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Research Article

Keratinase Production by Three *Bacillus* spp. Using Feather Meal and Whole Feather as Substrate in a Submerged Fermentation

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Three *Bacillus* species (*B. subtilis* LFB-FIOCRUZ 1270, *B. subtilis* LFB-FIOCRUZ 1273, and *B. licheniformis* LFB-FIOCRUZ 1274), isolated from the poultry industry, were evaluated for keratinase production using feathers or feather meal as the sole carbon and nitrogen sources in a submerged fermentation. The three *Bacillus* spp. produced extracellular keratinases and peptidases after 7 days. Feather meal was the best substrate for keratinase and peptidase production in *B. subtilis* 1273, with 412 U/mL and 463 U/ml. The three strains were able to degrade feather meal (62–75%) and feather (40–95%) producing 3.9–4.4 mg/ml of soluble protein in feather meal medium and 1.9–3.3 mg/ml when feather medium was used. The three strains produced serine peptidases with keratinase and gelatinase activity. *B. subtilis* 1273 was the strain which exhibited the highest enzymatic activity.

1. Introduction

Feather waste is a byproduct of the domestic poultry industry and is 90% keratin [1, 2]. However, the use of feather waste as a dietary protein supplement for animal feedstuffs is only carried out on a limited basis, due to its poor digestibility [3]. Keratin is an insoluble protein and is resistant to degradation by common peptidases, such as trypsin, pepsin, and papain [4, 5]. This resistance is due to the constituent amino acid composition and configuration that provide structural rigidity. The mechanical stability of keratin and its resistance to biochemical degradation depend on the tightly packed protein chains in α-helix (α-keratin) and β-sheet (β-keratin) structures. In addition, these structures are cross-linking by disulfide bridges in cystines residues [3, 4, 6].

A current value-added use for feathers is the conversion to feather meal using physical and chemical treatments. However these methods can destroy certain heat-sensitive amino acids, such as methionine, lysine, and tryptophan, generating other nonnutritive amino acids, for instance, lanthionine and lysinoalanine [7].

An alternative and attractive method for improving the digestibility of feathers or feather meal is biodegradation by keratinolytic microorganisms [8, 9]. A number of keratinolytic microorganisms can produce keratinases (E.C. 3.4.99.11), peptidases which are capable of degrading keratin. Various authors have reported that, among the keratinolytic microorganisms, some species of *Bacillus* [10–12], actinomycetes [9, 13, 14], and fungi [15–17] are able to produce these keratinases and peptidases.

Biodegradation of poultry waste by keratinases is an environment friendly biotechnological process, which converts this abundant waste into low-cost, nutrient-rich animal feed [18, 19]. Keratinolytic enzymes have applications in

the detergent, medical, cosmetic, and leather industries; they can also be used in prion degradation and as pesticides [11, 20, 21].

In the present work, the production of keratinases and peptidases by three *Bacillus* species isolated from poultry waste was investigated. Considering that the presence of keratinous substrates usually induces keratinase production, the main aim of the study was to compare the influence of feather or feather meal on the production of keratinolytic enzymes by three *Bacillus* spp.

2. Materials and Methods

2.1. Isolation, Selection, and Maintenance Procedures. Poultry residues, including feathers, feather meal, and other materials, obtained from a poultry industry (Rica Alimentos, Brazil), were added at 1% to modified Sabouraud (glucose 2.0%, peptone 1.0%, yeast extract 0.5%) or yeast extract-peptone-sucrose (yeast extract 0.5%, peptone 0.5%, KCl 2.0%, sucrose 2.0%) liquid media. After 48 hours at 28°C, a loopful of the growing microorganism was streaked on the same solid media above containing agar (2.0%) and incubated for 72 hours at 28°C. The isolated colonies were inoculated for 28 days at 28°C in tubes containing saline (NaCl 0.85%) and a single feather. The microorganisms which were able to grow in these conditions were transferred to yeast extract-peptone-sucrose solid medium slants, cultivated for 48 hours at 28°C and then maintained at 4°C.

