Keratinase Fermentation by *Bacillus licheniformis* KUB-K0006

Warintorn Eaksuree[1] Akkharadet Prachayakitti[2] Rutjawate Taharnklaew[3] Dietmar Haltrich[4]
Sunee Nitisinprasert[5] Suttipun Keawsompong[6*]

Abstract

Keratinase fermentation by *Bacillus licheniformis* KUB-K0006 was studied at pilot scale. The maximum activity (5.03 U/mL) and productivity (0.21 U/L h) were obtained at 5% (v/v) inoculum, 1.0 vvm aeration rate, without pH control (7.5 initial pH), agitation speed 300 rpm, at the temperature of 37°C for 24 h. During fermentation, controlled pH 7.5 gave similar enzyme activity to without controlled pH. This condition achieved its maximum productivity for keratinase production in 3 L fermentor. Fermentation scale-up was also studied by varying fermentor size at the volume of 19.5, 70 and 300 L. The profile of pH, growth and keratinase activity were similar in all fermentor sizes. The result suggested that the characteristics of the fermentors had little effect on keratinase production patterns in *B. licheniformis* KUB-K0006.

Keywords : Keratinase Production, *Bacillus*, Scale up, Feather

[1] Ph.D. student, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok, Thailand. e-mail: warintorne_eak@outlook.com

[2] Research and Development Center of BETAGRO Group, Science Park, Klong Nueng, Klong Luang, Pathum Thani, Thailand. e-mail: akkharadetp@betagro.com

[3] Research and Development Center of BETAGRO Group, Science Park, Klong Nueng, Klong Luang, Pathum Thani, Thailand. e-mail: Rutjawate@betagro.com

[4] Professor, Department of Food Sciences and Technology, Food Biotechnology Laboratory, BOKU-University of Natural Resources and Life Sciences, Vienna, Austria. e-mail: dietmar.haltrich@boku.ac.at

[5] Associate Professor, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok, Thailand. e-mail: fagisnn@ku.ac.th

[6] Assistant Professor, Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Chatuchak, Bangkok, Thailand; Special Research Unit: Probiotic and Prebiotics for Health, Center for Advanced Studies for Agriculture and Food (CASAF), Institute for Advanced Studies, Kasetsart University, Bangkok, Thailand. e-mail: fagisuk@ku.ac.th

** Corresponding author, e-mail: fagisuk@ku.ac.th*

Introduction

A feather-degrading *Bacillus licheniformis* KUB-K0006 was discovered and isolated by Nitisinprasert and Keawsompong (1997). This aerobic bacterial isolate possessed effective keratinase, with high feather digestibility at wide pH range and high temperature of up to 50°C. The keratinase was purified and characterised as a serine protease (Titapoka, 2003). Keratinase from *B. licheniformis* KUB-K0006 (which we call K6 keratinase) is endo-acting, (Nitisinprasert *et al.*, 1999).

Fermentation conditions, scale-up procedures, and gene regulation are all involved in successful production of an enzyme by fermentation. Optimization of parameters including medium composition, pH, dissolved oxygen, and temperature is important in developing the fermentation process. Due to a large divergence in physiological patterns found in *Bacillus* species, cultivation conditions promoting enzyme production vary widely (Wang and Shih, 1999).

In order to have sufficient quantities of keratinase for application research in feed, a scale-up fermentation process was determined for the production of this enzyme in a larger quantity than that obtained at the laboratory-scale. Fermentation parameters for enzyme production were optimized in 3 L fermentor. Effect of fermentor sizes for keratinase production were determined in 19.5, 70 and 300 L fermentor.

Objective

To study the effect of fermentor sizes for keratinase fermentation by *B. licheniformis* KUB-K0006

Materials and methods

Bacterial strain

Bacillus licheniformis KUB-K0006, was isolated from soil (Nitisinprasert and Keawsompong, 1997). This isolated strain of KUB-K0006 was stored at –80°C in Nutrient broth (NB) medium containing 20% (w/v) glycerol.

Fermentation medium and seed culture

The basic salt medium, prepared according to the modified method of Williams *et al.* (1990), contained (g/L) NH_4Cl (0.5), NaCl (0.5), $K_2HPO_4.3H_2O$ (0.354), KH_2PO_4 (0.4), $MgCl_2.6H_2O$ (0.24) and chicken feather meal (10).

