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# Characterizations of Two Bacterial Strains Showing High Keratinase Activities and Their Synergism in Feather Degradation

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## ABSTRACT

On the basis of physiological and biochemical tests using API LAB II plus computer programe analysis, two feather-degrading bacteria, KUB-K0006 and KUB-K0082 were identified and classified as *Bacillus licheniformis* and *B. pumilus*. Synergistic action of crude enzyme fluids from KUB-K0006 (CFL) and KUB-K0082 (CFP) with the ratio of 5:1 showed higher amount of digested feather than the one obtained from CFP and CFL alone at 3.04 and 2 times respectively, while the ratio of 1:5 showed higher amount of amino acid released than the one obtained from CFP and CFL alone at 1.13 and 1.77 times . The addition of 5 times, CFL however created the values of %pepsin digestibility to be 1.4 and 1.2 times of the action of CFP and CFL alone . When the suitable amount of enzyme was studied, it was found that 500-1000 units of CFL:CFP, 5:1, hydrolyzing feather provided the percentage of pepsin digestibility to be more than 75. Comparing to the commercial enzyme allzyme BPD, the comparative equally % pepsin digestbility obtained showed no significantly difference when 1,000 units was used.


**Key words :** *Bacillus*, keratinase, feather, feather lysate, amino acid

## INTRODUCTION

Feather waste was utilized on a limited basis as a dietary protein supplement for animal feedstuffs due to poor digestibility. Currently, steam pressure-cooked treatment was used to make the feather more digestible (Dalev, 1994). However, this process required significant energy and also destroyed certain amino acids (Papadopoulos and Ketelaars, 1986). Therefore biological treatment was another choice to improve the nutritional value of feather waste (William *et al*., 1990). In this study, two aerobic bacteria isolates possessing effective keratinase with high feather digestibility at wide pH ranges and high temperature up to 50°C were screened from feather waste and soil. These two isolates with different action namely K6 and K82 exhibited high feather digestion and free amino acid released respectively (Nitisinprasert & Kaewsompong, 1997). In this paper, the result of classification and synergistic action of enzymes from these two strains are reported.

## MATERIAL AND METHODS

### Bacterial strains

The keratinolytic strains K6 and K82 were isolated from chicken waste and soil respectively (Nitisinprasert & Keawsompong, 1997). Both strains K6 and K82 have been stored at the

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Department of Biotechnology under the new name as KUB-K0006 and KUB-K0082 respectively.

**Biological tests**

The API 20E and API 50CH Kit (API Laboratory products Ltd. biomerieus Saa, France) were used to determine biochemical and carbohydrate fermentation patterns respectively according to the manufacturer. The experiment was performed at 37°C under aerobic condition. The results of biochemical and carbohydrate fermentation were determined after 24 and 48 hrs. and analyzed by computer using API LAB II plus programe.

**Morphology and Motility**

Cell shape and gram stain of 24 h cultures grown on NA medium were examined by electron microscope (JSM 5410 LV, Joel). The motility was investigated by the method of Atlast (1995).

**Temperature growth determination**

One colony from a 1 d culture was inoculated into NB medium and grown for 24 and 48 h at 30, 40, 45, 50, 55, 60 and 65°C. The growth was measured by the turbidity at 660 nm (Koch, 1981)

**Culture conditions for enzyme production**

BBF medium, prepared according to the modified method of William *et al.* (1990), was used for the growth of the bacterial strains KUB-K0006 and KUB-K0082. It contained 0.5 (g/l) $NH_4Cl$, 0.5 NaCl, 0.354 $K_2HPO_4.3H_2O$, 0.4 $KH_2PO_4$, 0.24 $MgCl_2.2H_2O$, 0.1 yeast extract, and 10 ground feather. Crude enzyme preparation was prepared as the following: One colony from the overnight cultures was inoculated into 5 ml of NB medium and grown overnight at 37°C. The cultures were then transferred to a 500 ml of Erlenmeyer flask containing 100 ml of NB with 1% hammer-milled feather and grown on an incubator shaker (150 rpm) at 37°C for 5 days. The cultures were centrifuged for 15 min at ca. 5,000g and then the supernatant was used for feather digestion experiment.

