MICROBIOLOGY INDONESIA, August 2007, p 69-73
ISSN 1978-3477

Volume 1, Number 2

Screening of Proteolytic Enzymes of *Streptomyces* sp. Local Strain and Their Characterization

DERI YURATMOKO[‡], NISA RACHMANIA MUBARIK, AND ANJA MERYANDINI[*]

Department of Biology, Faculty of Mathematics and Natural Sciences, Institut Pertanian Bogor, Darmaga Campus, Bogor 16680, Indonesia

Protease of two *Streptomyces* sp. strain were chosen for characterization because of the large clear zone surrounding the colony in nutrient agar media containing 1% (w/v) skim milk. Extracellular protease from the two isolates SLW 8-1 and 451-3 were characterized following incubation of the isolate in Nutrient Broth media containing skim milk or chicken feather (1%). The optimum activity of the protease SLW 8-1 was at pH 9 and 80 °C, whereas that of the keratinase was at pH 6.5 and 70 °C. Protease of strain 451-3 showed its optimum activity at pH 7.5 and 50 °C whereas the keratinase was at pH 8.5 and 80 °C.

Key words: *Streptomyces* sp., protease, keratinase

Proteases are one of the important enzymes for industry. The sale of proteases constitutes around 60% of the total sales of industrial enzymes (Adinarayana *et al.* 2003) and around 500 tons of protease enzyme are produced every year to fulfill demand coming from industries (Crueger and Crueger 1984).

Many species of bacteria are known in relation to their ability to produce protease, such as *Bacillus subtilis*, *Escherichia coli*, *Clostridium bifermentans*, and *Streptomyces* sp. (Bockle *et al.* 1995; Rao *et al.* 1998; Petinate *et al.* 1999b; Adinarayana *et al.* 2003; Enggel *et al.* 2004). Besides protease, *Streptomyces* sp. is also able to produce keratinase, the enzyme that degrades keratin (Letourneaeu *et al.* 1998; Bressolier *et al.* 1999; Moreira *et al.* 2001). Keratin is abundant in the nature. It is usually found in feather, hair, nail, and horn (Ignatova *et al.* 1999). The presence of many cystein bridges or disulfide bonds, hydrogen bonds, and hydrophobic interactions in keratin structure make this substrate very stable, rigid, and it can hardly be degraded by common proteolytic enzymes (Lin *et al.* 1992; Bockle *et al.* 1995; Bressolier *et al.* 1999).

The test on keratinolytic activity is usually conducted by using flour made from chicken feathers. Lintang (2003) stated that the amino acid content in chicken feather flour is very similar to the amino acids contained in keratin, e.g. the amount of the amino acid serine, arginine, and proline. Keratin in chicken feathers contains some nutrients, which are 81% crude protein, 7% crude fat, 1% crude fiber, 0.33% calcium, and 0.55% phosphorus. The high nutrient content in chicken feathers suggest this is a good feeding substance. The constraints faced in using chicken feathers for feed is the difficulty in digesting the feathers because keratin is not water soluble. A treatment which is usually applied to make the feathers more easily digestable is to use high pressure and high temperature. This treatment, however, needs a very large amount of energy and causes a loss of a large amount of its amino acid contents (Ignatova *et al.* 1999). A biological approach to treat the waste of chicken feathers can be seen as an alternative solution.

MATERIALS AND METHODS

Screening of Proteolytic *Streptomyces* Strains. Culturing a strain from the Microbiology Laboratory, Department Biology, Institut Pertanian Bogor was conducted by growing the strains on nutrient agar with 1% skim milk (NAS media). The culture was then incubated for 7-8 days at room temperature. Strains which produced protease were characterized by formation of clear zone around their colony in NAS media.