A seed culture was prepared by propagating in fresh nutrient broth (NB) medium and streaked on nutrient agar medium. One colony cultured overnight was picked and inoculated into 5 mL of NB medium and grown overnight at 37°C on the incubating shaker at 250 rpm and inoculated to the next stage.

Fermentation and operation

Optimization of fermentation was carried out with a 3 L fermentor (BioFlo 110, New Brunswick Scientific, New Jersey, USA) containing 2 L culture volume. The bioreactor was equipped with an interface controller capable of monitoring and controlling the fermentation parameters, including pH, temperature, dissolved oxygen (DO), agitation, and foaming. Effect of fermentor sizes for keratinase production were accomplished with 19.5 L fermentor (BioFlo 415, New Brunswick Scientific, New Jersey, USA), 70 L Bioreactor (Applikon Biotechnology, Netherlands) and 300 L fermentor (FM-300A, B.E.Marubishi, Japan) containing 15, 30 and 200 L working volumes, respectively.

In BioFlo 110, the agitation range was limited to 300 rpm. The fermentations were carried out at various conditions aeration rates at 0.5-1.0 vvm at a temperature of 37°C for 24 h. The pH was automatically controlled at 7.5 with 1 M sodium hydroxide or 1 M orthophosphoric acid, compared with initial pH which was adjusted to 7.5 and not controlled. Inoculation was varied at 1-10% (Table 1). In FM-300A, the air flow rate was limited at 0.5 vvm.

The growth pattern, keratinase activity, and protein content were followed. The viable cell numbers were determined by standard plate count method and incubated 37°C for 16 h.

Table 1 Conditions for optimization keratinase production in 3 L fermentor

Treatments	Inoculums size (%)	Aeration rates (vvm)	pH
B1	1	1.0	initial pH 7.5
B2	5	0.5	initial pH 7.5
B3	5	1.0	initial pH 7.5
B4	10	1.0	initial pH 7.5
B5	5	1.0	control pH 7.5

Determination of keratinase activity

The keratinase activity was determined on the basis of the production of free amino groups using a modified method of Lin *et al.* (1992). A modified ninhydrin method (Rosen, 1957) was used for detecting free amino groups in reaction solutions. Five milligram of ground feather substrate was added to a 1.5 mL centrifuge tube with 0.8 mL of 50 mM potassium phosphate buffer pH 7.0. This mixture was stirred until the ground feather was homogeneously suspended. A volume of 0.2 mL of appropriately diluted enzyme solution was mixed with the mixture. After incubation at 50°C for 60 min, the reaction was stopped by the addition of 0.2 mL of 10% trichloroacetic acid (TCA). The reaction mixture was centrifuged to remove insoluble protein at

15,300 g for 30 min. A control was prepared by adding 10% TCA to a reaction mixture before adding the enzyme solution.

The supernatant was used for further free amino acid determination by ninhydrin method. One milliliter of solution was added into the mixture containing 0.5 mL of 0.2 mM cyanide-acetate buffer pH 5.3, and 0.5 mL of 3% ninhydrin solution in methyl cellosolve (2-methoxyethanol). After incubating at 100°C for 15 min, 5 mL of isopropanol:distilled water (1:1 v/v) was added immediately and mixed vigorously. The mixture was cooled and the absorbance was measured at 570 nm using a Bio-Rad model 680 microplate reader (California, USA). Measurement of free amino acid contents was based on the determination of free leucine. One unit of keratinase activity was defined 1 mL of enzyme solution as the amount of enzyme producing 1 µmole of free amino acid per hour under experimental conditions.

Results and discussion

Effect of inoculum size on keratinase production

Inoculum size is very important factor affecting cell growth and product formation. In this study, inoculum size was investigated with 1, 5 and 10%. As show in Figure 1, it was found that maximum activity was obtained in the presence of 5% inoculum (5.03 U/mL and productivity 0.21 U/mL·h). A higher inoculum of 10% (v/v) was found to reduce the production of keratinase. Therefore, high inoculum sizes do not necessarily give higher keratinase yield. Similar result was also reported by Mabrouk *et al.* (1999) who found the maximum production of protease by *B. licheniformis* ATCC21415 with an inoculum size of 5% (v/v). The increase in the production of protease using small inoculum sizes was suggested to be due to the higher surface area to volume ratio, which resulted in the increased production of keratinase. In addition, an improved distribution of dissolve oxygen and more effective uptake of nutrient also contributed to a higher protease production. If the inoculum sizes are too small, insufficient number of bacteria would then lead to a reduced amount of secreted protease (Abusham *et al.*, 2009). Moreover, the less keratinase production in small inoculum sizes of 1% possibly caused by lag phase of seed culture tends to prolong overall fermentation time due to insufficient activated cells. Bacterial inocula must be transferred in the log phase of growth, during which cells are still metabolically active (Wang and Shih, 1999).

