Brazilian Journal of Microbiology (2006) 37:395-399
ISSN 1517-8382

KERATINOLYTIC BACTERIA ISOLATED FROM FEATHER WASTE

Alessandro Riffel; Adriano Brandelli*

Laboratório de Bioquímica e Microbiologia Aplicada, Departamento de Ciência de Alimentos, ICTA, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brasil

Submitted: December 16, 2004; Approved: July 13, 2006

ABSTRACT

The aim of this study was to characterize keratinolytic bacteria isolated from feather waste. Four isolates were selected after growth on solid medium with feather meal as sole carbon and nitrogen source and screened for proteolytic activity on milk agar plates. Three isolates were Gram-negative (belonged to the genera *Burkholderia*, *Chryseobacterium* and *Pseudomonas*) and one was Gram-positive (*Microbacterium* sp.). These bacteria grew on diverse keratin wastes such as feather meal, raw feathers, chicken nails, hair and wool. Keratinase activity was detected during growth, but the complete degradation of these substrates was not always achieved. The proteolytic character of crude enzymes was assessed using azokeratin and azocasein as substrates. The keratinases were active on both substrates and were similar in keratin hydrolysis when compared with commercially available microbial peptidases. These novel keratinolytic isolates have potential biotechnological use in processes involving keratin hydrolysis.

Key words: feather, keratin, peptidase, proteolysis

INTRODUCTION

Feathers are produced in large amounts as a waste by-product of poultry processing plant. A current value-added use for feathers is the conversion to feather meal, a digestible dietary protein for animal feed, using physical and chemical treatments. These methods can destroy certain amino acids and decrease protein quality and digestibility (18,26).

The nutritional inferiority and insolubility of native feather protein derive from the composition and molecular configuration of constituent amino acids that ensure the structural rigidity of feathers (22). Resistance to proteolytic enzymes has been attributed to the complex structure of β-keratin filaments. In addition, disulfide cross-links produce a compact three-dimensional network (3), as a result of intermolecular disulfide bonds between rod domains and terminal domains of the constituent molecules (22).

The nutritional upgrading of feather meal through microbial or enzymatic treatment has been described. Feather meal fermented with *Streptomyces fradiae* and supplemented with methionine resulted in a growth rate of broilers comparable with those fed isolated soybean protein (5). The use of feather-lysate from *Bacillus licheniformis* with amino acid supplementation produced a similar growth rate in chickens when compared to chickens fed with a diet that included soybean meal (28). The crude keratinase enzyme produced by *B. licheniformis* significantly increased the total amino acid digestibility of raw feathers and commercial feather meal (12). This enzyme increased the digestibility of commercial feather meal and could replace as much as 7% of the dietary protein for growing chicks (20).

Keratinolytic microorganisms and their enzymes may be used to enhance the digestibility of feather keratin. They may have important applications in processing keratin-containing wastes from poultry and leather industries through the development of non-polluting methods (21). A number of keratinolytic microorganisms have been reported, including some species of *Bacillus* (1,27), actinomycetes (2,29) and fungi (11,25). Generally, an increase in keratinolytic activity is associated with thermophilic organisms, which require high energy inputs to achieve maximum growth and the

*Corresponding Author. Mailing address: ICTA-UFRGS, Av. Bento Gonçalves, 9500. 91501-970, Porto Alegre, RS, Brasil. Fax: (+5551) 3316-7048. E-mail: abrand@ufrgs.br

decomposition of keratin wastes (6,19). Recently, feather-degrading activity was also observed in studies of Gram-negative bacteria (23,24). These bacteria can degrade raw feathers at mesophilic temperatures, and are therefore useful to develop efficient processes involving keratin substrates.

In this report, we describe the selection and characterization of mesophilic microorganisms showing keratinolytic activity isolated from a poultry processing plant at Porto Alegre, Brazil. Some properties of their keratinases were also determined.

MATERIALS AND METHODS

Microorganisms

Bacterial strains designated as kr7 to kr14 were isolated from feather waste as described elsewhere (23,24). A previously characterized *Chryseobacterium* sp. strain kr6 that presented keratinolytic activity (23) was used for comparison. Bacterial identification was based on colony morphology, microscopic examination of Gram-stained cells, and biochemical tests (7,16), comparing the data with standard species. Additionally, an API 20E kit was used and the data was analyzed by automated interpretation with the APILAB Plus software (Bio-Mérieux, Marcy-l'Étoile, France).