2.2. Bacillus sp. Identification. The microorganisms were identified at the Bacterial Physiology Laboratory, Bacteriology Department, Fundação Oswaldo Cruz, Brazil, by Jeane Quintanilha and Dr. Leon Rabinovitch, based on cell morphology, physiological characteristics, and biochemical analysis. They were deposited at the *Coleção de Culturas do Gênero Bacillus e Gêneros Correlatos,* CCGB (which is affiliated to the World Federation of Culture Collections) located at the Oswaldo Cruz Foundation, Brazil.

2.3. Inoculum Preparation. Erlenmeyer flasks (125 mL) containing 25 ml of yeast extract-peptone liquid media were inoculated with a loopful of each strain and incubated at 26 ± 1°C and 300 rev/min for 72 hours. After centrifugation (2000 g/20min at 26°C) and washing twice with saline, each pellet was further used.

2.4. Keratin Substrate. Chicken feathers obtained from poultry waste were washed extensively with water, and anionic detergent, dried at 60°C overnight, delipidated with chloroform: methanol (1 : 1, v/v), and dried again at 60°C. Feather meal was provided by Rica Alimentos industry.

The keratin for analytical analyses was obtained by the method described by Wawrzkiewicz et al. [22] modified. Briefly, 10g of feathers were heated with a reflux condenser at 100°C for 80–120 min with 500 mL of DMSO. Keratin was then precipitated by the addition of two volumes of acetone and maintained at 4°C for 24–48 hours. The keratin precipitates were collected by centrifugation (2x

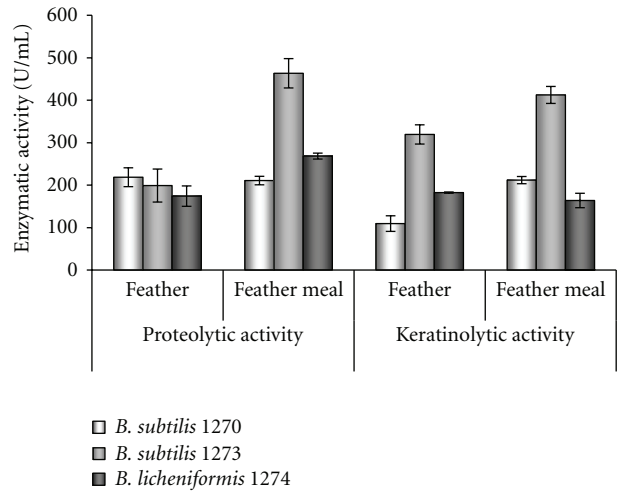

Figure 1: Proteolytic and keratinolytic activity of *B. subtilis* 1270, *B. subtilis* 1273, and *B. licheniformis* 1274 after being grown (7 days at 28°C) in feathers or feather meal media.

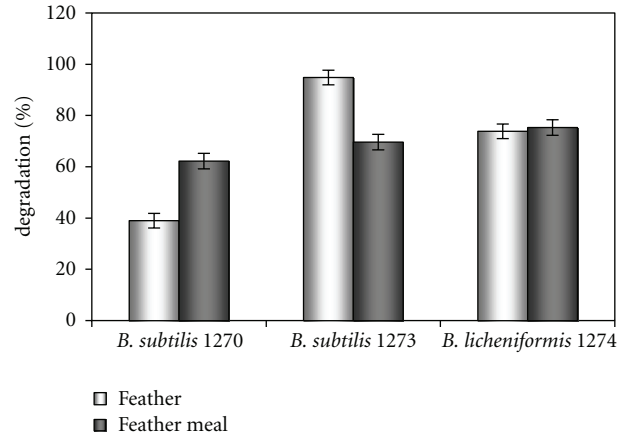

Figure 2: Substrate degradation by *B. subtilis*1270, *B. licheniformis* 1274, and *B. cereus*1274 after 7 days of cultivation at 28°C in feathers or feather meal media.