In addition, an improved distribution of dissolve oxygen and more effective uptake of nutrient also contributed to a higher protease production. If the inoculum sizes are too small, insufficient number of bacteria would then lead to a reduced amount of secreted protease. However, higher inoculum sizes could lead to or cause a lack of oxygen and depletion of nutrient in the culture media (Abusham *et al.*, 2009).

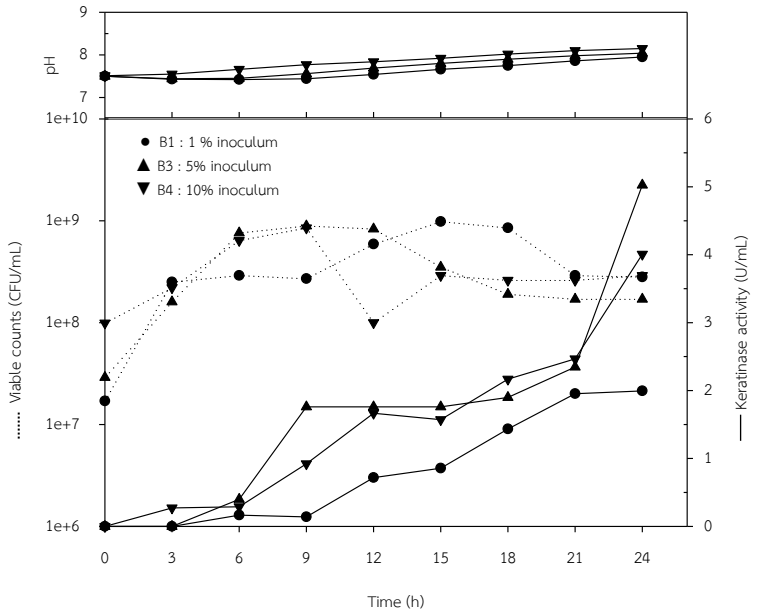

Figure 1 Effect of inoculum size on keratinase production, cell growth and pH during fermentation. All cultures were grown at initial pH 7.5, 37°C for 24 h, agitation speed 300 rpm and aeration rate 1.0 vvm.

Effect of aeration rate on keratinase production

Aeration rate was varied at 0.5 and 1.0 vvm. In Figure 2, a maximum keratinase activity of 0.5 and 1.0 vvm were 3.74 and 5.03 U/mL, at 21 and 24 h, respectively and cell grow more as the aeration increased. The highest keratinase production and bacteria growth were obtained when aerated at 1.0 vvm. At this speed, the aeration of the culture medium was increased, and this further led to a sufficient supply of dissolved oxygen in the media (Kumar and Takagi, 1999). This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis (Abusham *et al.*, 2009).

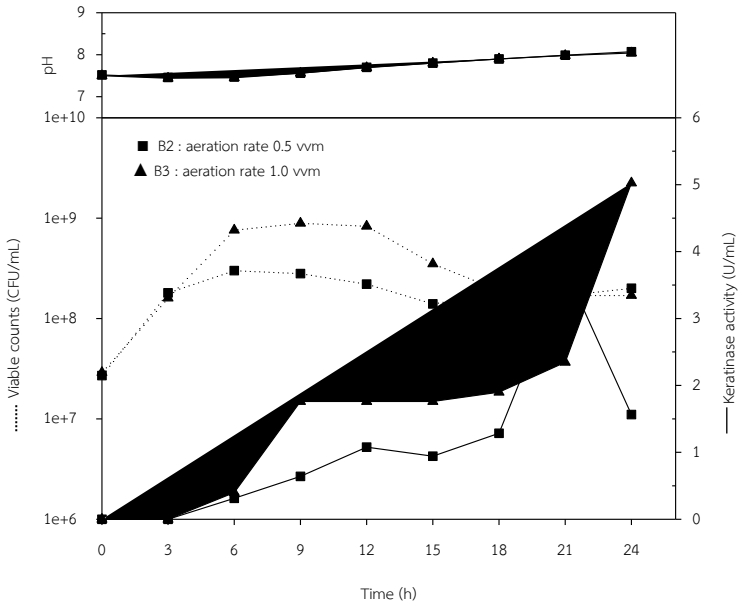

Figure 2 Effect of aeration rate on keratinase production, cell growth and pH during fermentation. All cultures were grown at 5% inoculum, initial pH 7.5, 37°C for 24 h and agitation speed 300 rpm.