Effect of temperature on growth and proteolytic activity

Milk agar plates (5 g L^{-1} peptone, 3 g L^{-1} yeast extract, 100 mL L^{-1} sterile UHT non-fat milk, and 12 g L^{-1} agar) were prepared for primary screening of proteolytic activity. Bacteria were inoculated onto plates and incubated at 22, 30, 37, 46, and 55ºC for 24 h. Strains that produced clearing zones in this medium were selected.

Enzyme assay

Keratinase activity was assayed with azokeratin as substrate (24). The reaction mixture contained 100 mL of enzyme preparation and 500 mL of 10 g L^{-1} azokeratin in 50 mM tris buffer, pH 8. The mixture was incubated at 45ºC for 15 min and the reaction was the stopped by the addition of trichloroacetic acid to a final concentration of 60 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 for 15 min at 45ºC. A similar protocol was used to determine enzyme activity on azocasein (Sigma, St. Louis, USA). Azokeratin was synthesized based on the methodology described for azoalbumin (23).

Degradation of keratin wastes

The capacity of degradation of keratin substrates was tested on medium containing 0.5 g L^{-1} NaCl, 0.3 g L^{-1} K_2HPO_4, 0.4 g L^{-1} KH_2PO_4 and 10 g L^{-1} of either feather meal, raw feathers, bovine hair, human hair, wool, or chicken nails powder. Degradation of substrates was visually inspected and aliquots were removed for keratinase activity assay.

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 30ºC. Enzymes were obtained by centrifugation at 10,000 g for 5 min, and culture supernatants were used as crude enzyme extracts. Keratinase activity was assayed at different cultivation times.

Comparison with commercial enzymes

Commercial enzymes (pronase E, papain and trypsin form Sigma-Aldrich, St. Louis; alcalase from Novo Nordisk, Denmark) were dissolved at 1 mg mL^{-1} in 50 mM tris pH 7.5 and then assayed for azoproteins as described previously.

RESULTS

Characterization of keratinolytic strains

Eight isolates were able to grow on medium containing feather meal as sole carbon and nitrogen source. The strains kr8, kr9, kr10, and kr14 produced clearing zones when tested for proteolytic activity on milk agar (Fig. 1). The largest clearing zones were observed for isolate kr10, with a zone diameter similar to the keratinolytic strain *Chryseobacterium* sp. kr6. Milk agar plates were incubated at several temperatures, and the results are summarized in Table 1. After 24 h, kr8 was able to grow from 22 to 46ºC and peptidase production occurred between 22 and

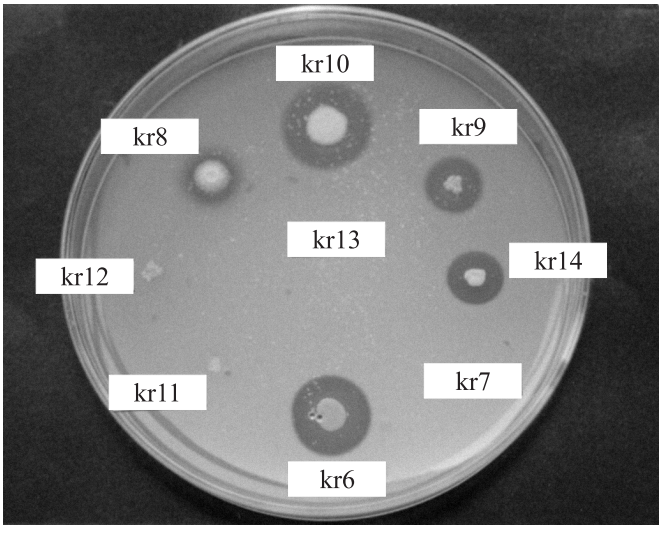

Figure 1. Production of clearing zones in milk agar plates by keratinolytic bacteria. Microorganisms were inoculated by stick and plates were incubated at 30ºC for 24 h.