Enzyme Production. Pure cultures of the strains were transferred into the NAS media and incubated for 7-8 days at room temperature. The colonies which grew in the NAS media were then harvested using cookborers (with 0.5 cm of diameter) and inoculated to 100 ml of Nutrient Broth media (3 g beef extract and 5 g peptone in 1 l of destilled water) that contained 1% (w/v) skim milk (NBS) in Erlenmeyer sized 500 ml. The cultures were incubated at room temperature using a shaking incubator with an agitation speed of 240 rpm. From the 3rd day of incubation, the protease activities of the cultures were assessed every 24 h. The results, which were conducted until day 14th, were aimed at determining the optimum time to yield the crude protease activity produced. The crude extracts of protease were separated from the cell mass by centrifugation (8,000 g for around 5 min).

Protease Activities. Protease activities were determined using a modified Walter (1984) method. As much as 100 ml crude protease extract was added with 0.5 ml of 1% (wt/vol) casein or 1% (wt/vol) chicken feather and 0.5 ml of 200 mM buffer Tris-HCl pH 7.5. The solution was then incubated at 37 °C for 10 min. The reaction was stopped by adding 1 ml of 10% (wt/vol) trichloroacetic acid (TCA) with continued incubation at 10 °C for 10 min. The next step was centrifugation of the reaction solution at 8,000 g for 10 min. The supernatant (0.75 ml) was added to 2.5 ml Na_2CO_3 0.5 M and 0.5 ml Folin Ciocalteau reagent (1:2), strongly shaken, and incubated for 10 min at room temperature and its absorbance read at 578 nm. One unit (IU) of the enzyme

[‡]Present address, PT. Panjang Jiwo, Jalan Daan Mogot Km. 19, Tangerang, Tanah Tinggi 15122, Indonesia
[*]Corresponding author, Phone/Fax: +62-251-622833, E-mail: ameryandini@yahoo.com

activity is defined as the amount of enzyme that produced 1 µmol soluble tyrosine per min. One unit is equal to 16.67 nkat (Dybkaer 2001).

Protein Concentration. Amounts of protein (mg ml^{-1}) were determined by the Bradford method (Bradford 1976). The protein standard was bovine serum albumin (BSA). Specific activity of the protease (nkat mg^{-1} protein) is the ratio between protease activity (nkat ml^{-1}) and total protein (mg ml^{-1}) of an extract.

Enzyme Characterization. The determination of optimum pH for protease and keratinase activities was conducted by assessing the extract of crude enzyme from pH 5 until pH 9 with interval of 0.5 units. If the optimum activity of the crude enzyme was pH 9, the assessment was continued to pH 10, 11, and 12. The buffer that was used included 200 mM citric acid (pH 5.0-6.5), 200 mM Tris-HCl (pH 7.0-9.0), 200 mM glycine-NaOH (pH 10.0), and 200 mM phosphate-NaOH (pH 11 and 12). The effect of pH on enzyme activities for each kind of substrate was monitored by using an extract of crude enzyme, based on the highest activities in their activity curve.

The determination of optimum temperature of protease and keratinase of the crude extract was conducted through assessment of enzyme activity with various incubation temperatures, which started from 30 °C until 90 °C using 10 °C intervals. The analyses were done at optimum pH.

RESULTS

Proteolytic Strains. A description of the six isolates of *Streptomyces* sp. grown in NAS media is presented in Table 1. Based on the data shown, isolates SLW 8-1, and 451-3 had a bigger clear zone compared to that of the other four isolates.

Protease Activities. On the 3th day, the strain SLW 8-1 had not shown any protease activity. The activity started to be detected on the 4th day. The highest activity was on the 7th day and started to decline on the 9th day, and with a bump in activity on the 10th day (Fig 1).

The strain 451-3 had a less varied patterns for protease activity. On day 3, the enzyme activities of this strain had been detected. The peak of the activity was on day 4, continued with the declining activities until day 6, and increased again until day 11 (Fig 1).

Enzyme Characterization. The extract of crude protease from strain SLW8-1 shows activity at pH between 8.5 and 10.0. The highest activity, which is 0.59 nkat mg^{-1} protein, falls at pH 9 (Fig 2). The highest activity of keratinase for this strain (3.337 nkat mg^{-1} protein) falls at pH 6.5 (Fig 3).