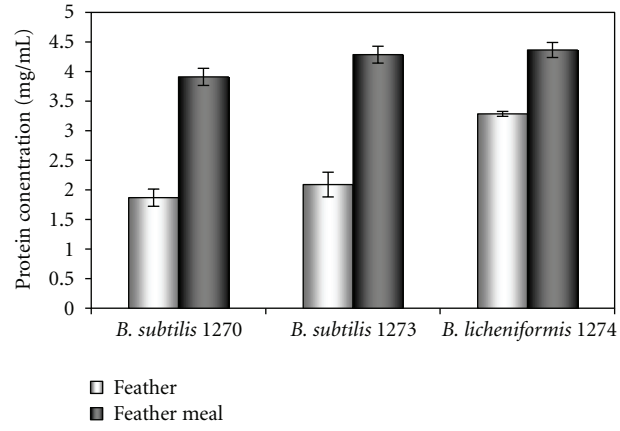

Figure 3: Protein concentration in culture supernatant of *B. subtilis* 1273, *B. licheniformis* 1274, and *B. cereus* 1268 grown in feathers and feather meals media at 28°C for 7 days.

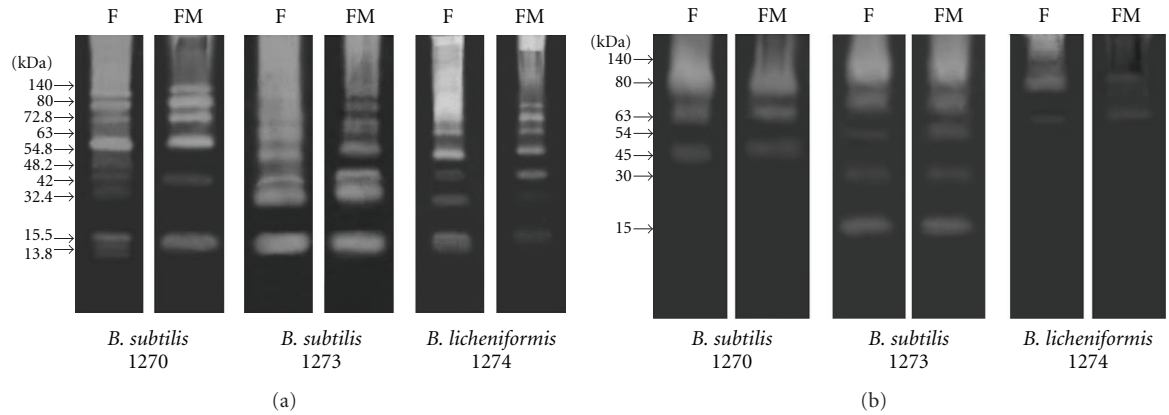

Figure 4: Gelatin (a) and keratin (b) zymogram analysis of extracellular peptidases from *B. subtilis* 1270, *B. subtilis* 1273, *B. licheniformis* 1274 grown in feather (F) or feather meal medium (FM). Gel strips containing concentrated culture supernatant were incubated for 48 h at 37°C in citric acid buffer pH 5.0. The approximate molecular mass of the peptidases is shown on the left.