Table 1. Effect of temperature on growth an proteolytic activity of bacteria isolated from a poultry industry.[a]

Isolate	22ºC	30ºC	37ºC	46ºC	55ºC
kr6	1	5	5	+	-
kr7	-	+	-	-	-
kr8	1	2	4	+	-
kr9	+	4	-	-	-
kr10	3	4	5	3	+
kr11	+	+	-	-	-
kr12	+	+	-	-	-
kr13	-	+	-	-	-
kr14	+	4	4	-	-

[a] Proteolytic activity was estimated by the distance (mm) between haloes and colonies after 24h. (+) Growth but not clearing zones were observed. (-) No growth or clearing zones were observed.

37ºC. The isolate kr10 was able to grow form 22 to 55ºC, but produced proteolytic activity between 22 and 46ºC. The strain kr14 grew between 22 and 37ºC and produced proteolytic activity between 30 and 37ºC. The other isolates exhibited inferior results in growth and proteolytic activity.

The identification of the keratinolytic bacteria was based on cell morphology, colony morphology, and several biochemical tests. Isolates kr8, kr9 and kr14 were determined to be Gram-negative coccobacilli by microscopic observation, whereas isolate kr10 was determined to be a Gram-positive rod. The isolate kr14 formed golden-yellow colonies on feather meal agar plates. Together with the results of physiological tests and API 20E, the characteristics indicated that kr8 is a *Bulkholderia* sp., kr9 is a *Chryseobacterium* sp., and kr14 is a *Pseudomonas* sp. The strain kr10 is a *Microbacterium* sp.

Hydrolysis of keratin wastes

The strains were tested for their capacity to degrade diverse keratin wastes. Cultivation on all substrates resulted in the production of keratinase, although maximum values were obtained on feather meal and feathers (Fig. 2). Strains kr6 and kr10 degraded chicken feathers completely. The strain kr14 disintegrated feather barbules but not all rachises. Minor feather degradation was achieved by strains kr8 and kr9. Although keratinase activity was detected during growth on the different substrates, no important degradation of wool or hair was consistently observed (data not shown).

Keratinolytic activity

Keratinolytic activity of the isolates was monitored during growth in feather meal broth. The keratinolytic activity of isolates kr6 and kr10 increased in the first days, while other isolates showed a maximum keratinase activity later (Fig. 3). The highest keratinolytic activity was consistently observed from isolate kr6. An increase in pH was always observed during growth on feather meal broth.

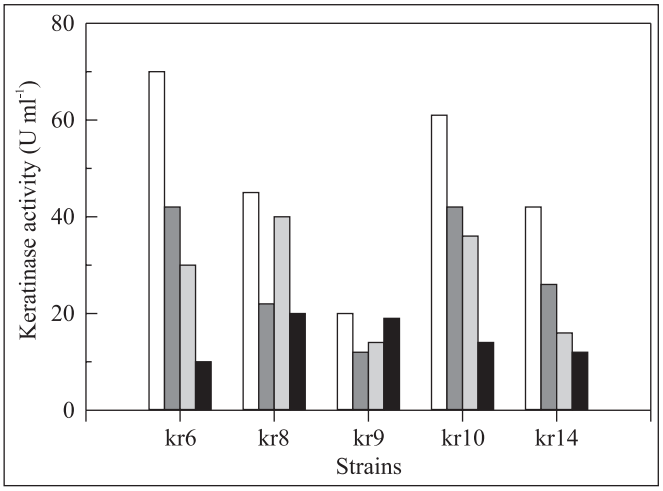

Figure 2. Keratinase activity during growth of keratinolytic bacteria on keratin wastes. Activity was measured after growth for 48 h at 30ºC in medium containing 10 g l^{-1} of either raw feathers (white bars), powdered chicken nails (dashed bars), bovine hair (gray bars), or wool (black bars).