**Determination of feather digestion for synergistic action studies**

50 ml of enzyme solution with various ratios giving total enzyme activity of 50 units was added into 500 ml Erlenmeyer flask containing 200 ml of 50 mM Potassium phosphate buffer pH 7 and 2.5 g ground feather. The digestion reaction was incubated on shaker with the velocity of 150 rpm at 50°C for 1 h.

50 ml of 10% Trichloroacetic acid was used to stop the reaction. The residual of feather lysate was determined by filtering 20 ml of the reaction mixture through No. 4 (Whatman) filter paper. The residual feather was collected and determined for the digested feather according to Helrich (1990). The filtrate solution (solution A) was used for further free amino acid determination.

**Analysis of free amino acid content**

Free amino acid (FA) contents were determined by using a modified method of Rosen (1957). Solution A (1 ml) was added into the solution containing 0.2 mM cyanide-acetate buffer pH 5.3, 0.5 ml and ninhydrin solution, 0.5 ml. After incubating at 100°C for 15 min, 5 ml of isopropanol-water was added immediately and shaken vigorously. The mixture was cooled and the absorbance was measured at 570 nm by uv-visible spectrophotometer UV1201 (Shimazu). Measurement of free amino acid contents was based on the determination of free leucine.

**Determination of %pepsin digestibility**

Pepsin digestibility was carried out according to the method of Helrich, AOAC Official Methods of Analysis (1990).

**Statistic analysis**

Data were analyzed as a completely randomized design. Treatment means were separated using a significant F-test (Steel and Torrie, 1960)

## RESULT AND DISCUSSION

**Characteristics of the isolates**

KUB-K0006 and KUB-K0082, which showed strong protease and feather degradation activities, were identified as gram positive, subterminal sporeforming aerobic bateria (Nitisinprasert and Keawsompong, 1997) . Therefore, they were classified as the members of the genus *Bacillus* (Krieg and Holt, 1984).

KUB-K0006 grown on NA medium was straight rods, 0.5 by 2.0 μm, occuringly singly or in pairs and motile, whereas KUB-K0082 was swollen rod, 0.5 by 1.5 mm, occurring singly and motile. The colonies surface of both isolates observed after 24 h on NA medium were irregular raised and curld. However, they were different with colony characterization. The colonies of KUB-K0006 were opaque and cream color while those of KUB-K0082 were convex, undulate and white color.

The physiological properties of these two isolates were studied as the following results. Both of them could grow at the temperature of 30, 40, 45 and 50°C, but not at 55 - 65°C. This indicated that they did not belong to *B. acidocaldarius, B. brevis, B. coagulans, B. schlegelii* and *B. stearothermophilus* (Krieg and Holt, 1984).

According to fermentation test (Table 1), both isolates were different among growing on sorbose, rhamnose, inositol, sorbitol, α-methyl-D-mannoside, glycerol, α-methyl-D-glucoside, N-acetyl-glucosamine, maltose, lactose, melibiose, inulin, starch, glycogen and D-turanose. With fourteen biochemical tests, it was found that both isolates were able to produce β-galactosidase, catalase and hydrolyze gelatin but none of them was able to utilize citrate and produce hydrogen sulphide, arginine dihydrolase, lysine decarboxylase, urease, tryptophane desaminase, ornithine decarboxylase and indole. They were also different in production of acetoin, oxidase activity and nitrate reduction as shown in Table 2.

Due to computer analysis using API LAB II plus software (Berkeley and Goodacre, 1992), the isolates KUB-K0006 and KUB-K0082 were classified as *Bacillus licheniformis* and *B. pumilus*, respectively.

**Effect of different ratios of crude enzymes from *B. licheniformis* KUB-0006 (CFL) and *B. pumilus* KUB-0082 (CFP) on synergistic activity**

