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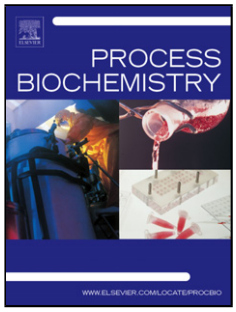

Title: Purification and characterization of a keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876

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Highlights

► This is the first report of a serine protease with keratinolytic activity from *P.lilacinus LPS* #876.

► Enzyme stability in broad pH range, and up to 65 °C, suggests its suitability as a detergent additive.

► Oxidant/detergent stability strengthens the enzyme's potential application as laundry additive.

► The production of this enzyme could be an alternative for solid waste management processes, an added valued product for tanneries

Purification and characterization of a keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876

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ABSTRACT

A keratinolytic serine protease secreted by *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*) upon culture in a basal medium containing 1% (w/v) hair waste as carbon and nitrogen source was purified and characterized. After purification the keratinase was resolved by SDS- PAGE as a homogeneus protein band of molecular mass 37.0 kDa. The extracellular keratinase of *P. lilacinum* was characterized by its appreciable stability over a broad pH range (from 4.0 to 9.0), and up to 65 °C, along with its strong inhibition by phenylmethylsulphonyl fluoride among the protease inhibitors tested (98.2% of inhibition), thus suggesting its nature as a serine protease. The enzyme was active and stable in the presence of organic solvents such as dimethylsulfoxide, methanol, and isopropanol; certain surfactants such as Triton X-100, sodium dodecylsulfate, and Tween 85; and bleaching agents such as hydrogen peroxide. These biochemical characteristics suggest the potential use of this enzyme in numerous industrial applications.

Keywords: Enzyme purification, Keratinase, Serine protease, Hair waste, *Purpureocillium lilacinum*

1. Introduction

Keratins are insoluble proteins highly cross-linked with disulfide bonds, which linkage in addition to a tightly packed supercoiled polypeptide chain results in high mechanical stability and resistance to proteolytic hydrolysis [1]. Keratin sources such as feather, horn, nails, and hair are abundantly available in nature as wastes. In particular,

hair waste—a solid refuse generated by the hair-saving unhairing processes—constitutes a troublesome biodegradable product that is produced in large quantities by tanneries. With feather waste a considerable portion is converted to feather meal and is used as a dietary supplement for animal feed [2]; but with hair-waste, its disposal is the only option at the present time. Therefore, the bioconversion of this kind of residue is an attractive possibility of biotechnological interest since such a utilization might represent an alternative means of waste management that could result in the production of valuable products such as slow-release nitrogen fertilizers, cosmetics, and biodegradable films [3] in addition to being a source of useful enzymes. With respect to this latter possibility, the keratinases produced when keratin-containing wastes are used as substrates could have practical biotechnologic uses: for example, unhairing capabilities of these enzymes would avoid the environmental problems caused by traditional methods such as treatment with sulfide in the leather industry, while the keratinases could also prove useful in the detergent and cosmetics industries [4].

Alkaline keratinases from different microorganisms, and with different biochemical properties, have been extensively purified and characterized [4]. The optimal activity of these keratinases lies in the neutral to alkaline pH range, *i. e.*, 7.0–9.0. A few keratinases exhibiting extremely alkalophilic pH optima (*e. g.*, 11.0), however, have been reported [5,6]; but those hydrolases have proven not to be resistant to bleach and detergents.

In order to determine the extent of its potential industrial application, the study of the properties of the *Purpureocillium lilacinum* keratinase is fundamental in order to design a biocatalyst suitable to endure industrial conditions, thus making large-scale applications ultimately feasible. *P. lilacinum* LPS # 876 was found to produce keratinase activity when it was grown in liquid cultures with chicken feathers as

substrate [7]. In the present investigation, the purification and characterization of a keratinolytic serine protease secreted by this same fungus, but with hair waste as the substrate, is described.

2. Materials and Methods

2.1. Microorganism and culture conditions

P. lilacinum LPS # 876 (formerly *Paecilomyces lilacinus*), isolated from soils in public places in the city of La Plata, Argentina [8], is a non-pathogenic fungal strain, which was deposited at the Spegazzini Institute fungal culture collection (La Plata National University, Argentina). It was maintained in tubes containing potato-dextrose agar under mineral oil at 4 ºC. Cultures were established in 1,000-ml Erlenmeyer flasks in 200 ml of hair basal medium containing (per liter) 10 g hair waste, 496 mg NaH_2PO_4, 2.486 g K_2HPO_4, 16 mg $FeCl_3.6H_2O$, 13 mg $ZnCl_2$, 10 mg $MgCl_2$, and 0.11 mg $CaCl_2$ (pH 7.0) [9]. Hair waste, obtained from a local tannery, was washed extensively with tap water; dried at 60 ºC for 2 days; and used as the source of carbon, nitrogen, and sulfur. The culture flasks were autoclaved at 121 ºC for 15 min for sterilization and then, after cooling, inoculated with 2 x 10^6 conidia per ml. The cultures were incubated in an orbital shaker at 200 rpm and 28 ºC for 117 h. Samples of 5 ml were withdrawn at regular intervals, centrifuged (5,000 ×g, 20 min, 4 °C) and the supernatant was used for pH, protein content and enzyme activities determinations. When purification of the enzyme was achieved, all the contents of each flask withdrawn and centrifuged at 5,000 ×g and 4 ºC for 20 min in order to precipitate the fungal biomass. The supernatant was then used for enzyme purification.

2.2. Enzyme-activity determination

After each purification step the keratinolytic and proteolytic activities were both measured as described elsewhere [10], with the latter activity determination being used for enzyme characterization.

2.3. Protein determination

Proteins were quantified after Bradford [11] with bovine-serum albumin (Sigma) as a standard.

2.4. Scaning electron microscopy (SEM)

To characterize the degradation of hair waste by *P. lilacinum*, digested and undigested hair waste samples were freeze-dried and then coated with gold palladium. SEM was accomplished using a Jeol JSM-840 microscope at an accelerating voltage of 25kV.

2.5. Purification of keratinase

The culture supernatant was filtered through cheesecloth at 4 ºC and concentrated under reduced pressure at 30 ºC. Solid ammonium sulfate was then added to the concentrated extract to up to 85% saturation. The precipitated proteins were dissolved in 20 ml of Tris-HCl buffer (20 mM, pH 7.0; TB) and then applied to a Sephadex G-25 gel-filtration column (XK 16/60, General Electric Little Chalfont, UK) equilibrated with TB and eluted isocratically with the same buffer at a flow rate of 1.0 ml min^{-1}. Fractions of 5 ml were collected and those exhibiting keratinase activity pooled, concentrated by lyophilization, resuspended in the same buffer up to 4 ml, and loaded onto a DEAE-Sephadex (XK 26/10, General Electric) anion-exchange column. The column was washed with TB, and the bound proteins were then eluted with a linear gradient of NaCl (0.0–1.0

M) in TB over 10 column volumes at a flow rate of 2 ml min^{-1}. Fractions (5 ml) were collected and analyzed for keratinase activity. Those fractions with keratinase activity were pooled and loaded onto a Sp-Sepharose-FF column (Hiload 16/10, General Electric) preequilibrated with TB. The proteins were eluted on a linear gradient of NaCl (0.0–1.0 M) in TB over 10 column volumes at a flow rate of 2 ml min^{-1} in the same chromatographic system. The fractions (10 ml) containing keratinase activity were pooled, concentrated by lyophilization, resuspended in TB to up to 2 ml, loaded onto a Superdex-75 column (XK 16/60, General Electric) equilibrated with Tris-HCl buffer (20 mM Tris, 0.15 M NaCl; pH 7.0), and eluted isocratically with the same buffer at a flow rate of 0.75 ml min^{-1}. The purified enzyme was stored at –20 ºC and used for further biochemical characterization. All chromatographic steps were carried out on an Amersham FPLC-U900 system (General Electric).

