces freundii [2], Streptomyces DSM 40540 [3] and Streptomyces thermophilus [4], Streptomyces flavis 2BG [5]. Though mechanism of keratin degradation are different in each microorganism, researchers generally believe that keratinase plays a key role. Onifade summarized three degradation mechanisms about the degradation pathway of keratin, mechanical keratinolysi, sulphitolysis and proteolysis [6].

These two thermophilic bacteria in our study may be ideal targets for comparative study of feather-degrading characteristics and mechanisms at high temperature. We would try to identify the key factors affecting the degradation of keratin by the two thermophilic bacteria, with feather be used as substrate. With analyzing the specific role of cells and proteases in the degradation process, the difference of feather degradation between the two strains was compared. Microscopic observation of feather degradation process be monitored to evidence for the mechanism of keratin degradation.
2. Material and method

2.1. Bacterial strains, media and growth conditions

Bacillus sp. WF146 and Thermoactinomyces sp. CDF were both isolated from the campus soil of Wuhan University, China. E. coli DH5α and E. coli BL21 (DE3) were used as the hosts for cloning and expression, respectively, and were grown at 37°C in Luria-Bertani (LB) medium supplemented. The components of the culture media used in this study were listed in Table 1. Chicken feathers collected from a local market were cleaned by washing with tap water, soaking in 70% ethanol for 1 h, rinsing in dH2O and air-drying, and then added to the medium. Culture media were sterilized by autoclaving at 121°C for 20 min. Strain WF146 and CDF was grown in 5 ml of LB medium at 55°C for 16-18 h with rotary shaking at 150 rpm and used as seed culture. Then, 0.5 ml of the seed culture was inoculated to 50 ml of a fresh LB, LBF, dLB or dLBF medium (Table 1) in a 250-ml flask and cultivated aerobically with rotary shaking at 150 rpm at 55°C. For cultivation of strain WF146 and CDF in YN or YNF medium (Table 1), cells from 1 ml of the seed culture was collected by centrifugation at 10,000 × g for 10 min, washed three times with sterile normal saline [0.9% (w/v) NaCl], and suspended in 1 ml of YN medium. Then the cell suspension was inoculated to 50 ml of a fresh YN or YNF medium and cultivated as described above. Bacterial growth was monitored by measuring colony forming unit (CFU) on LB agar plates incubated at 55°C for 16 h.

Table 1. Culture media used in this study

| Medium | Peptone | Yeast extract | NaCl | Feather |
|--------|---------|---------------|------|---------|
| LB     | 1       | 0.5           | 1    | -       |
| LBF    | 1       | 0.5           | 1    | 0.4     |
| dLB    | 0.1     | 0.05          | 1    | -       |
| dLBF   | 0.1     | 0.05          | 1    | 0.4     |
| YN     | -       | 0.05          | 1    | -       |
| YNF    | -       | 0.05          | 1    | 0.1     |

2.2. Purification of extracellular protease produced by strain WF146 and CDF

The culture supernatant of strain CDF grown at 55°C for 24 h in LB medium was recovered by centrifugation at 10,000 × g for 10 min. After dialysis against buffer A [50 mM Tris-HCl (pH 8.0), 5 mM CaCl2], the supernatant was subjected to affinity chromatography on a bacitracin-Sepharose™ 4B (Amersham Biosciences) column (1.6 cm × 20 cm) equilibrated with buffer A. After washing with buffer A, the enzyme was eluted with the same buffer containing 25% isopropanol. The eluted fraction was dialyzed against buffer A to remove isopropanol.

2.3. Enzyme activity assay

The proteolytic activity of the enzyme against azocasein (Sigma-Aldrich, St. Louis, MO, USA) or BSA was conducted at 60°C or 80°C for 20 min in 400 μL of reaction mixture containing 0.5% (w/v) azocasein or BSA and 200 μL of enzyme sample in buffer A. The reaction was terminated by the addition of 400 μL 40% (w/v) trichloroacetic acid (TCA). After incubation at room temperature for 15 min, the mixture was centrifuged at 13,000 × g for 10 min, and the absorbance of the supernatant was measured at 335 nm (A335) for azocasein or at 280 nm (A280) for BSA in a 1 cm cell. One unit (U) of activity was defined as the amount of enzyme required to increase the A335 or A280 by 0.01 U/min under the conditions described above.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of King and Laemmle [7]. To prevent self-degradation of the protease during sample preparation (boiling) or electrophoresis, the sample was precipitated by 20% TCA, and then washed.
with acetone before being subjected to SDS-PAGE. The gel was stained by Coomassie Brilliant Blue G-250.