Protease of strain 451-3 shows activity between pH 6.5-8, with the highest activity of 0.49 nkat mg^{-1} protein at pH 7.5 (Fig 4). The highest keratinase activity of strain 451-3 is 27.59 nkat mg^{-1} protein, which was obtained at pH 8.5. This strain also shows high keratinase activity at pH 7.0. The activity is 24.31 nkat mg^{-1} protein (Fig 5).

As a function of temperature, protease activity produced by strain SLW8-1 has three peaks i.e 0.63 nkat/mg protein (40 °C), 0.56 nkat mg^{-1} protein (50 °C), and 0.73 nkat mg^{-1} protein (80 °C) (Fig 6). Keratinase produced by strain strain SLW8-1 has optimum temperature at 70 °C with an activity of 4.559 nkat mg^{-1} protein (Fig 7).

Table 1 Description of 6 isolates grown nutrient agar with 1% skim milk media after 12 days of incubation

Isolate	Clear zone	Proteolytic index
PD 3-27	yes	< 1.00
SLW 8-1	yes	2.46
234P-16	no	-
KS-1	yes	< 1.00
PS-4-11	yes	1.30
451-3	yes	1.50

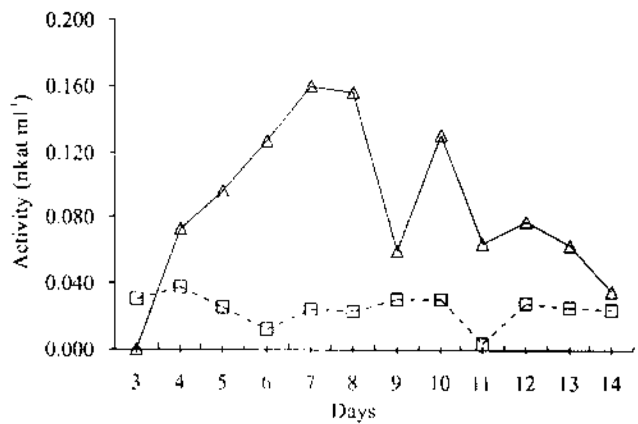

Fig 1 Activity curves from SLW8-1 and 451-3 proteases measured at pH 7.5 and 37 °C. -□- Protease 451-3, -△- Protease SLW8-1 (U ml^{-1}).

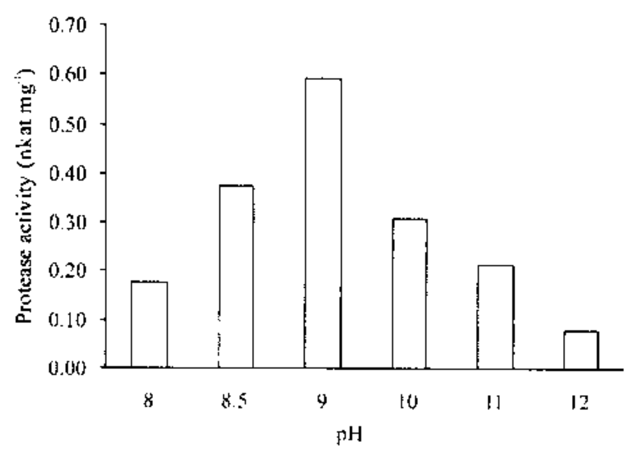

Fig 2 Protease activities from strain SLW8-1 as a function of pH at 37 °C.