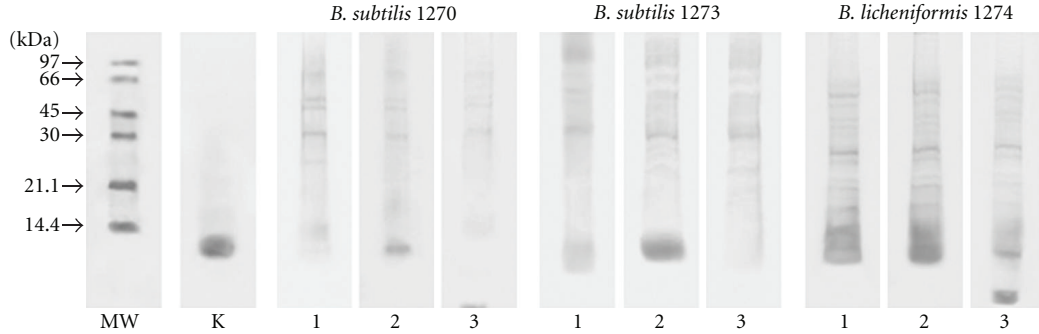

Figure 5: Cleavage of keratin substrate by extracellular peptidase activities of *Bacillus* sp. analysed by SDS-PAGE. K keratin; 1: supernatant control; 2: reaction mixture containing keratin and enzymatic extract before incubation; 3: reaction mixture after 1 hour of incubation at 37°C. Numbers on the left indicate relative molecular mass markers (kDa). Phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as molecular mass standards.

2000 g/15 min), washed twice with distilled water and dried at 4°C. The white powder obtained correspond to the keratin substrate [11].

2.5. Keratinase Production. Fermentations were carried out by seeding the inoculum preparation (item 2.3) into Erlenmeyer flasks (250 mL) containing a liquid medium (100 mL) composed of a mineral salt solution ($Na_2HPO_4.7H_2O$ 0.06M 72 mL, KH_2PO_4 0.06M 28 mL, pH 7.2), 0.01% yeast extract and two substrates as the main carbon and nitrogen source: feathers or feather meal at 1%. After incubation for 7 days at 26 ± 1°C under shake conditions (300 rpm) the media were centrifuged (2000 g/20 min). The supernatant was the source of enzymatic extract and was used for keratinase and gelatinase activity assays and in the zymographic and enzymographic analysis. For electrophoresis experiments supernatants were concentrated twenty times in dialyzing membranes (cut off 9000 Da) against polyethylene glycol 4000, overnight at 4°C.

2.6. Feather and Feather Meal Degradation. The percentage of substrate degradation was measured by weight loss. The feathers and feather meal still present in medium after the cultivation period were filtered through Whatman n°1 filter paper, thoroughly washed, with 70% alcohol, oven dried at 60°C for 48 hours, and finally weighed to determine weight-loss.

2.7. Enzymatic Activity. Keratinase activity was measured according to Grzywnowicz et al. [23]. The reaction mixture contained 1.0 mL of the culture supernatant diluted five times and 1.5 mL of 0.67% (w/v) keratin suspension in phosphate buffer 0.1 M, pH 7.4. After 1 hour of incubation at 37°C the reaction was interrupted by the addition of 1ml trichloroacetic acid 10% and placed in a refrigerator at 4°C for 30 minutes. An enzyme control was prepared by the addition of 1ml trichloroacetic before incubation. The reaction mixture was centrifuged (2000 g/10 min) and read at 280 nm in a spectrophotometer. One unit of keratinase activity was defined as the amount of enzyme required to produce an absorbance increase of 0.01 under the described assay conditions.

Gelatinase activity was measured in systems containing 100 μL culture supernatant, 400 μL phosphate buffer 0.1 M