During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Oxygen acts as a terminal electron acceptor for oxidative reactions to provide energy for cellular activities. Then mixing is important in the microbial synthesis of protease enzyme in free cell bioreactor and can be imparted by means of aeration and agitation. It is also largely dependent on higher oxygen mass transfer and lesser shear forces on microorganisms. For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation (Potumarthi *et al.*, 2007). Moreover, variation in the agitation speed has been found to influence the extent of mixing in the shake flasks or the bioreactor, and also affect the nutrient availability (Abusham *et al.*, 2009).

Effect of pH control on keratinase production

The effect of pH control on keratinase production was observed by comparing keratinase activity with (7.5) and without pH control (7.5 initial) during cultivation. Figure 3 showed the growth under a controlled pH 7.5 gave similar enzyme activity to without controlled pH. Thus, uncontrolled pH is to be preferred for optimum production of K6 keratinase. Similar result was also reported by Wang and Shih (1999) who studied fermentation scale-up for the production of keratinase by *Bacillus licheniformis* PWD-1, the parent strain, and *B. subtilis* FDB-29, a recombinant strain. During the batch fermentation by both strains, the pH changed from

7.0 to 8.5 while the keratinase activity and productivity stayed at high levels. The uncontrolled pH operation was more favorable than the controlled pH operations. Control of pH, therefore, is not necessary.

In 2 L fermentor, the most suitable fermentation condition for K6 keratinase production were found to be 5% (v/v) inoculum, aeration rate at 1.0 vvm and without pH control (7.5 initial) at 300 rpm, 37°C for 24 h. This condition achieved its maximum productivity for keratinase production in small scale fermentor (Table 2). Therefore treatment B3 was chosen for enzyme production in large scale fermentor.

Table 2 Keratinase activity and productivity of various conditions in 2 L fermentor (300 rpm, 37°C, 24 h)

Treatment	Conditions	Keratinase activity (U/mL)	Productivity (U/mL•h)
B1	1% inoculum, 1.0 vvm, initial pH 7.5	1.99	0.08
B2	5% inoculum, 0.5 vvm, initial pH 7.5	1.56	0.07
B3	5% inoculum, 1.0 vvm, initial pH 7.5	5.03	0.21
B4	10% inoculum, 1.0 vvm, initial pH 7.5	4.01	0.17
B5	5% inoculum, 1.0 vvm, control pH 7.5	4.56	0.19

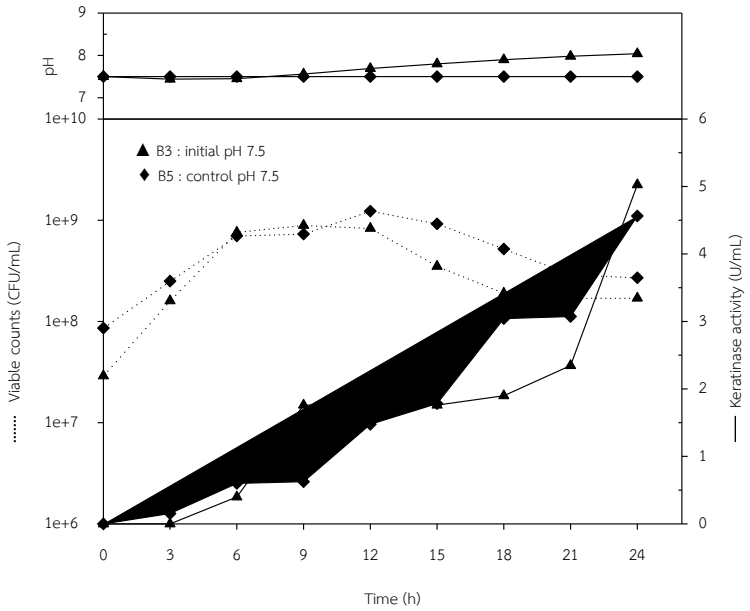

Figure 3 Effect of pH on keratinase production, cell growth and pH during fermentation. All cultures were grown at 5% inoculum, 37°C for 24 h agitation speed 300 rpm and aeration rate 1.0 vvm.