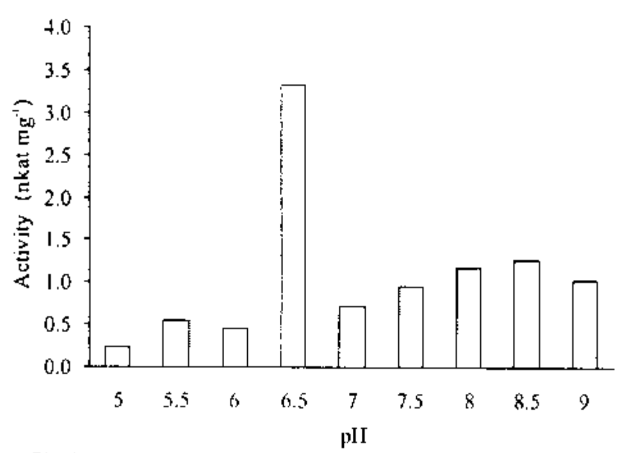

Fig 3 Keratinase activity from strain SLW8-1 as a function of pH at 37 °C.

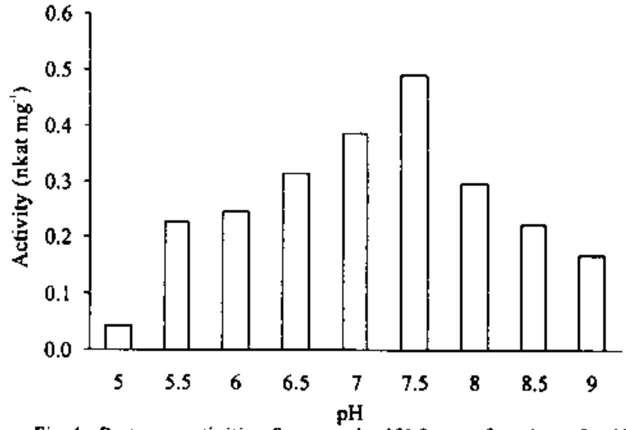

Fig 4 Protease activities from strain 45I-3 as a function of pH at 37 °C.

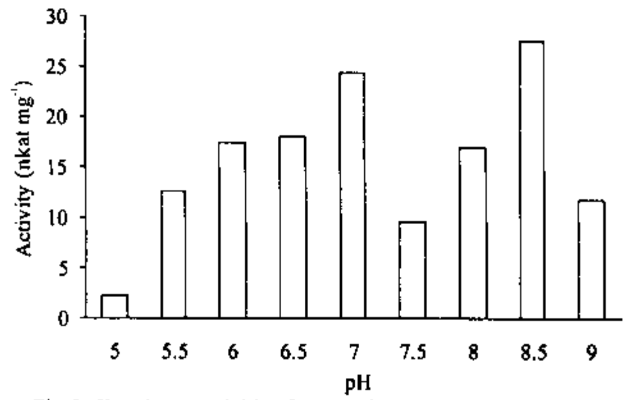

Fig 5 Keratinase activities from strain 45I-3 as a function of pH at 37 °C.

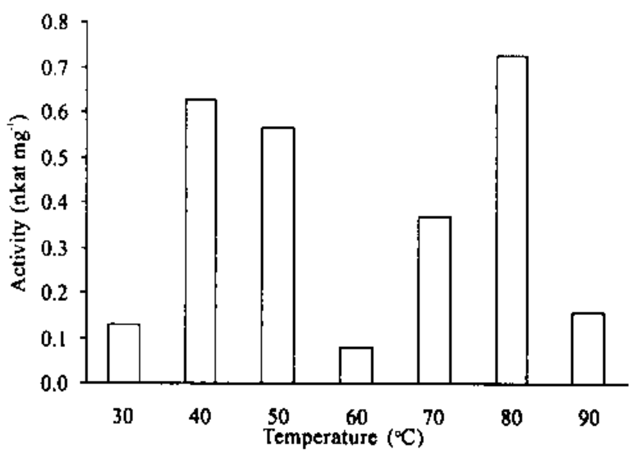

Fig 6 The effect of temperature on protease activity from strain SLW8-1 at pH 9.

Protease of strain 45I-3 has optimum temperature at 50 °C with protease activity 0.48 nkat mg^{-1} protein (Fig 8). Keratinase produced by strain strain 45I-3 has optimum temperature at 80 °C with activity 71.65 nkat mg^{-1} protein (Fig 9).

