Comparison of Two Feather-Degrading *Bacillus Licheniformis* Strains

Xiang Lin[1], Soo-Won Lee*, Hee Dong Bae, Jim A. Shelford[1] and Kuo-Joan Cheng[1]

Dept. of Food and Life Science, Sungkyunkwan University, Suwon 440-746, Korea

ABSTRACT : *Bacillus licheniformis* strains L-25 and PWD-1 are two thermophilic feather-degrading bacteria. Despite isolated from different environmental conditions, they were both capable of breaking down chicken feathers and growing in a medium in which feather was the only source of carbon and nitrogen. A 1.46-kb keratinase gene (*ker B*) was isolated from strain L-25 by a polymerase chain reaction (PCR) using L-25 genomic DNA as templates. Sequencing results reveal that *ker B* shares great sequence identity with a previously published keratinase gene of *B. licheniformis* PWD-1 (*ker A*). Only two amino acids differences were found in the deduced amino acid sequence between the keratinases from L-25 and PWD-1. However several nucleotide changes were found upstream of the putative promoter region. Protease inhibition studies indicated that neutral protease activity accounted for approximate 25 to 30% of total extracellular proteolytic activity produced by strain L-25 in the feather medium. In contrast, no measurable neutral protease activity was produced by strain PWD-1 in the feather medium. When glucose (1%), a common catabolic repressor, was added into the feather medium, L-25 was still able to grow and produce keratinase. Strain PWD-1 produced no neutral protease activity and its growth was severely inhibited in the feather medium containing glucose. L-25 produced an enhanced level of keratinase in the feather medium in comparison with PWD-1. *(Asian-Aust. J. Anim. Sci. 2001. Vol 14, No. 12 : 1769-1774)*

Key Words : *Bacillus*, Protease, Feather and Keratinase

INTRODUCTION

Keratin is resistant to common proteolytic enzymes and poorly digested by most organisms. However, keratin does not accumulate in nature. Biological processes may be presumed to accomplish its removal. Studies on keratinolytic microorganisms have been carried out by several laboratories in the past of few decades (Daniels, 1953; Deshmukh and Agraval, 1982; Koh et al., 1958; Noval and Nikerson, 1958; Wawrzkiewicz et al., 1987; Williams and Shih, 1989). A number of microorganisms have been reported to degrade different sources of keratin, such as human and animal hair, hoof, feather and wool. Noval and Nickerson (1958) and Nickerson et al. (1963) isolated a *Streptomyces fradiae* strain that hydrolyzes feathers. Koh et al. (1958) reported that keratinolytic enzymes were produced by *Aspergillus* strains. Williams and Shih (1989) and Williams et al. (1990) isolated a feather-degrading *B. licheniformis* strain from an anaerobic poultry waste digester. Some dermotophytes, such as *Microsporum canis* (Daniels, 1953), *M. gypseum* (Deshmukh and Agraval, 1982), *Trichophyton gallinae* (Wawrzkiewicz et al., 1987) and *T. Mentagrophytes* (Yu et al., 1968), also hydrolyze variable sources of keratins. It is understood that extracellular proteolytic enzymes or keratinase executed keratin degradation.

As a by-product of the poultry industry, feather is an inexpensive feed constituent with a very high protein content (~ 90% vs. ~ 45% in soybean). To a large extent, feathers are collectable from poultry processing plants. With proper treatments, feathers can be a significant alternative protein resource to meet the increasing demands for dietary protein in animal industry (Sullivan and Stephenson, 1957). So far, feathers are converted into feather meal and used as a supplement in diets for chicken and swine. However, the digestibility of feather keratin is still low due to its insolubility in aqueous solvents and resistance to most proteolytic enzymes (Lin et al., 1992; Lee and Moon, 1997). Keratinolytic bacteria and their keratinases offer an alternative to convert feather keratin into digestible feed protein for animals (Shih and Lee, 1993). Data from Shih's laboratory indicated that feather-lysate, a product of fermented feather by *B. licheniformis* PWD-1, was digestible, with a nutritional value comparable to soybean meal in support of chicken growth (Shih and Lee, 1993; William et al., 1991). Crude preparation of keratinase from PWD-1 enhanced the digestibility of feather meal when only 0.1% (w/w) of the keratinase was incorporated into broiler diets (Shih and Lee, 1993).

This paper presents evidence from the studies of the keratinase gene, protease production and profile, and other features of these two *B. licheniformis* strains, which further supports that diverse microorganisms may contribute to the removal of feathers in nature.