2.6. Molecular-weight determination

After a prior desalting step, the molecular weight of the protein isolate was estimated by sodium-dodecylsulfate–polyacrylamide-gel electrophoresis (SDS-PAGE) in a 12% (w/v) gel calibrated with low-molecular-weight markers (LMW Kit, General Electric) according to Laemmli [12]. The protein bands were stained with Coomassie brilliant blue R-250.

2.7. Biochemical characterization of purified keratinase

2.7.1. Effect of pH on enzyme stability and activity

The pH stability of the purified enzyme was studied over a range of 3.0–13.0, while the pH optimum of the protease activity was determined within the range 6.0–13.0 with azocasein as substrate (Azocasein is insoluble at pHs <6.0.). For measurement of the

pH stability, the enzyme was incubated at a given pH for 1 h at 37 °C and the residual protease activity determined under standard assay conditions. In both instances, a mixture of buffers (glycine, 2-(*N*-morpholino)ethanesulfonic acid, and Tris-HCl; 20 mM each) adjusted to the required pH was used.

2.7.2. Effect of temperature on enzyme stability

The thermostability of the purified enzyme was examined through incubations at different temperatures (40–65 ºC) for 180 min. Aliquots were withdrawn at regular time intervals and the protease activity measured under standard assay conditions. The activity remaining at each time point was expressed as a percent of the value recorded with the unheated crude protease.

2.7.3. Effect of inhibitors and metal ions on protease stability

The effect of the following inhibitors of protease activity was investigated: phenylmethylsulphonyl fluoride (PMSF, 1 mM), iodoacetate (10 mM), ethylendiaminetetraacetate (5 mM), 1,10-phenanthroline (1 mM) and Pepstatin A (chlorambucil, 100 µg ml^{-1}). The keratinase was preincubated in the presence of each inhibitor for 1 h at room temperature (20 ºC) and the protease activity remaining during subsequent assay expressed as a percent of the control value with enzyme not exposed to inhibitor.

The effect of different metal ions (at a concentration of 1 mM) on protease activity was studied by addition of the cations Ca^{2+}, Mg^{2+}, Zn^{2+}, K^{+}, and Hg^{2+} to the enzyme solution followed by incubation for 1 h at room temperature. The protease activity remaining upon subsequent assay was expressed as a percent of the control value with enzyme not exposed to cations.

185

186 *2.7.4. Effect of surfactants and oxidizing agents on protease stability*

187 The keratinase stability towards selected surfactants (SDS, Triton X-100, Tween
188 20, and Tween 85), and oxidizing agents (H_2O_2, sodium perborate) was tested by
189 incubating the enzyme with each additive for 1 h at room temperature. The remaining
190 protease activity was determined under standard conditions and expressed as percentage of
191 the control value with enzyme not exposed to an oxidizing agent.

192

193 *2.7.5. Determination of kinetic parameters*

194 The kinetic constants K_m and V_{max} of the purified enzyme were calculated by
195 fitting the activity data at increasing substrate concentrations (azocasein; [13]) to a linear
196 regression after Hanes-Hultin transformation [14].

197

198 *2.8. Protein identification*

199 Peptide mass fingerprinting of selected protein spots was carried out by in-gel
200 trypsin (Sequencing-grade, Promega) treatment during an overnight electrophoresis at 37
201 ºC. The trypsinized peptides were extracted from the gels with 60% (v/v) acetonitrile in
202 0.2% (w/v) trifluoroacetic acid, concentrated by vacuum-drying, and desalted on C18
203 reverse-phase microcolumns (OMIX pipette tips, Varian). The peptides from the
204 microcolumn were eluted directly onto the mass-spectrometer sample plates in 3 µl of
205 matrix solution (α-cyano-4-hydroxycinnamic acid in 60% (v/v) aqueous acetonitrile in
206 0.2% (w/v) trifluoroacetic acid. Mass spectra of the digestion mixtures were generated in a
207 4800 MALDI-TOF/TOF instrument (Applied Biosystems) in reflector mode and were
208 externally calibrated by means of a mixture of peptide standards (Applied Biosystems).
209 Collision-induced MS/MS dissociations of selected peptides were performed. Proteins

were identified by NCBInr database by searching with peptide m/z values through the MASCOT program and by means of the search parameters monoisotopic mass tolerance, 0.08 Da; fragment-mass tolerance, 0.2 Da; and methionine oxidation as possible modifications with one missed tryptic cleavage being allowed.

2.9. N-terminal sequencing

The N-terminal–amino-acid sequence of the purified keratinase was determined with a ABI 494 protein sequencer at Tufts University, Boston, MA USA.

3. Results and Discussion

3.1. Keratinase production during the growth of P. lilacinum

Figure 1A shows the time course of the production of extracellular keratinase activity in hair basal medium containing 1% (w/v) hair waste. A maximum production of protease and keratinase activities of 2.46 U/ml and 25 U/ml were achieved by between 111 and 117 h of incubation, respectively. Both activities increased in parallel, thus maintaining a constant keratinolytic:proteolytic (K:P) activity ratio of 11.32 ± 1.06. Because of this constant production ratio, the proteolytic activity was chosen as an indirect estimation of keratinolytic activity for the purpose of the biochemical characterization of the purified enzyme. During the whole fermentation the pH of the medium increased because of the ammonia produced by the deamination of peptides and aminoacids derived from keratin solubilization. On the other hand, soluble protein concentration showed a similar behavior to that of the proteolytic activity except for the fact that the maximum peak was reached a few days later (Fig. 1A). Since keratin is used as medium component, keratinase production is accompanied by subsequent degradation of keratin substrate

leading to an increase in soluble protein concentration. The increment of soluble protein has been reported as a measure for keratin degradation [15]. The decrease of soluble protein may be due to an increment of the proteolysis degree, thus releasing peptides which were not detected by Bradford's reagent.

A comparable kinetics of keratinase production has been reported for microorganisms such as *Bacillus subtilis* MTCC (9102) with horn-meal as the substrate [16] and *Microbacterium* sp. strain kr10 grown in feather-meal medium [17]. In cultures of *Aspergillus fumigatus* [18], *A. oryzae* [19], and *Trichophyton vanbreuseghemii* [20], the keratinase production reached a maximum after 21 days of incubation.

The extent of degradation of hair waste by *P. lilacinum* during culture was established by SEM. Fig. 1(B) and Fig. 1(C) shows SEM images of uninoculated hair fibres after hair-saving unhairing process using sodium sulfite/lime as unhairing agent and hairs fibres after 5 days of submerged culture, respectively. It could be observed a considerable degradation and disorganization of the fibre due to the fungal attack during the culture and also fungal aggregates with an extracellular matrix, adhered to degraded surfaces.