Most large organic compounds such as cellulose, hemicellulose and starch are actively degraded by mixed enzyme components which play either exo- or endo-acting to give mono- or disaccharides (Enari and Niku-Paavola, 1987; Eriksson and Wood, 1985). The previous paper presented the keratinases from these two strains to be different in feather digestion and free amino acids released. *B. licheniformis* KUB-K0006 showed high feather digestibility of 91.83 % and low free amino acid released of 28.47 μmole (as leucine) whereas *B. pumilus* KUB-0082 exhibited lower feather digestibility of 77.85 %but higher free amino acid released of 63.63 μmole (as leucine) (Nitisinprasert and Keawsompong, 1997). Therefore it was interesting to figure out the action of these two enzyme components. The mixture of these two enzyme components varying CFL : CFP ratios of 0:1, 1:5 , 1:1, 5:1 and 1:0 studied are shown in Figure 1. It was found that % digested feather obtained by using enzyme solution from the strain KUB-K0006 alone (1:0) was higher than that of the one from the strain KUB-K0082 (0:1) for 1.55 times. However, the value of amino acid released from the former was oppositely lower than that of

**Table 1** Comparison on fermentation test of the strains KUB-K0006 and KUB-K0082.

| Carbon sources | KUB-K0006 | KUB-K0082 |
|---|---|---|
| Glycerol | + | + |
| Erytritol | - | - |
| D-Arabinose | - | - |
| L-Arabinose | + | + |
| Ribose | + | + |
| D-Xylose | + | + |
| L-Xylose | - | - |
| Adonitol | - | - |
| β-Methyl-D-xyloside | - | - |
| Galactose | + | + |
| Glucose | + | + |
| Fructose | + | + |
| Mannose | + | + |
| Sorbose | + | - |
| Rhamnose | + | - |
| Dulcitol | - | - |
| Inositol | + | - |
| Mannitol | + | + |
| Sorbitol | + | - |
| α-Methyl-D-mannoside | - | + |
| α-Methyl-D-glucoside | + | - |
| N-Acetyl-glucoside | - | + |
| Amygdalin | + | + |
| Arbutin | + | + |
| Esculin | + | + |
| Salicin | + | + |
| Cellobiose | + | + |
| Maltose | + | - |
| Lactose | + | - |
| Melibiose | + | - |
| Sucrose | + | + |
| Trehalose | + | + |
| Inulin | + | - |
| Melezitose | - | - |
| Raffinose | + | + |
| Starch | + | - |
| Glycogen | + | - |
| Xylitol | - | - |
| Gentibiose | + | + |
| D-Turanose | + | - |
| D-Lyxose | - | - |
| D-Tagatose | + | + |
| D-Fucose | - | - |
| L-Fucose | - | - |
| D-Arabitol | - | - |
| L-Arabitol | - | - |
| GlucoNaTe | - | - |
| 2-Keto-Gluconate | - | - |
| 5-Keto-Gluconate | - | - |

+, positive result on fermentation; -, negative result on fermentation.

**Table 2** Comparison of biochemical test between KUB-K0006 and KUB-K0082.

| Biochemical Test | KUB-K0006 | KUB-K0082 |
|---|---|---|
| α-Galactosidase | + | + |
| Arginine dihydrolase | - | - |
| Lysine decarboxylase | - | - |
| Citrate utilization | - | - |
| Hydrogen sulfide production | - | - |
| Urease | - | - |
| Tryptophane deaminase | - | - |
| Indole production | - | - |
| Acetoin production | + | - |
| Gelatin hydrolysis | + | + |
| Catalase | + | + |
| Oxidase | + | - |
| Nitrate reduction | + | - |
| Ornithine decarboxylase | - | - |

+, positive result on production and utilization; -, negative result on production and utilization

the strain KUB-K0082 for 1.56 times. These results are the same as those reported in the previous study (Nitisinprasert & Kaewsompong, 1997). Comparing to the action of CFP alone (0:1), the addition of CFL with the CFL:CFP ratio of 1:1 and 5:1 clearly resulted in higher activities of both contents of digested feather for 1.4 and 3.04 times, and pepsin digestibility for 1.1 and 1.4 times but lower activities in amino acid released for 1.12 and 1.31 times respectively. On the other hand, comparing with CFL alone, the CFL:CFP ratios of 1:1 and 1:5 gave higher activities in amino acid released for 1.39 and 1.77 times respectively. Therefore, adding either of them clearly showed the synergistic activity by increasing of digested feather, pepsin digestion or amino acid released. The highest digested feather (28.6 mg) and pepsin digestibility (58.15%) were obtained at the CFL:CFP ratio of 5:1 as well as the highest amino acid released (10.13 μmole) obtained at the CFL:CFP ratio of 1:5.