Both isolates produced protease and keratinase with a higher keratinase activity if cultured on NAS media (Fig 10).

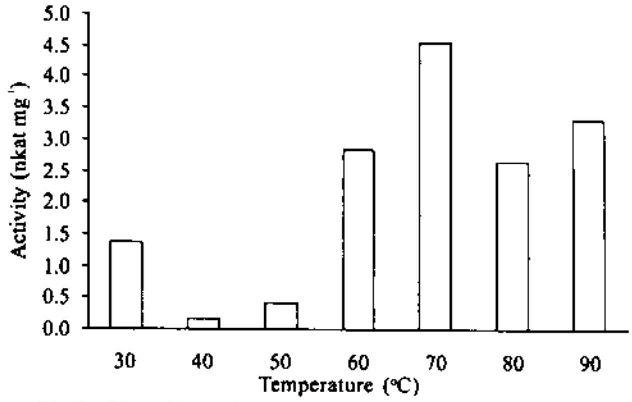

Fig 7 The effect of temperature on keratinase activity from strain SLW8-1 at pH 6.5.

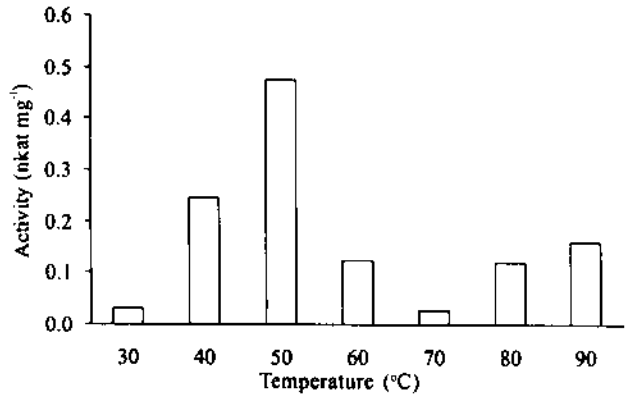

Fig 8 The effect of temperature on protease activity strain 45I-3 at pH 7.5

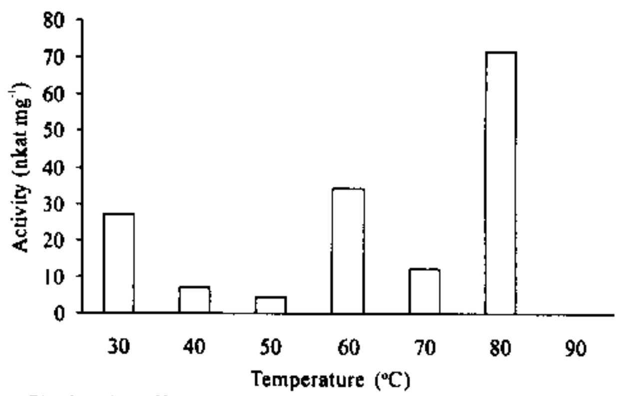

Fig 9 The effect of temperature on keratinase activity strain 45I-3 at pH 8.5.

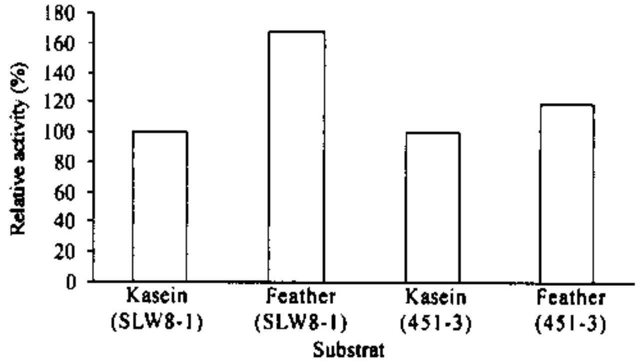

Fig 10 Relative protease and keratinase activity (%) from *Streptomyces* sp. SLW8-1 and 45I-3 measured at optimum pH and temperature of each enzyme.