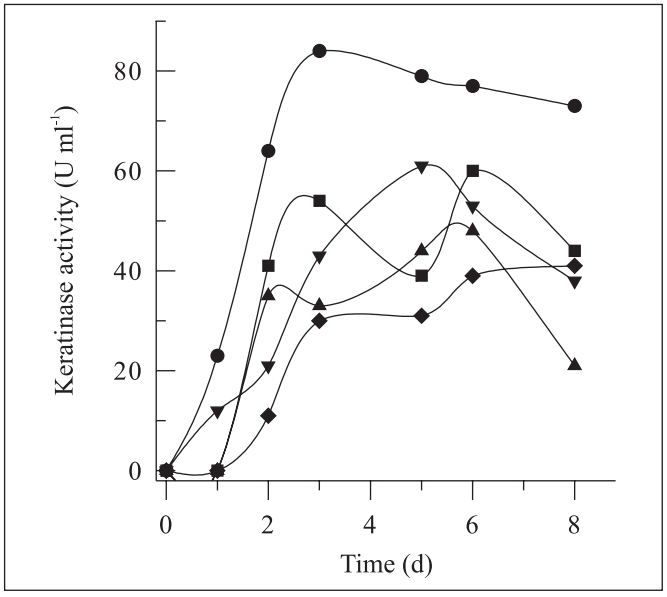

Figure 3. Keratinase activity during growth of keratinolytic bacteria in feather meal broth. Enzyme activities were measured using azokeratin as substrate. Each point represents the mean of three independent experiments. (●) kr6, (■) kr9, (▲) kr14, (▼) kr10, (◆) kr8.

The proteolytic activities of keratinolytic strains were compared with commercial enzymes by determining the hydrolysis of azokeratin and azocasein. Keratinase produced by strain kr10 exhibited higher specific activity degrading azocasein and azokeratin when compared to other enzymes (Table 2). The enzyme preparations were similar in hydrolysis of keratin substrate compared with commercially available microbial peptidases such as pronase, derived from *Streptomyces griseus*, and alcalase, form *B. licheniformis*.

DISCUSSION

Bacteria were isolated from a poultry processing plant, that owned keratinolytic activity and ability to degrade keratin wastes. These bacteria present different characteristics, such as a broad temperature range of growth. The optimal proteolytic activities were detected between 30 and 37ºC, whereas previously described keratinolytic bacteria mostly have feather-degrading activity at elevated temperatures (1,6,17). However, these strains behave similar to a *Vibrio* strain kr2, previously isolated from decomposing feathers (24). An optimum keratin-degrading activity at mesophilic temperatures should be a desirable characteristic because these microorganisms may achieve hydrolysis with reduced energy input.

Preliminary identification tests indicate that strains kr8, kr9 and kr14 belong to the Proteobacteria or *Cytophaga-Flavobacterium* group, and that strain kr10 is an actinomycetes. In agreement with this data, Lucas *et al.* (15) noted that feather-degrading Gram-negative bacteria isolated from soils belonged to the Proteobacteria or *Cytophaga-Flavobacterium* group. The most studied keratinolytic bacteria are *Bacillus* spp., which have been described to possess feather-degrading activity (9,14). *Bacillus licheniformis* is a well known keratinolytic organism, possessing the gene *ker*A, which has been cloned and sequenced (13). However, data on Gram-negative bacteria are relatively scarce and feather-degrading activity has been described only recently for *Vibrio* sp. (24), *Chryseobacterium* sp. (23) and *Xanthomonas* sp. (4).

An increase in pH values was observed during feather degradation, a trend similar to other microorganisms with large keratinolytic activities (8,24). This trend may be associated with proteolytic activity, consequent deamination reactions and the release of excess nitrogen as ammonium ions. The increase in pH during cultivation is pointed as an important indication of the keratinolytic potential of microorganisms (8).

Microorganisms growing on medium containing feather meal as a unique carbon and nitrogen source presented variable activity on azokeratin, suggesting that this enzyme may be inductive. Substrate levels in the medium may regulate enzyme secretion. Strain kr6 showed to be more adapted to keratinase production using keratin as substrate, since the maximum keratinase activity of the isolate was observed during early growth, and the strain displayed a higher total activity during incubation. The azokeratin/azocasein hydrolysis ratio was higher for strains kr6 and kr14, suggesting preferred utilization of keratin as substrate.

Through the strategy of isolation of keratinolytic microorganisms utilized in this work, bacteria presenting high keratinolytic activity were selected. Considering that feather protein has been showed to be an excellent source of metabolizable protein (10), and that microbial keratinases enhance the digestibility of feather keratin (12,20), these keratinolytic strains could be used to produce animal feed protein. In addition, the selected isolates were able to grow and display keratinolytic activity in diverse keratin wastes. This would be beneficial for the utilization of these residues. These novel isolates present potential biotechnological use in processes involving keratin hydrolysis.