It was interesing to note that the CFL:CFP ratio of 5:1 increased twice the content of digested feather, as comparing to the CFL alone (1:0). To consider the potential use of feather lysate by pepsin digestibility (Latshaw, 1989), the CFL:CFP ratio of 5:1 also exhibited the highest activity in pepsin digestibility. Therefore, *B. licheniformis* KUB-K0006 keratinase (CFL) was chosen to optimize its amount with fixing amount of CFP in the next experiment.

**Investigation on the suitable ratios of CFL and CFP**

A combination of CFL and CFP with total activity 357 units varied as follows: 0:1, 2.5:1, 5:1, 10:1 and 15:1 were carried out. These results are shown in Figure 2. It reveals the increasing of digested feather to 2.18, 3.14, 3.4, and 3.5 times respectively but the amino acid released was reduced

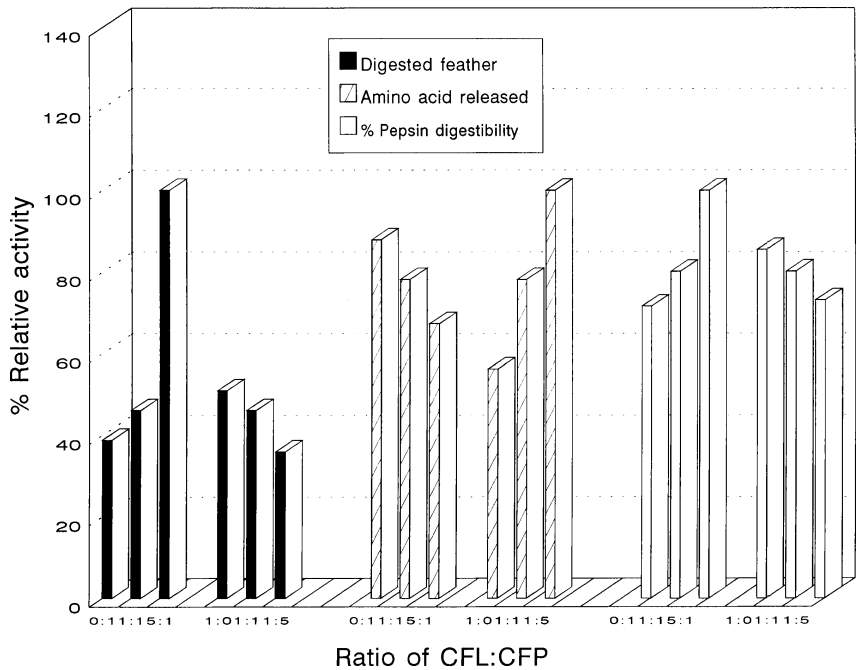


**Figure 1** The effect of various CFL and CFP ratios on feather digestion.

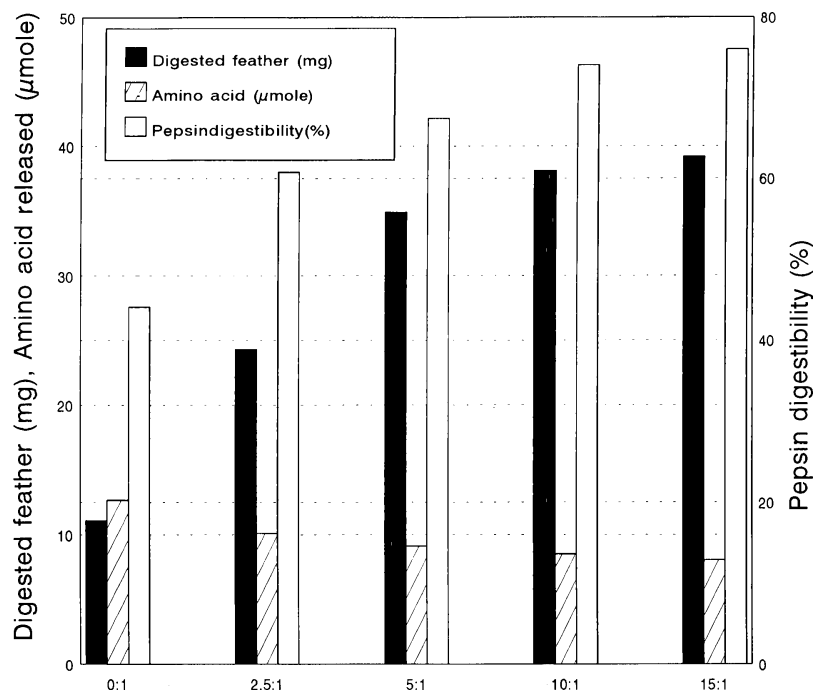


**Figure 2** The effect of the increasing portions of CFL on feather digestion.

to 1.5 times. Moreover pepsin digestibilities at the CFL:CFP ratios of 2.5:1, 5:1, 10:1 and 15:1 increased to 1.37, 1.53, 1.67 and 1.72 times of CFP alone respectively. The result clearly showed that the digested feather content and % pepsin digestibility increased rapidly at the ratio of 0:1, 2.5:1 and 5:1, and then the increment slowed down at that of 10:1 and 15:1. Therefore the ratio of 5:1 will be used for further study.

**Investigation on the suitable amount of keratinase**

According to the American Association of Food and Control Official (AAFCO), the available use of feather lysate should provide higher than 75% of pepsin digestibility (Davis *et al.*, 1961). Therefore, 357, 500, 700 and 1000 units of the combination CFL:CFP ratio of 5:1 were investigated as shown in Table 3. The result showed 500 - 1000 units of the combination to be able to digest 2.5 g of feather and provided higher than 75 % of pepsin digestibility.

**Comparison on the preparative enzymes and commercial enzyme solutions to feather digestion**

1000 units of the preparative enzyme (the combination of CFL : CFP ratio of 5:1) and the commercial enzyme were used to test for feather digestion under the same condition. It was found that the preparative enzymes released higher amount of amino acid but lower digested feather than the commercial enzyme did ($P>0.01$). However % pepsin digestibility values obtained from both sources were nonsignificant different ($P<0.05$) as shown in Table 4. Therefore, the preparative enzyme mixture might be used in substitution of the commercial enzyme imported for the future.