DISCUSSION

Although the diameter of the clear zone in solid media is not always correlated with the high activity of protease production in liquid media, the method is often used to precisely identify whether or not the isolates produce extracellular protease. Many bacteria are known for their ability to produce protease (Rao *et al.* 1998). Each of the bacteria isolates of the same genus produces a different quality and of protease having different characteristics. The addition of 1% (w/v) skim milk to the NA media was intended to induce the bacteria to synthesize protease.

Beyond its optimum time, protease activities continued to decline. The decrease was considered to result from autolysis process of the protease itself. Decreasing amounts of substrate, due to hydrolysis that occurred by extracellular protease produced previously, is also considered to contribute some effect to the decline of the protease activities beyond its optimum period.

Feather meal can be used to induce keratinase activity. *Vibrio* sp. strain Kr6 and *Chryseobacterium* sp. produced higher yields of keratinase using feather meal or raw feather as a casein substrate (Sangali and Brandelli 2000; Brandelli and Riffel 2005).

Protease of strain SLW8-1 belongs to the alkaline proteases, while its keratinase belongs to the pH neutral group of enzyme. Strain 45I-3, on the other hand produces, a neutral protease and an alkaline keratinase. A protease which is active in alkaline condition has also been reported by Petinate *et al.* (1999a) for enzymes produced by *Streptomyces cyaneus* (pH 9), Letourneau *et al.* (1998) for protease produced by *Streptomyces* sp. S.K_{1-02} (pH 10), and Adinarayana *et al.* (2003) for protease produced by *Bacillus subtilis* PE-11 (pH 10). Protease with optimum activity at a neutral pH was reported by Lin *et al.* (1992) for *B. licheniformis*, Bressolier *et al.* (1999) for *S. albidoflavus*, and Moreira *et al.* (2001) for *S. clavuligerus*.

The several peak of activities which occurred in the optimum temperature determination may be due to the presence of isozymes. Isozymes are enzymes which catalyze the same reaction but shows different physical and chemical characteristics (for example isoelectric point, optimum pH, substrate affinity, or inhibitor effect) when they are synthetised via different genes.

Letourneau *et al.* (1998) also reported that *Streptomyces* sp. S.K_{1-02} will rapidly produced a protease mixture with high keratinolytic activity if cultured on a simple medium supplemented with feather meal.

Some studies have also reported the ability of one strain to produce different types of protease such as *Streptococcus suis* which is able to produce 4 isozymes (Jobin and Grenier 2003). *Streptomyces albidoflavus* has been reported to produce at least 6 isozymes of protease after the exponential growth phase is completed. The production of several proteases in *Streptomyces* is perhaps due in-situ degradation of mycelium proteins by protease during different stages of morphological differentiation (Bressolier *et al.* 1999). Ginther (1979) also reported that in *Streptomyces lactamdurans* the production of an antibiotic and protease is closely associated with the sporulation process.

The protease of strain 45I-3 has optimum temperature at 50 °C with protease activity 0.48 nkat mg^{-1} protein (Fig 8). The similar result was reported by Moreira *et al.* (2001) for a protease partially purified from *S. clavuligerus* 3585, and Lin *et al.* (1992) for protease activity of *B. licheniformis*.

In our study, the protease of strain SLW 8-1 shows optimum activity at pH 9 and 80 °C, while its optimum keratinase activity is reached at H 6.5 and 70 °C. Also pH 7.5 and 50 °C is needed by strain 45I-3 to show its optimum protease activity, and pH 8.5 and 80 °C are needed for its optimum keratinase activity.

ACKNOWLEDGMENT

The authors wish to thank Yulin Lestari for the isolates.

REFERENCES

Adinarayana K, Ellaiah P, Prasad DS. 2003. Purification and partial characterization of thermostable serine alkaline protease from a new isolated *Bacillus subtilis* PE-11. *AAPS Pharm Sci Tech* 56:1-9.