3.2. Enzyme purification

The extracellular keratinase produced by *P. lilacinum* cultivated on hair-waste medium was purified from the concentrated culture extract (72 U/mg protein) by ammonium-sulfate precipitation, gel filtration, and ion-exchange chromatography to obtain a 19.8-fold enrichment and a specific activity of about 1,430 U/mg protein at a yield of 1.3% (Table 1). Although purification resulted in a low total yield, the procedure was chosen in order to obtain a homogeneous fraction of keratinase for the purpose of biochemical characterization. In accordance with that objective, analysis of the enzyme on SDS-polyacrylamide gels revealed a single band of apparent molecular weight 37 kDa

(Fig. 2). Similar molecular weight values were found for proteinases secreted by *P. lilacinum* strains (33.5 kDa, [21] and 33 kDa, [22]), *Bacillus licheniformis* (33 kDa, [23]), and *Trichophyton vanbreuseghemii* (37 kDa, [20]).

3.3. Biochemical characterization of purified keratinase

Enzymes with keratinolytic activity have constituted a focus of interest in various studies because of their wide spectrum of potential industrial applications—*e. g.*, in the catalysis required in the production of fertilizers or animal feed, as additives in detergent formulation, and as dehairing agents in tanneries [4,24 - 26]. Keratinases can also be used in skin-care cosmetics and for feather-waste degradation in the poultry industry [27]. As a rule, naturally available enzymes are not optimally suitable for such industrial applications, and this incompatibility often stems from the lack of stability of those proteins under the conditions of the particular process needed. Although sometimes an adaptation of industrial processes to mild and environmentally benign conditions can be suitable, the use of extreme conditions is often unavoidable. For example, proteolytic enzymes incorporated into detergent formulations should exhibit certain special characteristics: activity and stablity at alkaline pHs and/or at relatively high temperatures (40–50 ºC or more) and compatiblity with other detergent components such as surfactants, perfumes, bleaches, and oxidizing and sequestering agents [28]. In general, the majority of commercially available enzymes are not stable in presence of bleaching or oxidizing agents. Regardless of the conditions of the process in question, the stability of the biocatalyst is often a relevant economic consideration.

The pH stability of the *P. lilacinum* enzyme was tested at values between 3.0 and 13.0. The enzyme was fully stable over a wide pH range (from 4.0 to 9.0). Outside this range, the keratinase catalysis was only moderately stable, retaining 50% of the native

activity at pH 3 and 40% at pH 12 (Fig. 3A). Keratinases have occasionally exhibited this degree of stability—*e. g.*, the keratinases from *Kocuria rosea* within the range of pH 10.0–11.0 [29]; from *Norcardiopsis* sp. TOA-1, at pH 12.0 [5]; and from *Bacillus* sp. AH-101, between pH 11.0 and 12.0 [6]. The notably wide pH range throughout which the *P. lilacinum* keratinase is both active and stable may enhance its biotechnological applications, especially in the leather and detergent industries.

Fig. 3B shows the effect of pH on enzyme activity. The *P. lilacinum* keratinase was found to be active at pHs ranging from 6.0 to 13.0 with a constant maximum activity between pHs 7.0 and 12.0.

The thermal stability of the keratinase was evaluated by incubating the purified enzyme at different temperatures (between 40–65 °C) for 180 min (Fig. 4). The enzyme was stable below 50 °C and also retained more than 40% of the initial activity after 3 h of incubation at that temperature. The half-life of the enzyme was estimated at 137 min at 50 °C and 68 min at 55 °C. In comparison, the keratinase from *Bacillus* sp. P7 had a half-life of 53 min at 50 °C and less than 10 min at 55 °C [30]; while the keratinase from *K. rosea* remained fully active after 1 h of incubation at 10–60 °C, with 40% of the initial activity remaining after 1 h at 90 °C [29]. We therefore conclude that the keratinase from *P. lilacinum* exhibits moderate thermotolerance and thermostability, which features might be conducive to the efficient use of the enzyme in processes involving protein hydrolysis [31]. Moreover, a recent trend in the detergent industry has resulted in the requirement of alkaline proteases that remain active at washing temperatures (between 20 and 30 ºC), with that prerequisite aimed at maintaining fabric quality along with low energy demands [32]. As indicated by additional assays, the *P. lilacinum* keratinase proved to be completely active over this temperature range, thus pointing to the enzyme's usefulness within that specific industrial context (data not shown).

Most of the keratinases that have been reported belong to the serine or metalloprotease classes [33]. In the present study, the enzymatic activity was strongly inhibited by PMSF, a serine-protease inhibitor; whereas other inhibitors assayed affected the enzyme activity only slightly (Table 2). Accordingly, from the inhibition characteristics observed for the *P. lilacinum* keratinase—a 98.2% inhibition with PMSF and a 92.1% inhibition in the presence of Hg^{2+}—this keratinase is highly likely to be a thiol-dependent serine protease [22].

After purification of the enzyme from the fungus Ca^{2+} was found to slightly decrease keratinase catalysis (Table 2). Since the enzyme´s stability therefore does not depend on the presence of Ca^{2+}, the likelihood of the keratinase's usefulness in the detergent industry is increased, mainly because in that process—it commonly employing alkaline proteases—chelating agents are included to avoid the problem of hardness in the water. In the presence of such chelating agents, Ca^{2+} could be easily removed, thus greatly affecting the activity of a Ca^{2+}-dependent hydrolase. For this reason, enzymes without any metal-ion requirement for stability offer considerable potential for use in the manufacture of detergents.

The organic solvents dimethylsulfoxide, isopropanol, methanol, and ethanol, in the concentrations tested, had no effect on keratinase activity (Table 2), as had been reported to be true for the keratinases from *K. rosea* [29], from *Bacillus* sp. P7, and from *Nocardiopsis* sp. TOA-1 [5]. The purified keratinase also proved highly stable in the presence of nonionic surfactants, retaining 100% of its initial activity in the presence of 1% (v/v) Triton X-100, 1% (v/v) Tween 20, and 1% (v/v) Tween 85 after 1 h of incubation at room temperature. SDS at 0.5% (w/v), a strong anionic surfactant, produced only a minor inhibition of enzyme activity, with the keratinase retaining approximately 70% of the initial levels after 1 h of incubation at room temperature. This percent retention

was greater than the figure of 45.8% that had been reported for a keratinase from *Chryseobacterium* L99 sp. nov. after a 1-h incubation with only 0.2% (w/v) SDS [34].

In the inactivation process of proteins by oxidizing agents, methionine residues have been identified as primary targets. All subtilisins (serine proteases) contain a Met residue next to the Ser of the catalytic site so that they are strategically positioned for the enzyme to undergo oxidative inactivation in the presence of oxidizing agents such as hydrogen peroxide. Thus, many of the available alkaline proteases have been found to exhibit a low activity and stability towards the oxidants that are common ingredients in modern bleach-based detergents. To overcome these shortcomings, several attempts have been made to enhance enzyme stability through protein engineering [35]. In addition, the search for enzymes with a high stability against surfactants and oxidants for industrial applications has gained an equally high priority. Accordingly, when the *P. lilacinum* keratinase was incubated in the presence of 1% (v/v) H_2O_2 or 1% (w/v) sodium perborate for 1 h at room temperature, no inactivation occurred. This substantial stability toward oxidizing agents was similar to the properties of the proteases from *B. licheniformis* NH1, with those hydrolases retaining 85% and 80% of the initial activity after 1 h of incubation at 40 ºC with 0.5% (v/v) H_2O_2 and 0.2% (w/v) sodium perborate, respectively [36]. Moreover, the *P. lilacinum* keratinase proved to be more stable than the *B. licheniformis* RP1 proteases, where those retained only 48% of their activities after a 1-h incubation at 40 ºC in the presence of this same concentration of sodium perborate [28].