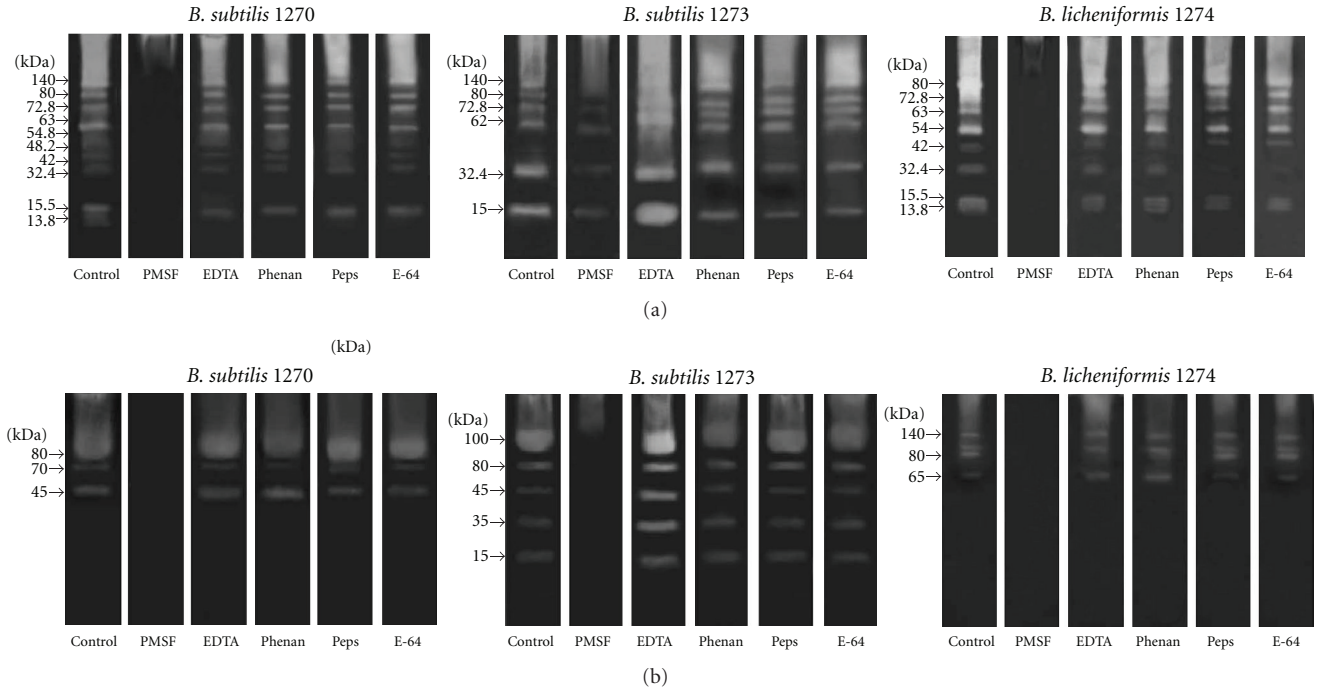

Figure 6: Effect of proteolytic inhibitors on the extracellular gelatinases (a) and keratinases (b) of *Bacillus* sp. grown in feather medium for 7 days at 28°C. Gel strips containing concentrated culture supernatant were incubated for 48 h at 37°C in proteolysis buffer (0.1 M citric acid buffer pH 5.0) in the absence (control) or in the presence of different proteolytic inhibitors: 3 mmoL l^{-1} phenylmethylsulfonyl fluoride (PMSF), 0.26 mmoL l^{-1} ethylenediaminetetraacetic acid (EDTA), 10 mmoL l^{-1} 1,10 phenanthroline (Phenan), 10 μmoL l^{-1} pepstatin A (Peps), and 5 μmoL l^{-1} M transepoxysuccinyl L-leucylamido-(4-guanidino) butane (E-64).

pH 7.4, and 750 μL gelatin 1%. After incubation at 37°C for 30 minutes, 375 μL of this solution was mixed with 500 μL isopropanol, refrigerated at 4°C for 15 minutes, centrifuged (2000 g/15 min), and 100 μL of the supernatant was used to measured the amount of protein by the Lowry method [24, 25]. One unit of gelatinase activity was defined as the amount of enzyme required to produce 1 μg of peptides under the described assay conditions.

2.8. Protein Content. This was determined in the culture supernatants according to Lowry et al. [25], using albumin bovine serum as the standard. Readings were carried out in a spectrophotometer at 660 nm.