MATERIALS AND METHODS

Bacterial strains, vectors and culture media

B. licheniformis PWD-1 (ATCC 53757) was kindly provided by Dr. Jason C. H. Shih of North Carolina State

University. *B. licheniformis* L-25 (ATCC202179) was isolated by this laboratory as previously described (Williams et al., 1991). *E. coli* competent cells INV F' and PCR cloning vector were purchased from Invitrogen Corporation (San Diego, CA). Protease-deficient strain *B. subtilis* DB104 (Kawamura and Doi, 1984) and plasmid pUB18-P43 were provided by Dr. S.-L Wong of the University of Calgary. The feather medium contains basal salts (NaCl, 0.5 g/l; MgCl$_2$·6HO, 0.1 g/l; CaCl$_2$, 0.05 g/l; KH$_2$PO$_4$, 0.35 g/l; K$_2$HPO$_4$, 0.7 g/l; pH7.0) and 1% feathers (W/V). A milk-agar plate (NaCl, 0.5 g/l; MgCl$_2$·6H$_2$O, 0.1 g/l; CaCl$_2$, 0.02 g/l; KH$_2$PO$_4$, 0.35 g/l; K$_2$HPO$_4$, 0.7 g/l; skim milk, 25 g/l; agar, 18 g/l; pH 7.0) was employed to select clones that produce proteolytic activity.

Growth of bacteria and proteolytic activity assay

Strains L-25 or PWD-1 were inoculated and grown in test tubes (20 × 1.5 cm) containing 5 ml of nutrient broth as seed culture. After grown for 6 to 8 h, this seed culture was inoculated into 200 ml feather medium (2% inoculum). This feather medium was cultivated at either 37 or 50°C with shaking (180 rpm). Samples were taken at 12 hours interval for activity assay. Both strains were also grown in feather medium supplemented with 1% glucose (W/V) to test the repressive effect of glucose on protease expressions.

Two methods were employed for the determination of extracellular proteolytic activities. Keratinolytic activity was measured by the hydrolysis of azokeratin, a keratin derivative. Azokeratin was prepared by reacting ball-milled feather powder with sulfanilic acid and NaNO$_2$ using the method described by Tomarelli et al. (1949) for the preparation of azoalbumin. Five mg of azokeratin was used to assay keratinolytic activity at 50°C as described by Lin et al. (1992). Caseinolytic activity was determined as described Fontecha et al. (1996) by a reaction using azocasein (Sigma Chemical Co., St. Louis, MO) as a substrate. This assay was carried out in a tube containing 0.1 ml of culture supernatant (or appropriate diluted sample) and 0.4 ml of azocasein solution (5 mg/ml in 50 mM potassium phosphate buffer, pH 7.5) for 15 min at 50°C. The reaction was then terminated by the addition of 0.5 ml of 4% trichloroacetic acid solution (W/V). After centrifugation (14,000 rpm, 5 min), the absorbency of supernatant was read at 450 nm. For better comparison, one unit of keratinolytic or caseinolytic activity was defined as an increase of 0.01 absorbency unit at 450 nm per min at 50°C.

Proportions of serine and neutral proteases

Culture supernatants taken at peak activity were used to analyze the combination of proteases produced. Two common protease inhibitors, PMSF and EDTA were employed to eliminate serine and neutral protease activities

in the culture supernatants. The working concentrations of PMSF and EDTA were used as recommended: PMSF, 1 mM; EDTA, 5 mM (Salvesen et al., 1989). The control treatment consisted of azocasein without inhibitors. The percentage of each type of protease was calculated as follows:

% of specific protease = A − Ai / A × 100

Where A is the total activity measured by azocasein hydrolysis with absence of any inhibitors, and Ai is caseinolytic activity determined when a specific inhibitor was present.

Isolation and cloning of keratinase gene (*ker* B) from strain L-25

Strain L-25 was grown overnight in 50 ml of nutrient broth at 50°C and the genomic DNA extracted by standard methods (Ausubel et al., 1987). On the basis of keratinase gene sequence published by Lin et al. (1995), two primers (primer1: 5'-CTCCTGCCAAGCTGAAGC-3'; primer2: 5'-GATCATGGAACGGATTC-3') were prepared for a polymerase chain reaction (PCR) using L-25 genomic DNA as templates. Amplified keratinase gene was directly cloned into a cloning vector pCRII (Invitrogen Co.). Both M13 reverse (17-mers) and T7 promoter primers (20-mers) (Promega Co. Madison, WI) that flank the insert were employed to analyze the *ker B* sequence. The nucleotide sequence was analyzed at University Core DNA Services at the University of Calgary, Alberta, Canada.