3.4. Kinetic parameters

The kinetic parameters K_m and V_{max}, measured with azocasein as substrate, were 0.72 mg/ml and 3.6 U/min, respectively (Fig. 5). This K_m value resulted to be similar to that reported by Silveira et al. [37] for *Chryseobacterium* sp. strain kr6 (Km, 0.75 mg/ml) and lower from those reported by Ghosh et al. [38] from *Bacillus cereus* DCUW (Km, 0.161 mg/ml) and by Daroit et al. from *Bacillus* sp. P45 (Km, 2.85 mg/ml)[39].

3.5. Protein identification and N-terminal–sequence analysis

After trypsin hydrolysis and MALDI-TOF/TOF analysis, a search in the NCBI nr database identified peptide similarities (52% homology, including both N- and C-terminal peptides) to a previously reported *P. lilacinus* serine protease (Swiss-Prot Accesion No. Q01471; NCBI Accesion 3F7O_A), indicating the similarity of the keratinolytic protease purified in this work to that earlier described *P. lilacinus* enzyme [21]. Table 3 shows the amino acid sequence coverage of *P. lilacinum* serine protease obtained from micro sequencing and MALDI/TOF MS data. Additional results concerning MALDI-TOF/TOF results can be obtained from http://www.matrixscience.com/cgi/protein_view.pl?file=../data/20110406/FttpInSTt.dat&hit=1.

The N-terminal–amino-acid sequence of the enzyme was A-Y-T-Q-Q-P-G-A-I, thus showing complete identity to the N-terminal–amino-acid sequence of the serine protease from still another *P. lilacinus* strain (CBS 243.75; [21]), but did not match the N-terminal sequence of a thiol-dependent serine protease (G-A-T-T-Q-G-A-T-G/I-Xxx-G) isolated from a fourth *P. lilacinus* strain (VKM F-3891;[22]).

4. Conclusions

A keratinolytic serine protease from *Purpureocillium lilacinum* LPS # 876 would appear to be a protease with significant industrial possibilities as a result of its catalytic stability over a broad pH and temperature range in addition to its tolerance to bleaching and chelating agents. The enzymatic properties of the enzyme suggest its potential use in detergent formulations and the leather industry (*i. e*., for the processes of dehairing and bating). These characteristics of the fungal keratinase and its prospective application in other commercial contexts—such as in the cosmetic and pharmaceutical industries—are indeed promising.

The production of the keratinase from *P. lilacinum* is a simple process and amenable to a scaling-up since the enzyme is excreted into the extracellular medium when the microorganism is cultured with hair waste as a sole nitrogen, energy, and carbon source. Finally, the production of the enzyme with such attractive biochemical characteristics from a cheap substrate constitutes an economically attractive process for industrial applications because of its low production cost.

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References

[1] Böckle B, Galunsky B, Müller R. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Appl Environ Microbiol 1995; 61:3705-3710.

[2] Odetallah NH, Wang JJ, Garlich JD, Shih JCH. Keratinase in starter diets improves growth of broiler chicks. Poultry Science 2003; 82:664-670.

[3] Schrooyen PMM, Dijkstra PJ, Oberthür RC, Bantjes A, Feijen J. Partially carboxymethylated feather keratins. 2. Thermal and mechanical properties of films. Journal of Agricultural and Food Chemistry 2001; 49:221-230.

[4] Gupta R, Ramnani P. Microbial keratinases and their prospective applications: an overview . Appl Microbiol Biotechnol 2006; 70:21-33.

[5] Mitsuiki S, Ichikawa M, Oka T, Sakai M, Moriyama Y, Sameshima Y, Goto M, Furukawa K. Molecular characterization of a keratinolytic enzyme from an alkaliphilic *Nocardiopsis sp*. TOA-1. Enzyme Microb Technol 2004; 34:482-489.

[6] Takami H, Akiba T, Horikoshi K. Production of extremely thermostable alkaline protease from *Bacillus* sp. no. AH-101. Appl Microbiol Biotechnol 1989; 30:120-124.

[7] Cavello I, Cavalitto S, Hours R. Biodegradation of a Keratin Waste and the Concomitant Production of Detergent Stable Serine Proteases from *Paecilomyces lilacinus*. Appl Biochem Biotechnol 2012; 167:945-958.

[8] Gortari C, Cazau MC, Hours R. Hongos nematófagos de huevos de *Toxocara canis* en un paseo público de La Plata, Argentina. Revista Iberoamericana de Micología 2008; 24:24-28.

[9] Galarza B, Goya L, Cantera C, Garro ML, Reinoso HM, Lopez LMI. Fungal biotransformation of bovine hair part I: Isolation of fungus with keratinolytic activity. J Soc Leather Technol Chem 2004; 88:93-98.

[10] Cavello IA, Hours R, Cavalitto SF. Bioprocessing of "Hair Waste" by *Paecilomyces lilacinus* as a source of a bleach-stable, alkaline, and thermostable keratinase with potential application as a laundry detergent additive: characterization and wash performance analysis. Biotechnology Research International 2012; 2012:1-12.

[11] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72:248-254.

[12] Laemmli UK. Cleavage of structural proteins during assembly of head of *bacteriophage* T4. Nature 1970; 227:680-685.

[13] Ageitos M, Vallejo JA, Sestelo ABF, Poza M, Villa TG. Purification and characterization of a milk-clotting protease from *Bacillus licheniformis* strain USC13. J Appl Microbiol 2007; 103:2205-2213.

[14] Hultin E. Statistical calculations of the accuracy of the Michaelis constant from viscosimetric determinations of polymetaphosphatase and dextranase activity. Acta Chemica Scandinava 1967; 21:1575-1580.

[15] Bálint B, Bagi Z, Tóth A, Rákhely G, Perei K, Kovács K. Utilization of keratin-containing biowaste to produce biohydrogen. Appl Microbiol Biotechnol 2005; 69:404-410.

[16] Balaji S, Kumar MS, Karthikeyan R, Kumar R, Kirubanandan S, Sridhar R, Seghal PK. Purification and characterization of an extracellular keratinase from hornmeal-degrading *Bacillus subtilis* MTCC (9102). World J Microb Biotech 2008; 24:2741-2745.

[17] Thys RCS, Brandelli A. Purification and properties of a keratinolytic metalloprotease from *Microbacterium* sp. J Appl Microbiol 2006; 101:1259-1268.

[18] Do JH, Anderson MJ, Denning DW, Bornberg-Bauer E. Inference of Aspergillus fumigatus pathway by computational genome analysis: tricarboxylic acid cycle (TCA) and glyoxilate shunt. J Microb Biotech 2004; 14:74-80.

[19] Jousson O, Lechenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni M, Monod M. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentation in the dermatophytes *Trichophyton* and *Microsporum*. Microbiology 2004; 150:301-310.

[20] Moallaei H, Zaini F, Larcher G, Beucher B, Bouchara JP. Partial purification and characterization of a 37 KDa extracellular proteinase from *Trichophyton vanbreuseghemii*. Mycopathologia 2006; 161:369-375.

[21] Bonants PJ, Fitters PF, Thijs H, den Belder E, Waalwijk C, Henfling JW. A basic serine protease from *Paecilomyces lilacinus* with biological activity against *Meloidogyne hapla* eggs. Microbiology 1995; 141:775-784.