Bockle B, Galunsky B, Muller R. 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. *Appl Environ Microbiol* 6:3705-3710.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantitaties of protein in utilizing the principle of protein-dye binding. *Ann Biochem* 72:248-254.

Brandelli A, Riffel A. 2005. Production of an extracellular keratinase from *Chryseobacterium* sp. growing on raw feathers. *Biotechnol* 8:35-42.

Bressollier P, Letourneau F, Urdaci M, Verneuil B. 1999. Purification and characterization of a keratinolytic serine protease from *Streptomyces albidoflavus*. *Appl Environ Microbiol* 65:2570-2576.

Crueger A, Crueger W. 1984. *Biotechnology: A Textbook of Industrial Microbiology*. In: Brock TD (ed). Sunderland: Minauer Associater Inc.

Dybkaer R. 2001. Unit "katal" for catalytic activity. *J Pure Appl Chem* 73:927-931.

Enggel J, Meryandini A, Natalia L. 2004. Karakterisasi protease ekstraseluler *Clostridium bifermentans* R14-1-b. *J Mikrobiol Indones* 9:9-12.

Ginther CL. 1979. Sporulation and the production of serine protease and cephamycin C by Streptomyces lactamdurans. *Antimicrob Agents Chemotherapy* 15:522-526.

Ignatova Z, Gousterova A, Spassov G, Nedkov P. 1999. Isolation and partial characterisation of extracellular keratinase from a wool degrading thermophilic actinomycete strain *Thermoactinomyces candidus*. *J Can Microbiol* 45:217-222.

Jobin MC, Grenier D. 2003. Identification and characterization of four proteases produced by *Streptococcus suis*. *FEMS Microbiol Lett* 220:113-119.

Letourneau F, Soussotte V, Bressollier P, Branland P, Verneuil B. 1998. Keratinolytic activity of *Streptomyces* sp. S.K_{1-02}: a new isolated strain. *Appl Environ Microbiol* 26:77-80.

Lin X, Lee C, Casale ES, Shih JCH. 1992. Purification and characterization of keratinase from a feather-degrading *Bacillus licheniformis* strain. *Appl Environ Microbiol* 58:3271-3275.

Lintang RAJ. 2003. Keratinase pendegradasi bulu ayam dari bakteri termofilik L-23 asal Sulawesi Utara [Thesis]. Bogor: Program Pascasarjana IPB.

Moreira KA, Calvalcanti MTH, Duarte HS, Tambourgi EB, de Melo EHM, Silva VK, Porto ALF, Filho JLdeL. 2001. Partial characterization of protease from *Streptomyces clavuligerus* using an inexpensive medium. *J Brazilian Microbiol* 32:215-220.

Petinate SDG, Branquinha MH, Coelho RRR, Vermelho AB, de Simone SG. 1999a. Purification and partial characterization of an extracellular serine-proteinase of *Streptomyces cyaneus* isolated from Brazilian cerrado soil. *Appl Microbiol* 87:557-563.

Petinate SDG, Martins RM, Coelho RRR, Meirelles MNL, Branquinha MH, Vermelho AB. 1999b. Influence of growth medium in proteinase and pigment production by *Streptomyces cyaneus*. *J Mem Inst Oswaldo Cruz Rio de Janeiro* 94:173-177.

Rao MB, Thanksale AM, Ghatge MS, Desphande VV. 1998. Molecular and biotechnological aspects of microbial protease. *J Microb Mol Biol Rev* 62:597-635.

Sangali S, Brandelli A. 2000. Feather keratin hydrolysis by a *Vibrio* sp. strain Kr2. *J Appl Microbiol* 89:735-743.

Walter HE. 1984. Proteinase (proteins substrates). Method with haemoglobin, casein and azocoll as substrate. In: Bergmeyer J, Grass M (eds). *Methods of Enzymatic Analysis*. 3th Ed. Weinheim Germany: Verlag Chemie. p 270-278.