Effect of fermentor sizes

After optimization studies in 2 L fermentors, scale-up production was studied on different sizes of fermentors in 19.5, 70 and 300 L fermentors with 15, 30 and 200 L working volumes, respectively. Fermentation was carried out with aeration at 1.0 vvm however 300 L fermentor was 0.5 vvm due to high pressure between processes running at 1.0 vvm. In 19.5 and 70 L fermentors, nutrient broth was used to prepare a seed culture for *B. licheniformis* KUB-K0006 in flasks (18 h) while the developed formula medium (1% feather meal in basic salt medium) was used in 300 L fermentor so as to reduce the cost. After 18 and 15 h, it was inoculated in 15, 30 and 200 L developed formula medium for keratinase production, respectively.

As shown in Figure 4, keratinase was produced from the late exponential or stationary phase of growth. The activities ranged from 4.9–5.24 U/mL with a productivity of 204–218 U/L h and medium pH increased to 7.99-8.13. The dissolved oxygen (DO) level in the 300 L fermentor was different from that observed in the 15 and 30 L fermentors. DO level has increased during fermentation in 300 L fermentor, whereas it has reduced in 15 and 30 L fermentors. On the other hand, 300 L fermentor can be maintained at DO level better than other fermentor sizes. However, the pattern of pH, growth curves and keratinase activity were similar in all fermentors. The performances of *B. licheniformis* at different sizes of fermentation are summarized and

compared in Table 3. From the results, the characteristics of the fermentors had little effect on their fermentation patterns in *B. licheniformis* KUB-K0006. Wang and Shih (1999) also reported that keratinase activity and keratinase productivity obtained from the scale up production of keratinase by *B. licheniformis* PWD-1 in 10 L (Bioflow III) and 100 L (IF-150, New Brunswick Scientific, USA) fermentation were similar, under optimum cultivation conditions at 37°C.

Table 3 Keratinase activity and productivity in various size of fermentor (300 rpm, 37°C, 24 h)

Model	Keratinase activity (U/mL)	Productivity (U/L•h)
BioFlo 110 (2 L)	5.03	209.58
BioFlo 415 (15 L)	5.12	213.33
70 L Bioreactor (30 L)	5.24	218.33
FM-300A (200 L)	4.90	204.17

All cultures were cultivated at 5% inoculum, initial pH 7.5, 37°C for 24 h, stirred at 300 rpm and aerated with 1.0 vvm except FM-300A with 0.5 vvm.