[22] Kotlova EK, Ivanova MN, Yusupova MP, Voyushina TL, Ivanushkina NE, Chestukhina GG. Thiol-Dependent Serine Proteinase from *Paecilomyces lilacinus*: Purification and Catalytic Properties. Biochemistry (Moscow) 2007; 72:117-123.

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

[24] Anbu P, Gopinath SC, Hilda A, Lakshmipriya T, Annadurai G. Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis*. Bioresour Technol 2007; 98:1298-1303.

[25] Godfrey G. Protease in waste treatment. In: Godfrey G,West S editors. Industrial Enzymology. London: Macmillan Press Ltd; 1996. p. 315-316.

[26] Macedo AJ, Beys da Silva WO, Gava R, Driemeier D, Pêgas Henriques JA, Termignoni C. Novel keratinase from Bacillus subtilis S14 exhibiting remarkable dehairing capabilities. Appl Environ Microbiol 2005; 71:594-596.

[27] Onifade AA, Al-Sane NA, Al-Musallam AA, Al-Zarban S. A review: 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 1998; 66:1-11.

[28] Sellami-Kamoun A, Haddar A, El-Hadj Ali N, Ghorbel-Frikha B, Kanoun S, Moncef N. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP 1 in commercial solid laundry detergent formulations. Microbiologial Research 2008; 163:299-306.

[29] Bernal C, Cairó J, Coello N. Purification and characterization of a novel exocellular keratinase from *Kocuria rosea*. Enzyme Microb Technol 2006; 38:49-54.

[30] Correa AP, Daroit DJ, Brandelli A. Characterization of a keratinase produced by *Bacillus sp*. P7 isolated from an Amazonian environment. Int Biodeter Biodegr 2010; 64:1-6.

[31] Rao MB, Tanksale A.M., Ghatge MS, Deshpande VV. Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev 1998; 62:597-635.

[32] Venugopal M, Saramma AV. Characterization of alkaline protease from *Vibrio fluvialis* strain VM 10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. Process Biochem 2006; 41:1239-1243.

[33] Brandelli A. Bacterial Keratinases: useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Bioprocess Technol 2008; 1:105-116.

[34] Lv LX, Sim MH, Li YD, Min J, Feng WH, Guan WJ, Li YQ. Production, characterization and application of a keratinase from *Chryseobacterium* L99 sp. nov. Process Biochem 2010; 45:1236-1244.

[35] Estell DA, Graycar TP, Wells JA. Engineering an enzyme by site-directed mutagenesis to be resistant to chemical oxidation. J Biol Chem 1985; 260:6518-6521.

[36] Hmidet N, El-Hadj Ali N, Haddar A, Kanoun S, Sellami-Kamoun A, Nasri M. Alkaline proteases and thermostable α-amylase co-produced by *Bacillus licheniformis* NH1: Characterization and potential application as detergent additive. Biochem Eng J 2009; 47:71-79.

[37] Silveira ST, Jaeger MK, Brandelli A. Kinetic data and substrate specificity of a keratinase from *Chryseobacterium* sp. strain kr6. Chemical Technology and Biotechnology 2009; 84:361-366.

[38] Ghosh A, Chakrabarti K, Chattopadhyay D. Degradation of raw feather by a novel high molecular weight extracellular protease from newly isolated *Bacillus cereus* DCUW. J Ind Microbiol Biotechnol 2008; 35:825-834.

[39] Daroit DJ, Corrêa AP, Segalin J, Brandelli A. Characterization of a keratinolytic protease produced by the feather-degrading Amazonian bacterium *Bacillus* sp. P45. Biocatalysis and Biotransformation 2010; 28:370-379.

Table 1

Steps involved in the purification of *P. lilacinus* keratinase

Purification step	Volume (ml)	Total protein (mg)	Total activity (U_k)	Specific activity (U_k/mg)	Yield (%)
Concentrated extract	290	503.8	36274.9	72.0	100
Precipitation	20	302.5	30982.8	102.4	85
G-25	120	37.2	3950.7	106	11
DEAE Sepharose	26.5	4.14	2759.8	625.8	7.6
Sp Sepharose FF	26.5	1.40	954.3	640	2.6
Superdex 75	13.25	0.32	458.5	1432.7	1.3

Table 2

Effect of protease inhibitors, metal ions, detergents and solvents on protease activity

Chemical	Concentration	Residual activity (%)
None		100
Inhibitors		
PMSF	1 mM	1.8 ± 0.5
Iodoacetate	10 mM	79.4 ± 0.6
EDTA	5 mM	93.0 ± 1.8
1,10-Phenantroline	1 mM	88.3 ± 1.0
Pepstatin A	100 µg/ml	88.7 ± 2.3
Metal ions		
Mg^{2+}	1 mM	72.7 ± 0.6
Zn^{2+}	1 mM	74.9 ± 1.5
Ca^{2+}	1 mM	81.7 ± 1.8
Hg^{2+}	1 mM	7.9 ± 0.4
K^{+}	1 mM	83.5 ± 1.0
Detergents		
Triton X-100	1 % (v/v)	100 ± 0.4
Tween 20	1 % (v/v)	98.3 ± 1.9
Tween 85	1 % (v/v)	101.5 ± 2.6
SDS	0.5 % (v/v)	69.5 ± 2.5
Bleaching agents		
H_2O_2	1 % (w/v)	99.4 ± 5.5
Sodium perborate	1 % (w/v)	99.7 ± 2.4
Solvents		
DMSO	1 % (v/v)	99.0 ± 0.6
Ethanol	1 % (v/v)	100 ± 5.8
Methanol	1 % (v/v)	100 ± 2.9
Isopropanol	1 % (v/v)	88.7 ± 5.5