2.9. Enzymography. Keratin degradation was evaluated by adding 20 μL of the concentrated supernatant (containing 5 μg proteins) to 5 μg keratin and 20 μL of distilled water. The reaction mixture was incubated for 1 hour at 37°C. The presence of keratin and other proteinaceous material was detected by SDS-PAGE. After incubation, the reaction mixture was diluted in 40 μL sample buffer (Tris-HCl 0.15 M pH 6.8, SDS 0.5%, glycerol 10.8% v/v, 3% 2-mercaptoetanol and bromophenol blue 0.1%), heated at 100°C for 5 minutes, and then 30 μL was applied to 15% polyacrylamide gel [26]. Runs were performed at 170 V for 2.5 hours at 26°C. The gels were stained with Coomassie Brilliant Blue R250 (5 mL stock solution of coomassie blue 2% w/v; 4 mL acetic acid; 20 mL methanol and 11 mL distilled water) overnight. Then the gels were decolorized with a methanol: acetic acid: water solution (50 : 10 : 40 v/v/v) under agitation (70 rpm), until the bands appeared.

2.10. Zymograms. The concentrated culture supernatant was mixed with the peptidase sample buffer [Tris-HCl 0.32 M, pH 6.8; glycerol 48% (v/v); SDS 8% (w/v) and bromophenol blue 0.06% (w/v)] in a 6 : 4 proportion. Samples were applied to 12.5% polyacrylamide gel (staking gel 5%) containing 0.1% (w/v) gelatin or keratin copolymerized [26, 27]. Runs were performed at 170 V for 2.5 hours at 4°C. After the runs the gels were washed twice with Triton-X 2.5% (v/v) for 15 minutes under agitation (70 rpm) to remove SDS and then incubated for 48 hours at 37°C in citric acid buffer (48.5 mL citric acid 0.1 M and 51.5 mL Na_2HPO_4 0.2 M), pH 5.0. For proteolytic detection the gels were incubated in coomassie blue as described above, overnight, and decolorized with methanol: acetic acid: water (50 : 10 : 40 v/v/v), under agitation, until clearing of the degrading bands.

2.11. Effect of Inhibitors on Peptidase and keratinase Activity. The concentrated culture supernatants were submitted to zymography, as described above, with gelatin or keratin as substrates copolymerized. The gels were incubated for 48 h in proteolysis buffer (0.1 M citric acid buffer pH 5.0) containing 3 mM phenylmethylsulfonyl fluoride (PMSF), 0.26 mM ethylenediaminetetraacetic acid (EDTA), 10 mM1

1,10 phenanthroline, 10 μM pepstatin A, and 5 μM trans-epoxysuccinyl l-leucylamido-(4-guanidino) butane (E-64).

3. Results and Discussion

3.1. Isolation, Selection, and Characterization of Keratinolytic Bacillus sp. Seventeen different keratinolytic microorganisms were isolated from poultry waste in decomposition. After cultivation in minimal medium with feather as the sole carbon and nitrogen source, three isolates, presenting a pronounced growth and feather degradation, were selected and identified as *Bacillus subtilis* LFB-FIOCRUZ 1270, *B. subtilis* LFB-FIOCRUZ 1273, and *B. licheniformis* LFB-FIOCRUZ 1274 and deposited in culture collection of Fundação Oswaldo Cruz (Coleção de Culturas do Gênero *Bacillus* e Gêneros Correlatos—FIOCRUZ), in Brazil.

3.2. Influence of Keratin Substrate on Keratinase and Peptidase Production. Extracellular keratinase and peptidases were obtained after growth of the *Bacillus* sp. on the culture medium containing feathers or feather meal as the sole carbon and nitrogen sources. Both keratin substrates present low digestibility however they could be an important protein source for animal feed after enzymatic hydrolysis of keratin [28]. The three microorganisms were able to produce keratinases and peptidases after 7 days of fermentation. Feather meal was the best substrate for keratinase production with *B. subtilis* 1270. No significant difference was observed in the keratinolytic activity when *B. licheniformis* 1274 was cultivated in feather or feather meal media. *B. subtilis* 1273 presented the highest level of keratinase and peptidase activity with 319 U/mL of keratinases in feathers and 412 U/mL in feather meal and 450 U/mL of peptidases in feather meal (Figure 1). Peptidase production using feathers was the same for the three *Bacillus* (around 200 U/mL) (Figure 1).