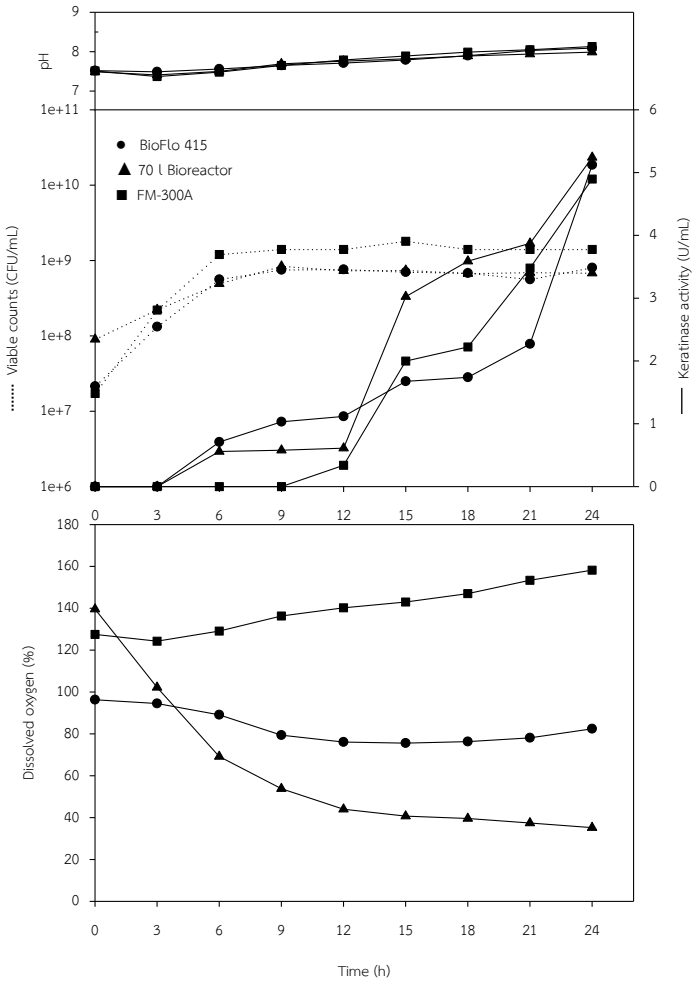

Figure 4 Effect of kearatinase production on various fermentor sizes. All cultures were grown at 5% inoculum, 37°C for 24 h agitation speed 300 rpm and aeration rate 1.0 vvm except FM-300A with 0.5 vvm.

Conclusion

The fermentation condition for K6 keratinase production was found to be 5% (v/v) inoculum, aeration rate at 1.0 vvm and without pH control (7.5 initial) at 300 rpm, 37°C for 24 h. This condition achieved its maximum productivity for keratinase production in 3 L fermentor. Fermentation was studied in vary fermentor sizes (3, 19.5, 70 and 300 L). The pattern of pH, growth curves and keratinase activity were similar in all fermentors.

Acknowledgments

This research was financially supported by a Royal Golden Jubilee (RGJ) grant, The Thailand Research Fund (TRF) and the Kasetsart University Research and Development Instisute (KURDI), Thailand. We also thank Department of Food Sciences and Technology, Food Biotechnology Laboratory, BOKU-University of Natural Resources and Life Sciences, Vienna, Austria and Betagro Science Center Co., Ltd. for technical support.

References

Abusham, R.A., R.N. Rahman, A.B. Salleh and M. Basri. (2009). Optimization of physical factors affecting the production of thermo-stable organic solvent-tolerant protease from a newly isolated halo tolerant *Bacillus subtilis* strain Rand. Microbial Cell Factories, 8 (20), 1-9.

Kumar, C.G. and H. Takagi. (1999). Microbial alkaline proteases: From a bioindustrial viewpoint. Biotechnology Advances, 17 (7), 561-594.

Lin, X., C.G. Lee, E.S. Casale and J.C. Shih. (1992). Purification and characterization of a keratinase from a feather-degrading *Bacillus licheniformis* strain. Applied and Environmental Microbiology, 58 (10), 3271-3275.

Mabrouk, S.S., A.M. Hashem, N.M.A. El-Shayeb, A.M.S. Ismail and A.F. Abdel-Fattah. (1999). Optimization of alkaline protease productivity by *Bacillus licheniformis* atcc 21415. Bioresource Technology, 69 (2), 155-159.

Nitisinprasert, S. and Kaewsompong, S. (1997). Screening and characterization of microorganisms for feather degradation from various sources in Thailand. Journal of Science, Khon Kaen University, 25, 65-75.

Nitisinprasert, S., W. Pornwirun and S. Keawsompong. (1999). Characterizations of two bacterial strains showing high keratinase activities and their synergism in feather degradation. Kasetsart Journal: Natural Science, 33, 191-199.

Potumarthi, R., S. Ch and A. Jetty. 2007. Alkaline protease production by submerged fermentation in stirred tank reactor using *Bacillus licheniformis* NCIM-2042: Effect of aeration and agitation regimes. Biochemical Engineering Journal, 34 (2), 185-192.

Rosen, H. (1957). A modified ninhydrin colorimetric analysis for amino acids. Archives of Biochemistry and Biophysics, 67 (1), 10-15.

Titapoka, S. (2003). Study on synergism of keratinase produced by *Bacillus licheniformis* kub-k0006 and *Bacillus pumilus* kub-k0082. Master's thesis, Kasetsart University.

Wang, J.J. and J. Shih. 1999. Fermentation production of keratinase from *Bacillus licheniformis* PWD-1 and a recombinant *B. Subtilis* fdb-29. Journal of Industrial Microbiology and Biotechnology, 22 (6), 608-616.

Williams, C.M., C.S. Richter, J.M. Mackenzie and J.C. Shih. (1990). Isolation, identification, and characterization of a feather-degrading bacterium. Applied and Environmental Microbiology, 56 (6), 1509-1515.