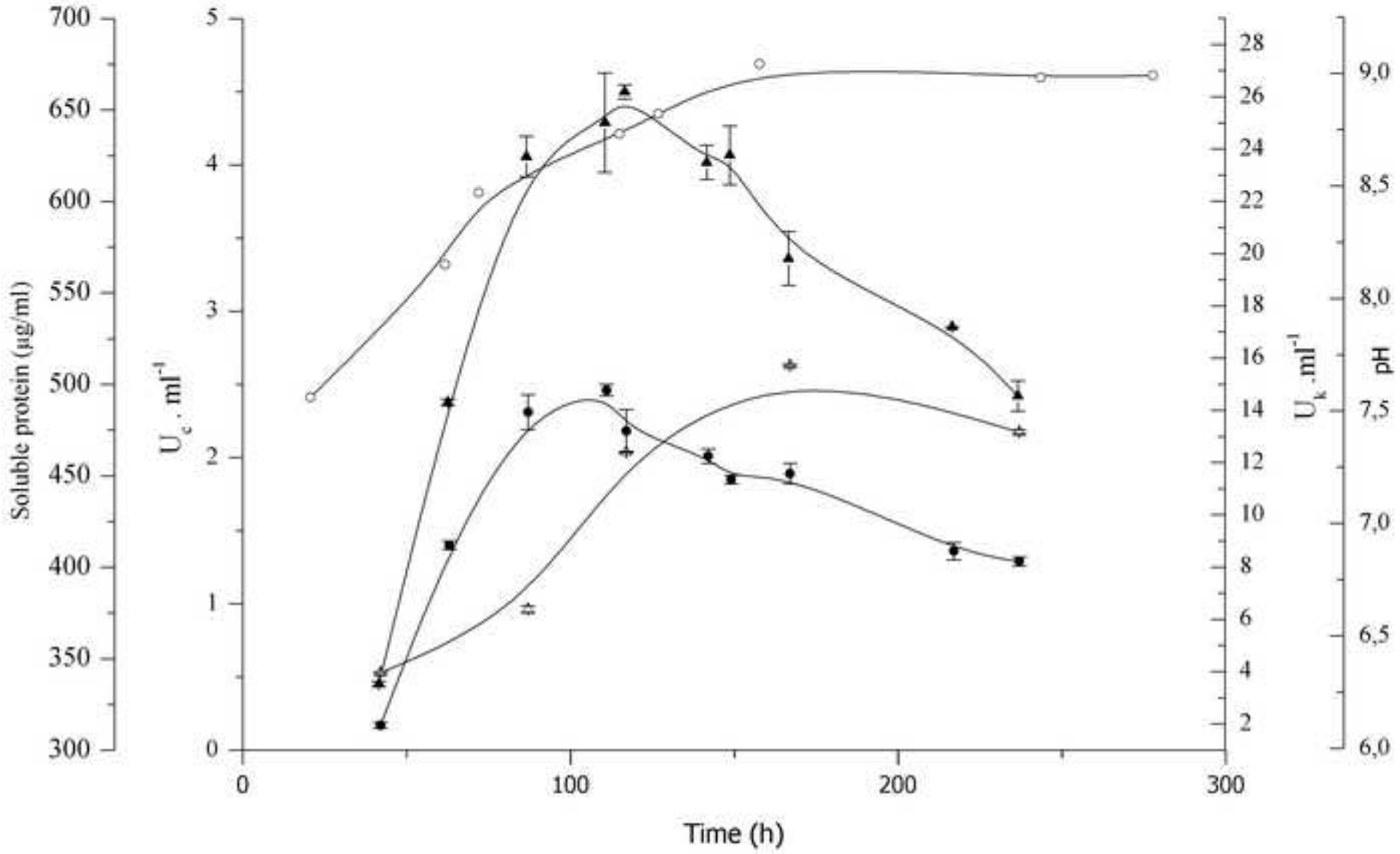
* Data are shown as residual activity (%) ± SD