There are only a few studies that have used feather meal as a substrate for microbial fermentation, and according to the results obtained this substrate is a keratin-rich substrate supporting microbial growth in culture medium [2, 29, 30]. Additionally the percentage of degradation of feather, or feather meal by the three stains, was evaluated. *B. subtilis* 1273 degraded 70% and 95% of feather meal and feather, respectively (Figure 2). *B. subtilis* 1273 supernatant presented the highest peptidase and keratinase activity with feather meal with 463 U/mL and 412 U/mL, respectively. Protein content was highest with feather meal (3.9–4.4 mg/mL). With feathers, 1.9–3.3 mg/mL were obtained for all *Bacillus* sp. (Figure 3).

Son et al. [31] investigated the keratinase production and keratin degradation by *B. pumilus* F3-4 after 7 days of incubation with different keratin substrates. Feather meal was the most degraded (97%) and induced higher keratinase activity. The ability of a microorganism to degrade keratin and the resulting levels of keratinase produced vary according to the specie, chemical composition, the molecular structure of keratin substrates, and the culture conditions [31, 32].

3.3. Gelatin and Keratin Zymograms. All *Bacillus* sp. showed major peptidases migrating at 13.8 to 140 kDa. The differences observed were quantitative (Figure 4(a)). Keratinases were detected in a range of 45–80 kDa in *B. subtilis* 1270, 15–100 kDa in *B. subtilis* 1273, and 63–140 kDa in *B. licheniformis* 1274 (Figure 4). *B. licheniformis* 1274 in feathers or feather meal presented the lowest keratinases activity (Figure 4(b)).

Keratinases with molecular masses ranging from 15 to 240 kDa have been reported [33]; however the majority of keratinases presented molecular masses varying from 20 to 50 kDa [34]. Our group previously showed multiple peptidases and keratinase (15–200 kDa) by other *Bacillus* sp. [11, 27].

Extracellular keratinases of other *Bacillus* such as *B. subtilis* strain KS-1, *B. pseudofirmus* strain FA30-01, *B. pumilus,* and *B. cereus* have been described secreting single polypeptides with a molecular mass of 25.4, 27, 65, and 45 kDa, respectively [8, 10, 12, 35].

Enzymographic analysis using keratin substrate (Figure 5) demonstrated that the enzymatic extract obtained from the *Bacillus* was able to hydrolyze keratin. Protein bands with a molecular mass of 10 kDa corresponding to feather keratin were detected in SDS-PAGE after incubation with the enzymatic extract.

3.4. Determination of Peptidase and Keratinase Classes. Inhibition tests demonstrated that all peptidases including keratinases are serine peptidases (Figure 6). Most of keratinases described, particularly those produced by *Bacillus,* are serine peptidase, and some of them belong to the subtilisin family [36]. Keratinases of *B. cereus* and *B. pseudofirmus* FA30-01, and *B. pumilus* were completely inhibited by PMSF [3, 10, 35]. In the *Bacillus* genus a secreted metallopeptidase has been described by Werlang and Brandelli [37].

4. Conclusions

In this work we described the isolation and identification of three new keratinolytic *Bacillus* sp. Feather and feather meal were used in a submerged fermentation in order to obtain the peptidases. It is interesting to note that the occurrence of peptidases was more prominent in the presence of feather meal. These results showed the potential of feather meal as a substrate for microbial fermentation. Microorganisms isolated in this study present potential for biotechnological uses. The *B. subtilis* 1273 was the most efficient in keratin degradation. Its enzymes could be used as additives in animal feed to improve feather meal digestibility.

Acknowledgments

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