Keratinase gene within pCRII were excised by *Xbal* - *Spel* digestion, and inserted into the *Xba* I site of Plasmid pUB18-P43 for expression. Resulting plasmid was transformed into *B. subtilis* DB104 competent cells using the procedure described by Gryczan et al. (1978). Transformants were first selected on tryptose blood agar base (TBAB) plate with kanamycin (10 mg/ml), and then placed on milk-agar plates for further identification. The colonies with clear haloes were chosen for plasmid isolation and PCR analysis.

RESULTS

Growth of bacteria and proteolytic activity assay

Strains L-25 and PWD-1 were able to grow in feather medium and hydrolyze feathers. At 37°C, both strains showed the highest keratinolytic activity between 60 to 72 h (figure 1), completely hydrolyzed feathers in 48 to 60 h. When measured by casein hydrolysis, the caseinolytic activity produced by L-25 and PWD-1 were comparable (figure 2a). However, keratinolytic activity of L-25 surpass that of PWD-1 by 27% (figure 2b). At 50°C, substantial reduced proteolytic activities by both strains were observed and confirmed by two different assays, azokeratin and azocasein hydrolyses (figure 2). Measured by azocasein
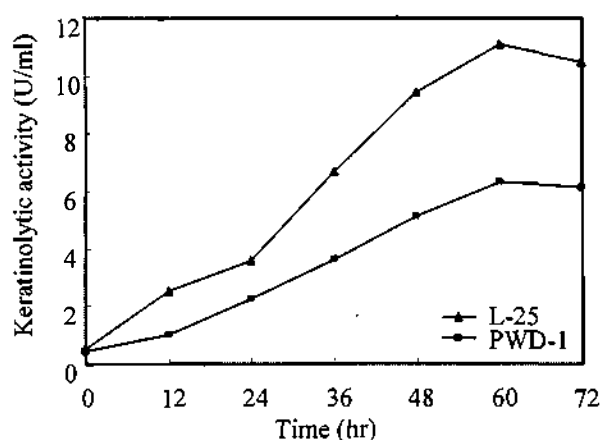
Figure 1. Production of keratinase by *B. licheniformis* L-25 and PWD-1 in feather Medium at 37°C

hydrolysis, L-25 and PWD-1 produced 6-fold and 9-fold caseinolytic activity, respectively, at 37°C in comparison with those at 50°C (figure 2a). Significant increases in keratinolytic activity were also observed when the growing temperature declined from 50°C to 37°C; L-25 increased by 70% and PWD-1 by 115% (figure 2b). When 1% glucose was supplemented into the feather medium, no proteolytic activity was produced by PWD-1 at 37°C (figure 3), and feather remained intact in the medium (figure 4). L-25, in contrast, generated 67% of its keratinolytic activity in the glucose containing feather medium when compared with its yield in the feather medium (figure 3). Feathers were completely hydrolyzed after 4 days of cultivation at 37°C.

Proportion of serine and neutral proteases

L-25 produced both serine and neutral proteases in the feather medium. Approximate 32% keratinolytic and 15 to 25% caseinolytic activities resulted from neutral protease (figure 2). When glucose (1%) was added into the feather medium, keratinolytic activity produced by L-25 was reduced, the proportion of keratinolytic activity from neutral protease slightly increased (figure 3). Strain PWD-1 produced no proteolytic activity when glucose (1%) was added into its medium (figure 3) At both 37 and 50°C, the predominant proteolytic activity produced by PWD-1 resulted from serine protease (figure 2 and 3).