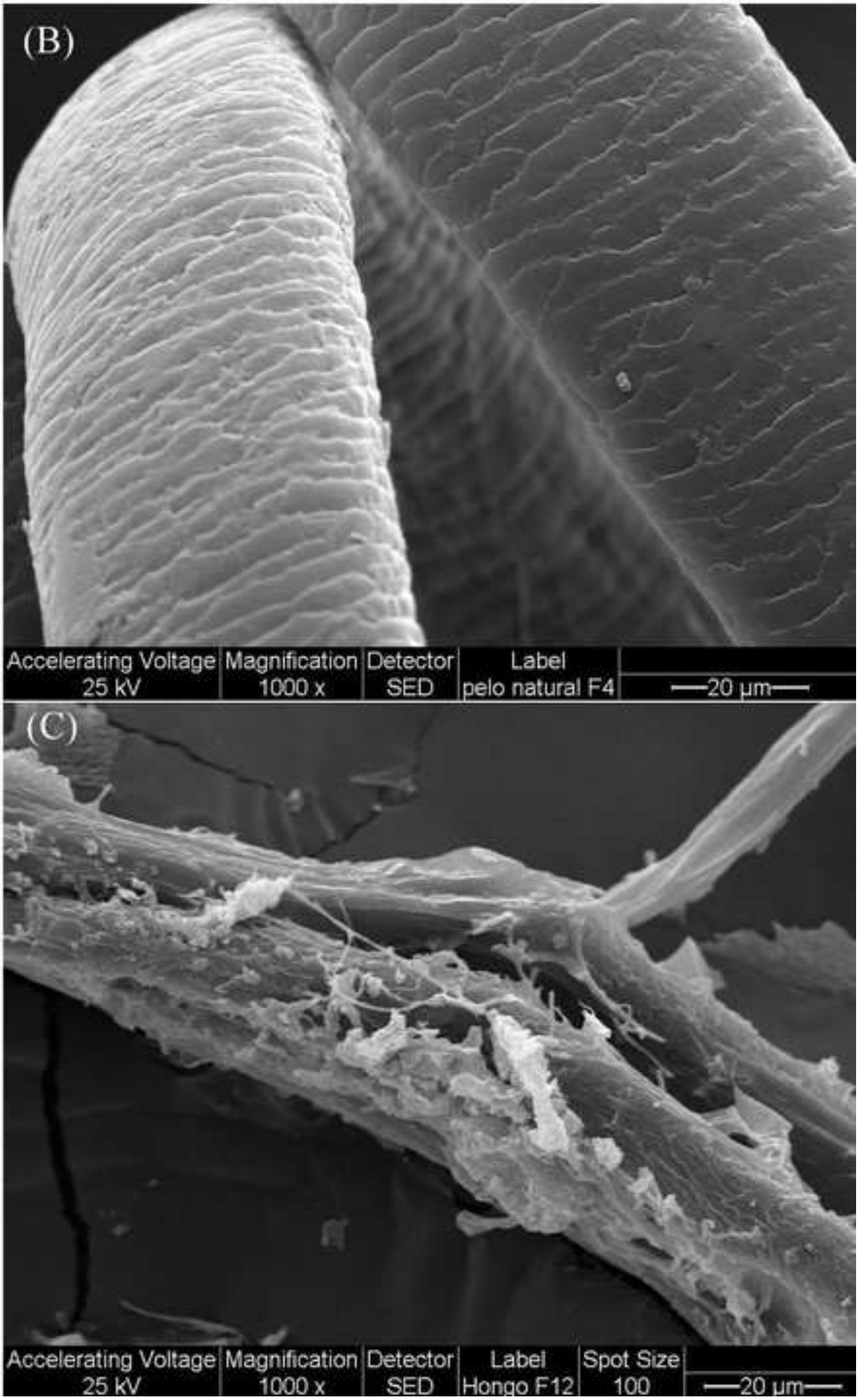
Table 3

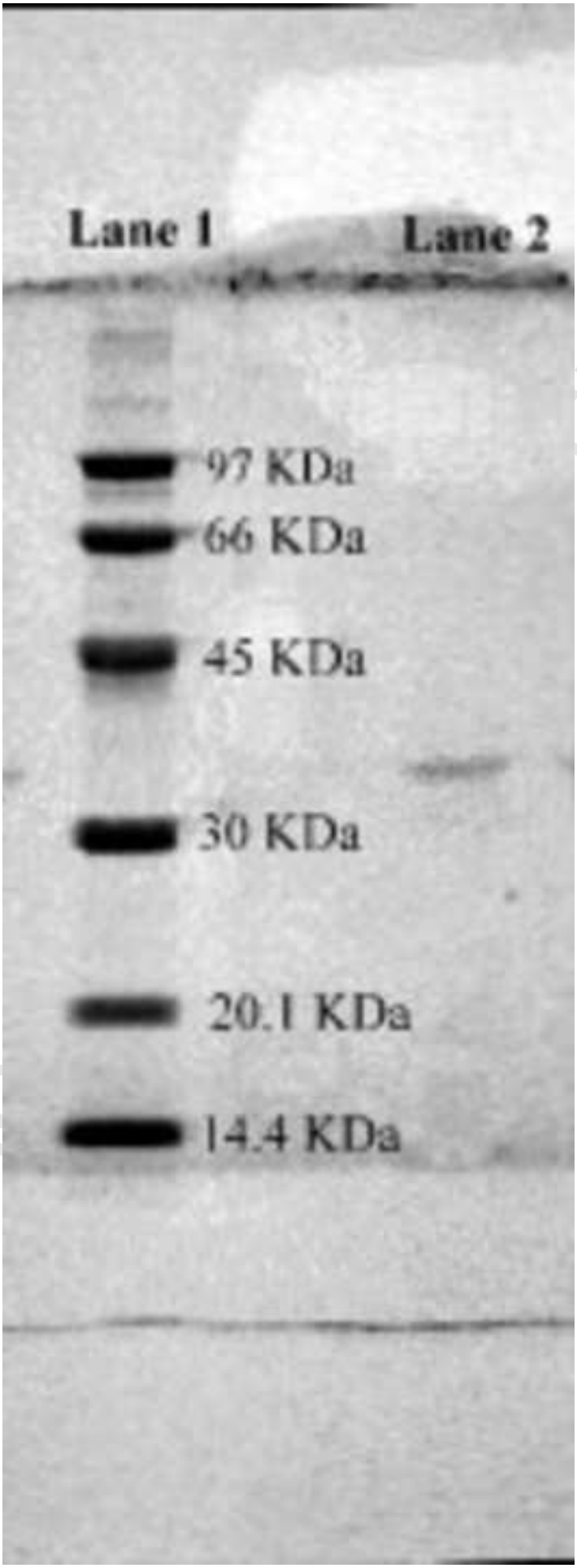
Amino acid sequence coverage of *P. lilacinum* serine protease obtained from MALDI/TOF MS data.

Peptide position	Matched masses	Matched Cys_PAM	Peptide sequence
1-14	1501.7534		AYTQQPGAPWGLGR
21-54	3543.5622	Cys36:3614.5857	GSTTYEYDTSGGSGTCAYVIDTGVEASHPEFEGR
98-122	2534.1885		VLDNSGSGSYSGIISGMDFAVQDSK
98-124	2777.2483		VLDNSGSGSYSGIISGMDFAVQDSKSR
141-153	1303.6455		AQSVNDGAAAMIR
154-192	3740.7996	Cys181:3811.8401	AGVFLAVAAGNDNANAANYSPASEPTVCTVGATTSSDAR
263-284	2192.0811		NVLTGIPSGTVNYLAFNGNPSG

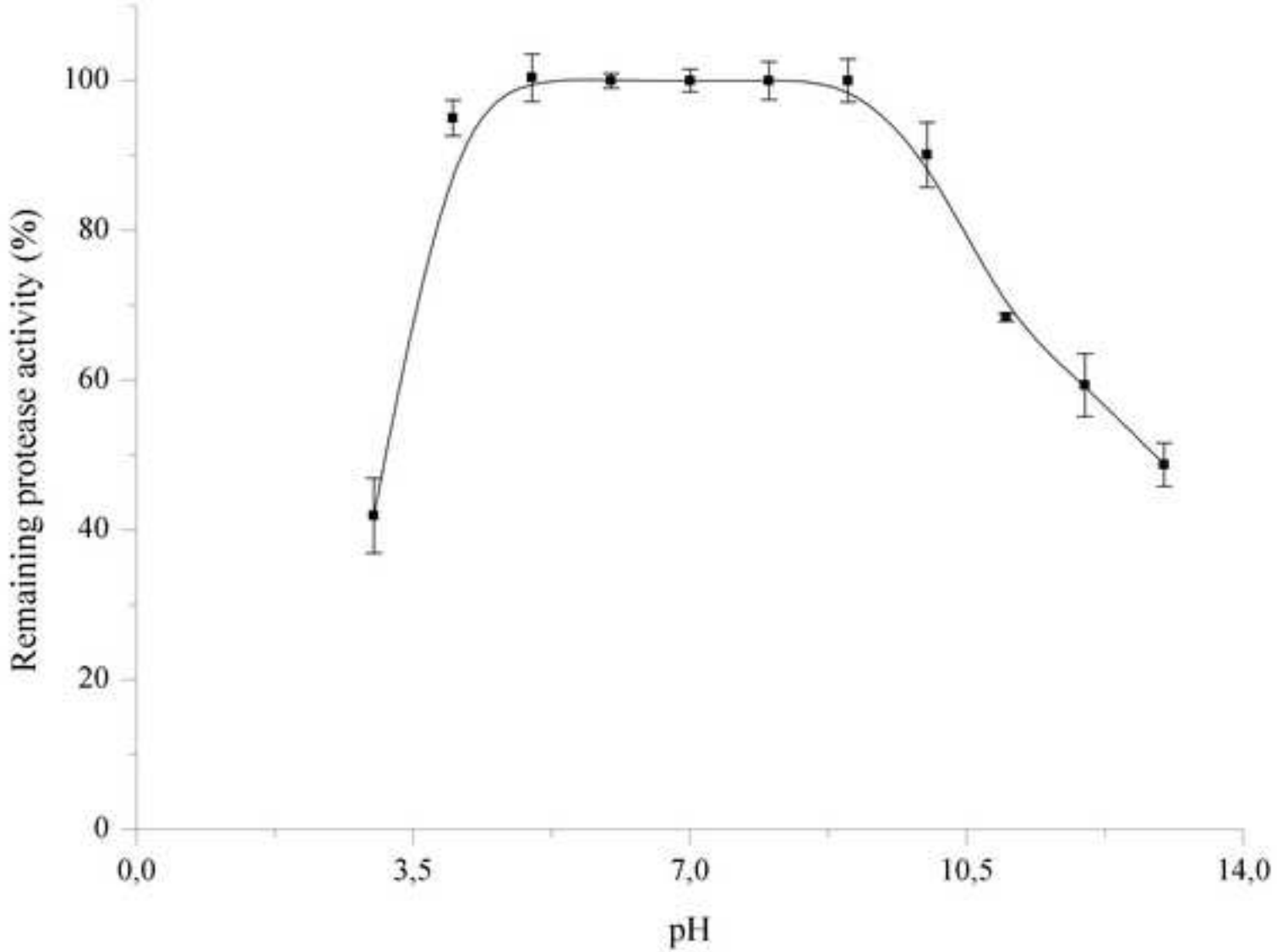
Figure(s)


Soluble protein (µg/ml)
700
650
600
550
500
450
400
350
300
U_c. ml^{-1}
5
4
3
2
1
0
U_k. ml^{-1}
28
26
24
22
20
18
16
14
12
10
8
6
4
2
pH
9,0
8,5
8,0
7,5
7,0
6,5
6,0
0
100
200
300
Time (h)