3. Conclusion
Growth and extracellular protease production under oligotrophic conditions
It is obviously nutritional deficiency in YN/YNF medium, compared with LB medium. Chicken feather is the main carbon and nitrogen source in YNF medium. The growth and extracellular protease production of Bacillus sp. WF146 and Thermoactinomyces sp. CDF are mainly affected by chicken feather substrate. The growth curve and extracellular protease activity were showed in Fig.1. Due to the lack nutrients, neither WF146 nor CDF can grow in medium YN, and few extracellular protease activity was detected. Both the two strains could grow in medium YNF, and displayed obviously protease activity.

![Figure 1](image-url)

**Figure 1.** Growth curves and extracellular protease activity were showed in this figure (Figure A is strain WF146 and figure B is strain CDF.). Strains were grown at 55°C in YN/YNF medium. Feather degradation by the strains under oligotrophic conditions. The data are expressed as means ± SD (bars) of three independent experiments.

3.1. Comparison of keratinolytic activities between protease WF146 and C2 in vitro
Protease WF146 and C2 from the two strains were recombinant expressed in E.coli BL21 (DE3) and purified [8, 9]. We adjust the concentration of these two enzymes that the samples with uniform Azocasein activity were obtained. The keratinase activity of these two proteases was compared under the same conditions as shown in Fig.3. Feathers was degraded by protease C2 under reducing conditions within 30 min, and the feathers completely disappear in 1 h or so. It required about 4 h to decompose feathers under reducing conditions by protease WF146. This difference indicated that protease C2 may has stronger substrate preference for feather than protease WF146.
Figure 2. Extracellular protein were showed in this figure. Strains were grown at 55°C in dLBF medium (Figure A is strain WF146 with CaCl2 added, and figure B is strain CDF). Feather degradation by the strains under oligotrophic conditions. Lane 1. Soluble components of the culture. Lane 2. Recombinant protease as control. Arrows show the mature enzymes of WF146 (A) or C2 (B).

3.2. Expression of the major protease of strain WF146 and strain CDF
To isolate keratinolytic proteases of strain WF146 and strain CDF, SDS-PAGE were used to detect the soluble components of the culture mediums (dLBF). As shown in Fig.2, a 32-kDa protein (named protease WF146) and a 30-kDa protein (named protease C2) were found, which the main protein in the culture was. According to previous studies [8, 9], they are both the major protease in WF146 and CDF cultures.

Figure 3. Degradation of chicken feathers by recombinant protease WF146 (A) or C2 (B). Sterilized chicken feathers (2 mg) were incubated in 0.8 mL of 50 mM Gly–NaOH (with 0.5%β-Me) at 60°C supplemented with 60 μg/mL protease WF146 or C2.
3.3. Comparison of feather degradation between strain WF146 and CDF in culture

The aggregation of cells around the feathers was showed in Fig.4. Initially, only a few cells aggregated. After 24 hours, a large number of cells surrounded the feather fragments, especially over the fragment breakage. The phenomenon was more significant that mycelium of CDF was obviously concentrated. Both strains WF146 and CDF can degrade feathers in culture, while reducing power may be indispensable in the degradation process for protease WF146 and C2 in vitro. It indicated that the strains WF146 or CDF provide something like reductive power by cells.

As the results above, the main protease components in the soluble components of the culture under oligotrophic conditions were WF146 and C2, they can degrade feathers prominently under reducing conditions. Both the strains can degrade feathers in dLBF and YNF medium, under the conditions without reductive force added also. We observed that these bacteria were concentrated at the break of feathers (Fig. 4). It may indicated the reduction force was provided by these cells. However, there are significant differences in cell morphology between the two strains that WF146 is a bacterium while CDF have mycelial structure. Considering the faster degradation process of feather by strain CDF, we indicated that strain CDF may contribute physical force in the affection of mycelium.

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