## CONCLUSION

Keratinases from *B. licheniformis* KUB-K0006 and *B. pumilus* KUB-K0082 clearly showed the different actions on high digested feather and amino acid released respectively. Comparing to cellulase system, exoglucanase and endoglucanase were attac hed to cellulose by exo- and endo- acting at the end chain and random respectively (Eriksson and Wood, 1985). Therefore, it is possible that *B. licheniformis* KUB-K0006 keratinase with fast feather degradation might act as an endo-acting, whereas *B. pumilus* KUB-K0082 keratinase with low feather degradation but high amino acid released might act as an exo-acting. The combination of these two kinds of keratinase clearly showed the

**Table 3** Effect of amount of enzymes with CFL:CFP = 5:1 on feather digestion, amino acid released and pepsin digestibility.

| Amount of enzyme (units) | Digested feather (mg) | Amino acid released (μmole) | Pepsin digestibility of residual feather (%) |
|---|---|---|---|
| 357 | 34.9 | 9.12 | 67.43 |
| 500 | 36.7 | 10.16 | 75.06 |
| 700 | 39.0 | 12.16 | 79.87 |
| 1000 | 41.2 | 13.61 | 85.70 |

Note: 2.5 g of ground feather was used for each reaction

**Table 4** Comparison of preparative enzyme mixture and commercial enzyme for feather digestion, amino acid released and pepsin digestibility.

| Source of enzyme | Digested feather (mg) | Amino acid released (μmole) | Pepsin digestibility of residual feather (%) |
|---|---|---|---|
| Preparative enzyme (CFL:CFP, 5:1) | 41.2a | 13.61c | 85.7e |
| Commercial enzyme (Allzyme PBD) | 50b | 10.27d | 89.4e |

Note: The enzymatic reaction was performed by using 1,000 units of enzyme carried out at 50°C for 1 h.
a and b showed significant difference of feather digestion at P>0.01
c and d showed significant difference of amino acid released at P>0.01
e showed nonsignificant difference in pepsin digestibility at P<0.05

synergism. The mixture of keratinolytic enzymes from KUB-K0006 and KUB-K0082 promised the potential use for feather lysate production from feather waste. However studies on optimization of enzyme production and enzyme system are still needed for industrial application.

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