Isolation and cloning of keratinase gene from Strain L-25

A 1.46kb keratinase gene (*ker B*) fragment was amplified by PCR priming by primer 1 and 2 and directly cloned into plasmid pCRII. The nucleotide sequence of *ker B* was analyzed by sequencing reactions using the universal primers, M13 reverse primer and T7 promoter. A pair of internal primers (primer 3,5'-CTGAATTCAAGCGGAA-GCGG-3', and primer 4,5'-CGATGGCATTCAGCGA-
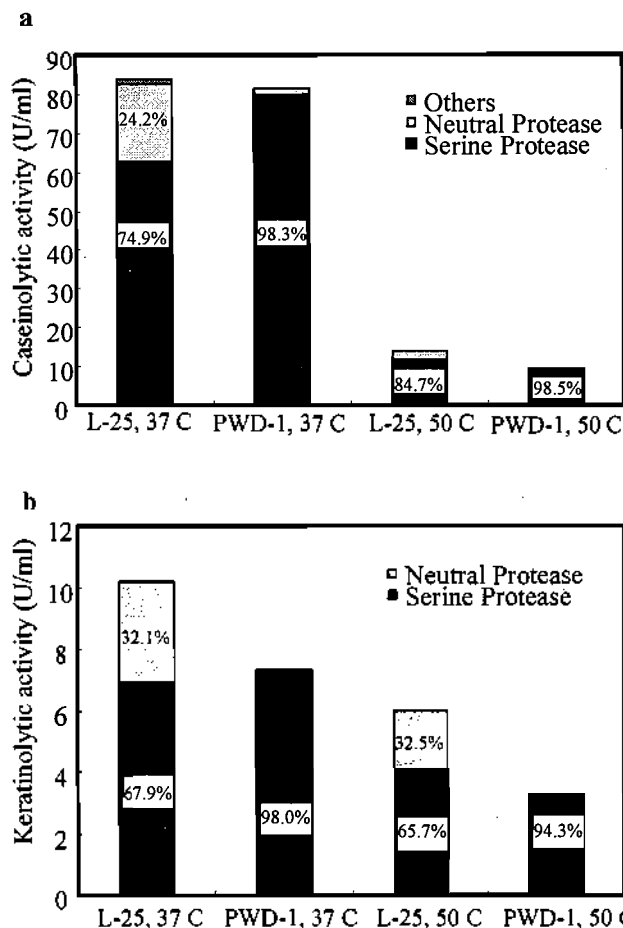
The numbers in boxed indicate the percentages of each protease contributing to total activity.

Figure 2. Comparison of caseinolytic (a) and keratinolytic activities (b) produced by *B. licheniformis* L-25 and PWD-1 at 37°C and 50°C
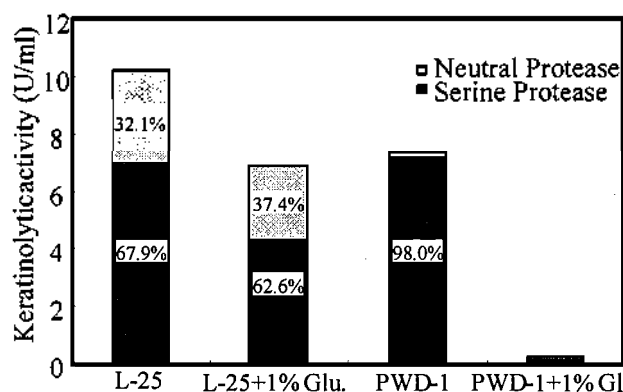

Figure 3. Production of keratinolytic activity in feather and feather-glucose Media by L-25 and PWD-1 at 37°C

TTCC-3') were prepared to facilitate the analysis of internal *ker B* sequence. The determined nucleotide sequence was confirmed by more than three independent sequencing
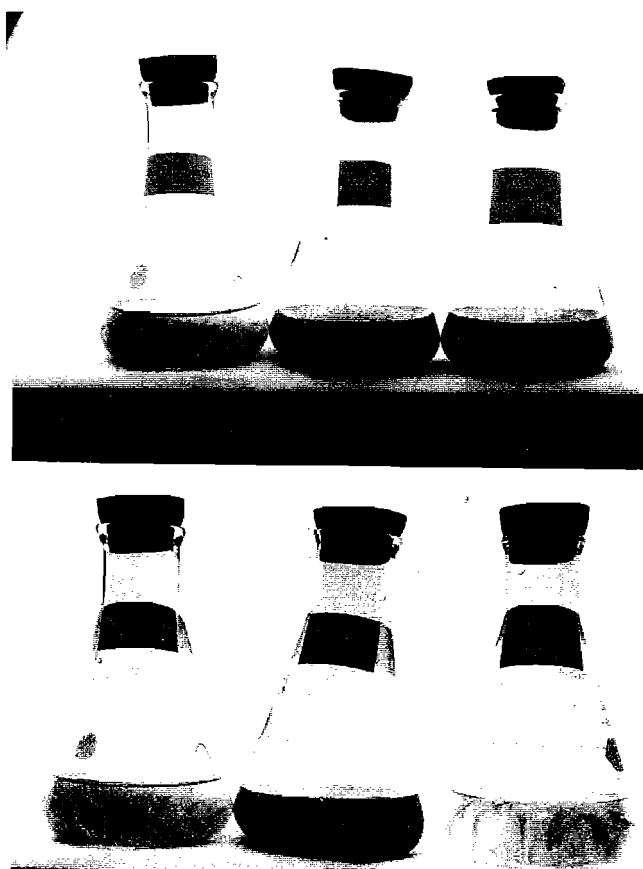
Figure 4. The comparison of feather meal hydrolyze by L-25 and PWD-1 with and without 1% of glucose