Table 2. Hydrolysis of azoproteins with peptidases.[a]

Enzyme	Activity on azocasein (U/mg protein)	Activity on azokeratin (U/mg protein)	Keratin: casein ratio
Culture supernatant of kr6	19.2	6.1	0.32
Culture supernatant of kr8	10.7	3.0	0.28
Culture supernatant of kr9	14.6	3.5	0.24
Culture supernatant of kr10	48.4	9.1	0.19
Culture supernatant of kr14	15.8	4.8	0.30
Trypsin	18.6	3.1	0.17
Papain	23.1	2.7	0.12
Pronase	34.1	10.5	0.30
Alcalase	29.3	6.9	0.24

[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.

ACKNOWLEDGEMENTS

This work was supported by CNPq and FAPERGS (Brazil).

RESUMO

Bactérias queratinolíticas isoladas de resíduos de penas

O objetivo deste estudo foi caracterizar bactérias queratinolíticas isoladas resíduos de penas. Quatro isolados foram selecionados após crescimento em meio sólido contendo farinha de pena como única fonte de carbono e nitrogênio e avaliados quanto a atividade proteolítica em placas de ágar leite. Foram identificadas três linhagens Gram-negativas (pertencentes aos gêneros *Burkholderia*, *Chryseobacterium* e *Pseudomonas*) e uma Gram-positiva (*Microbacterium* sp.). Estas bactérias cresceram em vários resíduos queratinosos como farinha de pena, penas de frango, unhas de frango, pelos e lã. A atividade queratinolítica foi observada durante crescimento, mas a degradação completa dos substratos não foi observada em todos os casos. O caráter proteolítico das enzimas foi determinado usando azoqueratina e azocaseína como substratos. As queratinases foram ativas em ambos substratos e apresentaram hidrólise de queratina comparável a peptidases microbianas disponíveis comercialmente. Estes novos isolados queratinolíticos apresentam potencial uso biotecnológico em processos relacionados com hidrólise de queratina.