reactions (figure 5).

Keratinase gene within pCRII were excised by XbaI-SpeI digestion, and inserted into the XbaI site of Plasmid pUB18-P43 for expression. The resulting plasmid was transformed into protease-deficient *B. subtilis* DB104 competent cells. More than 100 transformants were first selected on tryptose blood agar base (TBAB) plate with 10mg kanamycin/ml, and then placed on milk-agar plates for further identification. The colonies with clear haloes were chosen for plasmid isolation. The resulting plasmid pLK-8 was 5.5 kb in size. When pLK8 was used as template, the 1.46 kb *ker B* fragment was amplified in a PCR priming by primer 1 and 2 (Data not shown), indicating the existence of *ker B* in the new vector.

Strain FD-8 (DB104/pLK-8) was grown on feather medium (containing 10-mg kanamycin/ml). FD-8 cells grew rapidly and completely hydrolyzed all feathers in the medium. No feather hydrolysis occurred within the control DB104 culture, and the medium remained clear after 5 days of cultivation.

DISCUSSION

B. licheniformis PDW-1 was isolated from an anaerobic poultry waste digester in the southeast of the United States (Willaim and Shih, 1989; Williams et al., 1990) and L-25 from canola meal compost in Alberta, Canada (Lin et al., unpublished). Despite of differences in their native environment meteorologically and their enrichment conditions, both strains demonstrated potent keratinolytic activity toward feather keratin and were capable of growing at high temperature. For both strains, the yields of protease decreased drastically when the growth temperature was increased from 37°C to 50°C. The decreases may due to proteolytic autolysis was certainly more severe at 50°C than at 37°C.

Proteases produced by both strains showed comparable activities toward azocasein (figure 2), however, proteases produced by L-25 were more active to azokeratin (figure 3). If we consider the fact that L-25 produces considerable amounts of neutral protease, we infer that the neutral protease from L-25 plays an important role in feather hydrolysis. It might be a keratinolytic protease *per se*. Keratinolytic serine and neutral protease could act synergistically and be more effective toward feather keratin. To verify these, we have purified keratinolytic neutral protease for the study of feather hydrolysis. At present, we are unable to conclude whether or no PWD-1 possesses a similar neutral protease gene to L-25. Even PWD-1 has the neutral protease gene, it must be highly suppressed in the feather medium under given conditions.

The keratinase gene of L-25 (*ker B*) is highly similar to *ker A* of PWD-1, (Lin et al., 1995). Only two amino acids (Leu-15 of pre-region, Val-273 of mature enzyme) in the coding region of *ker B* differ from residues in *ker A*. The changes in pre-region (Phe-15 → Leu-15) and at the carboxyl end of the mature enzyme (Ala-273 → Val-273) remain hydrophobic residues in each position. Each change was resulted from a single nucleotide change in the codon; TTC (Phe) → TTA (Leu), GCT (Ala) → GTT (Val). Notable changes occur in the upstream region of the putative promoter, while 8 out of 70 nucleotides are different between these two keratinase genes (figure 5). It is likely that the nucleotide changes in the upstream region of the promoter may alter the binding affinity of some regulatory proteins, such as *hpr* and *sin*, two negative regulatory proteins (Gaur et al., 1986; Perego and Hoch, 1988). Results from other laboratories have evidenced that promoter and upstream of promoter regions of subtilisin gene (*apr E*) and neutral protease gene (*npr E*) are the targets for Hpr and Sin regulations (Henner et al., 1988; Kallio et al., 1991).

Catabolic repression of biosynthesis of extracellular enzymes by glucose and other readily metabolized carbon sources has long been documented in *Bacillus* species (Emanuilova and Toda, 1984; Ramesh and Lonsane, 1991;

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   1 ctcctgccaa gctgaagcgg tctattcata ctttcgaact gaacattttt ctaaaacagt
  61 tattaataac caaaaaattt taaattggcc ctccaaaaaa ataggcctac catataattc
 121 attttttttc tataataaat taacagaata attggaatag attatattat ccttctattt
 181 aaattattct gaataaagag gaggagagtg agtaatgatg aggaaaaaga gtttttggct
 241 tgggatgctg acggccttca tgctcgtgtt cacgatggca ttcagcgatt ccgcttctgc
 301 tgctcaaccg gcgaaaaatg ttgaaaagga ttatattgtc ggatttaagt caggagtgaa
 361 aaccgcatct gtcaaaaagg acatcatcaa agagagcggc ggaaaagtgg acaagcagtt
 421 tagaatcatc aacgcggcaa aagcgaagct agacaaagaa gcgcttaagg aagtcaaaaa
 481 tgatccggat gtcgcttatg tggaagagga tcatgtggcc catgccttgg cgcaaaccgt
 541 tccttacggc attcctctca ttaaagcgga caaagtgcag gctcaaggct ttaagggagc
 601 gaatgtaaaa gtagccgtcc tggatacagg aatccaagct tctcatccgg acttgaacgt
 661 agtcggcgga gcaagctttg tggctggcga agcttataac accgacggca acggacacgg
 721 cacacatgtt gccggtacag tagctgcgct tgacaataca acgggtgtat taggcgttgc
 781 gccaagcgta tccttgtacg cggttaaagt actgaattca agcggaagcg gatcatacag
 841 cggcattgta agcggaatcg agtgggcgac aacaaacggc atggatgtta tcaatatgag
 901 ccttggggga gcatcaggct cgacagcgat gaaacaggca gtcgacaatg catatgcaag
 961 aggggttgtc gttgtagctg cagcagggaa cagcggatct tcaggaaaca cgaatacaat
1021 tggctatcct gcgaaatacg attctgtcat cgctgttggt gcggtagact ctaacagcaa
1081 cagagcttca ttttccagtg tgggagcaga gcttgaagtc atggctcctg gcgcaggcgt
1141 atacagcact tacccaacga acacttatgc aacattgaac ggaacgtcaa tggtttctcc
1201 tcatgtagcg ggagcagcag ctttgatctt gtcaaaacat ccgaaccttt cagcttcaca
1261 agtccgcaac cgtctctcca gcacggcgac ttatttggga agctccttct actatgggaa
1321 aggtctgatc aatgtcgaag ctgccgctca ataacatatt ctaacaaata gcatatagaa
1381 aaagctagtg tttttagcac tagctttttc ttcattctga tgaaggttgt ccaatatttt
1441 gaatccgttc catgatc
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Figure 5. Comparison of Nucleotide Sequences of Keratinases from L-25

Weickert and Chambliss, 1989), and pose serious problems in the fermentation process. Mutants that resist to catabolic repression possess valuable features for industrial application. It has been found in *B. subtilis* that the upstream of the coding region of a number of genes possesses certain sites responsible for glucose repression (Weickert and Chambliss, 1989; Fujita and Fujita, 1986; Lin et al., 1997). On the basis of the results we obtained, we demonstrated that L-25 is able to express keratinase(s) under glucose catabolic repression. In contrast, keratinase expression in PWD-1 was highly suppressed in glucose containing feather medium. These results were in agreement with that in previous report. As indicated by Lin et al. (1997) and Wang and Doi (1984), when a vegetative growth promoter P-43 replaced the original *ker A* promoter, *ker A* was no longer repressed by glucose in the feather medium. At present, we can only speculate that the changes in L-25 upstream regions may be one of the factors that drive the expression of keratinase in the feather-glucose medium. Glucose may also stimulate the mRNA turnover rates of digestive enzymes to increase the nucleotide pool in favor of other enzyme biosynthesis.

Besides the differences in enzyme production and gene sequence, two obvious features distinguish these two strains. There are: (i) L-25 produces neutral protease and keratinolytic serine protease, PWD-1 yields only serinal keratinase under given conditions; (ii) L-25 is capable of producing keratinolytic proteases and hydrolyzing feathers under catabolic repression by glucose. Current results suggest that feather hydrolysis can be accomplished by a combination of keratinolytic enzymes. The multiple enzymes involved may have added effects on keratin degradation. We will continue to make an effort to purify the keratinolytic neutral protease, and isolate the neutral protease gene in L-25.

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