Palavras-chave: pena, queratina, peptidase, proteólise

REFERENCES

1. Atalo, K.; Gashe, B.A. Protease production by a thermophilic *Bacillus* species (P-001A) which degrades various kinds of fibrous proteins. *Biotechnol. Lett*., 15, 1151-1156, 1993.
2. Böckle, B.; Galunski, B.; Müller, R. Characterization of a keratinolytic serine protease from *Streptomyces pactum* DSM40530. *Appl. Environ. Microbiol*., 61, 3705-3710, 1995.
3. Bradbury, J.H. The structure and chemistry of keratin fibers. *Adv. Prot. Chem*., 27, 111-211, 1973.
4. De Toni, C.H.; Richter, M.F.; Chagas, J.R.; Henriques, J.A.; Termignoni, C. Purification and characterization of an alkaline serine endopeptidase from a feather-degrading *Xanthomonas maltophilia* strain. *Can. J. Microbiol*., 48, 342-348, 2002.
5. Elmayergi, H.H.; Smith, R.E. Influence of growth of *Streptomyces fradiae* on pepsin-HCl digestibility and methionine content of feather meal. *Can. J. Microbiol*., 17, 1067-1072, 1971.
6. Friedrich, A.B.; Antranikian, G. Keratin degradation by *Fervidobacterium pennavorans*, a novel termophilic anaerobic species of the order Thermotogales. *Appl. Environ. Microbiol*., 62, 2875-2882.
7. Holtz, J.D. *Bergey's Manual of Determinative Bacteriology*. Williams & Wilkins, Baltimore, 1997, 787p.
8. Kaul, S.; Sumbali, G. Keratinolysis by poultry farm soil fungi. *Mycopathologia*, 139, 137-140, 1997.
9. Kim, J.M.; Lim, W.J.; Suh, H.J. Feather-degrading *Bacillus* species from poultry waste. *Process Biochem*., 37, 287-291, 2001.
10. Klemersrud, M.J.; Klopfenstein, T.J.; Lewis, A.J. Complementary responses between feather meal and poultry by-product meal with or without rumminally protected methionine and lysine in growing calves. *J. Anim. Sci*., 76, 1970-1975, 1998.
11. Kushwaha, R.K.S. The in vitro degradation of peacock feathers by some fungi. *Mykosen*, 26, 324-326, 1983.
12. Lee, C.G.; Ferket, P.R.; Shih, J.C.H. Improvement of feather digestibility by bacterial keratinase as a feed additive. *FASEB J*., 59, 1312, 1991.
13. Lin, X.; Kelemen, D.W.; Miller, E.S.; Shih, J.C.H. Nucleotide sequence and expression of *ker*A, the gene encoding a keratinolytic protease of *Bacillus licheniformis* PWD-1. *Appl. Environ. Microbiol*., 61, 1469-1474, 1995.
14. Lin, X.; Inglis, G.; Yanke, L.; Cheng, K.J. Selection and characterization of feather-degrading bacteria from canola meal compost. *J. Ind. Microbiol. Biotechnol*., 23, 149-153, 1999.
15. Lucas, F.S.; Broennimann, O.; Febbraro, I.; Heeb, P. High diversity among feather-degrading bacteria from a dry meadow soil. *Microb. Ecol*., 45, 282-290, 2003.
16. MacFaddin, J.F. *Biochemical tests for identification of medical bacteria*. Lippincott, Williams & Wilkins, Baltimore, 2000, 912p.
17. Mohamedin, A.H. Isolation, identification and some cultural conditions of a protease-producing thermophilic *Streptomyces* strain grown on chicken feather as substrate. *Int. Biodeter. Biodegrad*., 43, 13-21, 1999.
18. Moritz, J.S.; Latshaw, J.D. Indicators of nutritional value of hydrolyzed feather meal. *Poultry Sci*., 80, 79-86, 2001.
19. Nam, G.W.; Lee, D.W.; Lee, H.S.; Lee, N.J.; Kim, B.C.; Choe, E.A.; Hwang, J.K.; Suhartono, M.T.; Pyun, Y.R. Native feather degradation by *Fervidobacterium islandicum* AW-1 a new isolated keratinase-producing thermophilic anaerobe. *Arch. Microbiol.*, 178, 538-547, 2002.
20. Odetallah, N.H.; Wang, J.J.; Garlich, J.D.; Shih, J.C.H. Keratinase in starter diets improves growth of broiler chicks. *Poultry Sci*., 82, 664-670, 2003.
21. Onifade, A.A.; Al-Sane, N.A.; Al-Musallan, A.A.; Al-Zarban, S. Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresour. Technol.*, 66, 1-11, 1998.
22. Parry, D.A.T.; North, A.C.T. Hard a-keratin intermediate filament chains: substructure of the N- and C-terminal domains and the predicted structure and function of the C-terminal domains of type I and type II chains. *J. Struct. Biol*., 122, 67-75, 1998.
23. Riffel, A.; Lucas, F.; Heeb, P.; Brandelli, A. Characterization of a new keratinolytic bacterium that completely degrades native feather. *Arch. Microbiol*., 179, 258-265, 2003.
24. Sangali, S.; Brandelli, A. Feather keratin hydrolysis by a *Vibrio* sp. kr2 strain. *J. Appl. Microbiol.*, 89, 735-743, 2000.
25. Santos, R.M.D.; Firmino, A.A.P.; Sá, C.M.; Felix, C.R. Keratinolytic activity of *Aspergillus fumigatus* Fresenius. *Curr. Microbiol*., 33, 364-370, 1996.
26. Wang, X.; Parsons, C.M. Effect of processing systems on protein quality of feather meal and hog hair meal. *Poultry Sci*., 76, 491-496, 1997.
27. Williams, C.M.; Richter, C.S.; MacKenzie, J.M.; Shih, J.C.H. Isolation, identification, and characterization of a feather-degrading bacterium. *Appl. Environ. Microbiol*., 56, 1509-1515, 1990.
28. Williams, C.M.; Lee, C.G.; Garlich, J.D.; Shih, J.C.H. Evaluation of a bacterial feather fermentation product, feather-lysate, as a feed protein. *Poultry Sci*., 70, 85-94, 1991.
29. Young, R.A.; Smith, R.E. Degradation of feather keratin by culture filtrates of *Streptomyces fradiae*. *Can. J. Microbiol*., 21, 583-586, 1975.