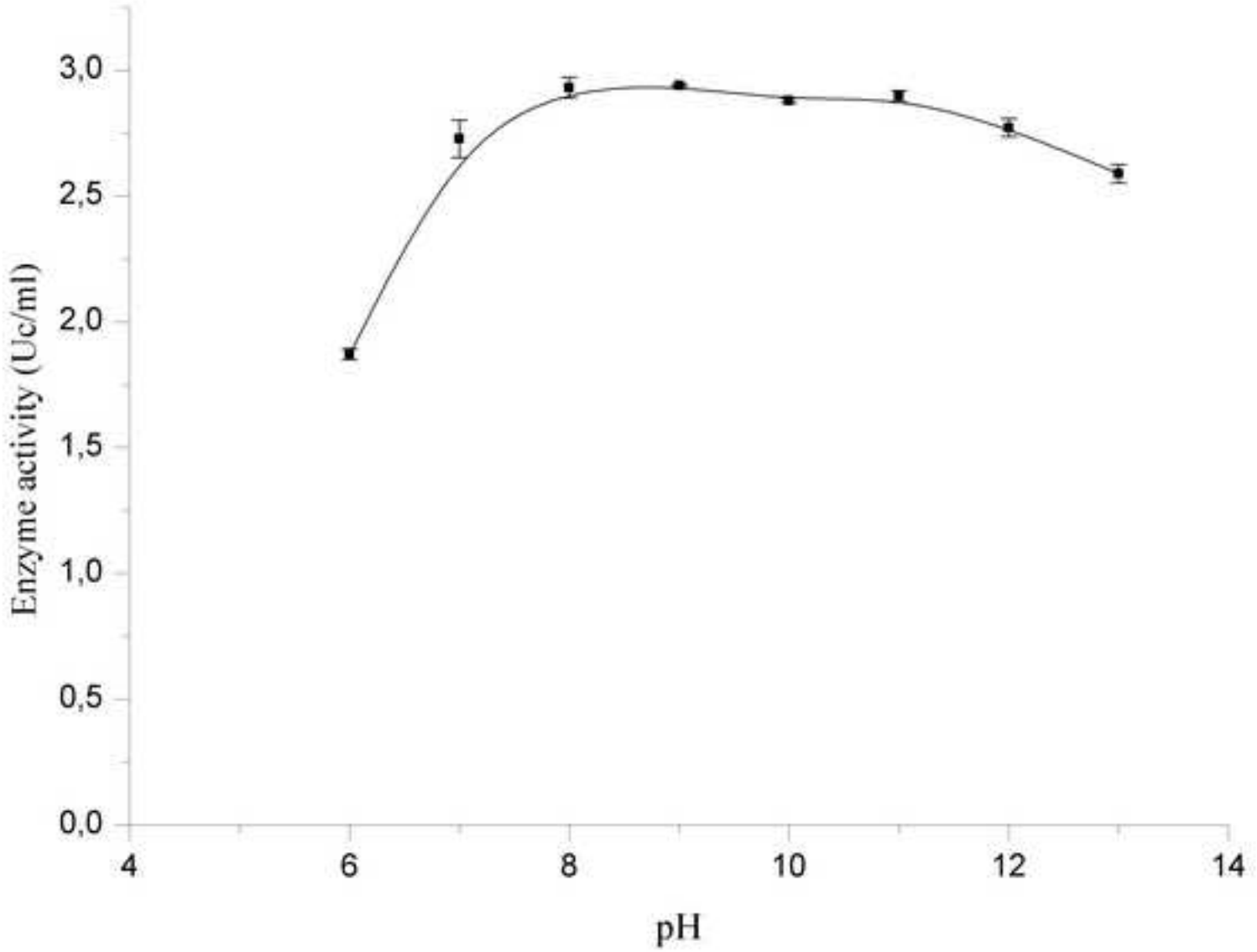




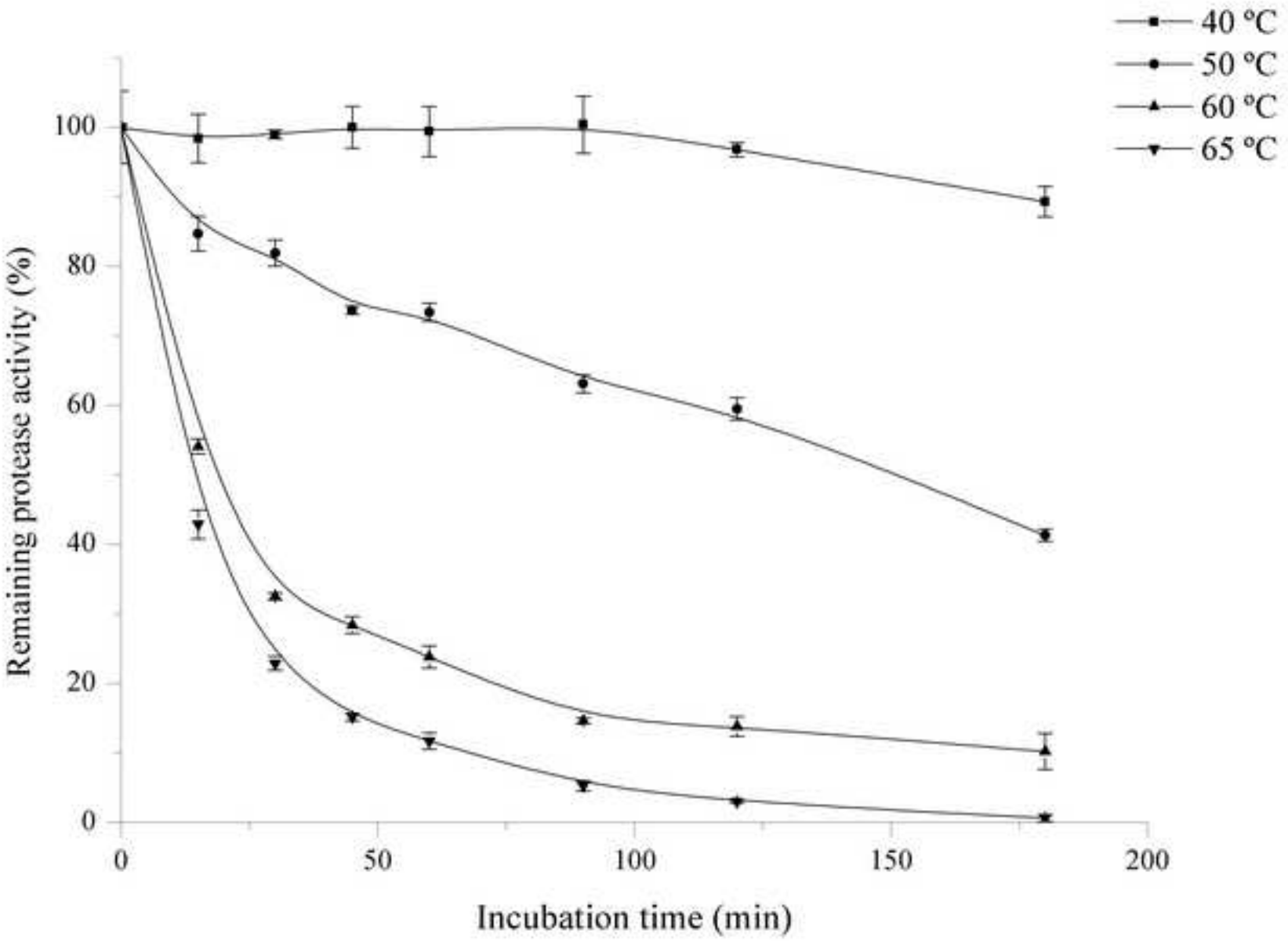
Figure(s)


Remaining protease activity (%)
100
80
60
40
20
0
0,0
3,5
7,0
10,5
14,0
pH

Figure(s)



Figure(s)


40 °C
50 °C
60 °C
65 °C
Remaining protease activity (%)
100
80
60
40
20
0
0
50
100
150
200
Incubation time (min)

Figure(s)

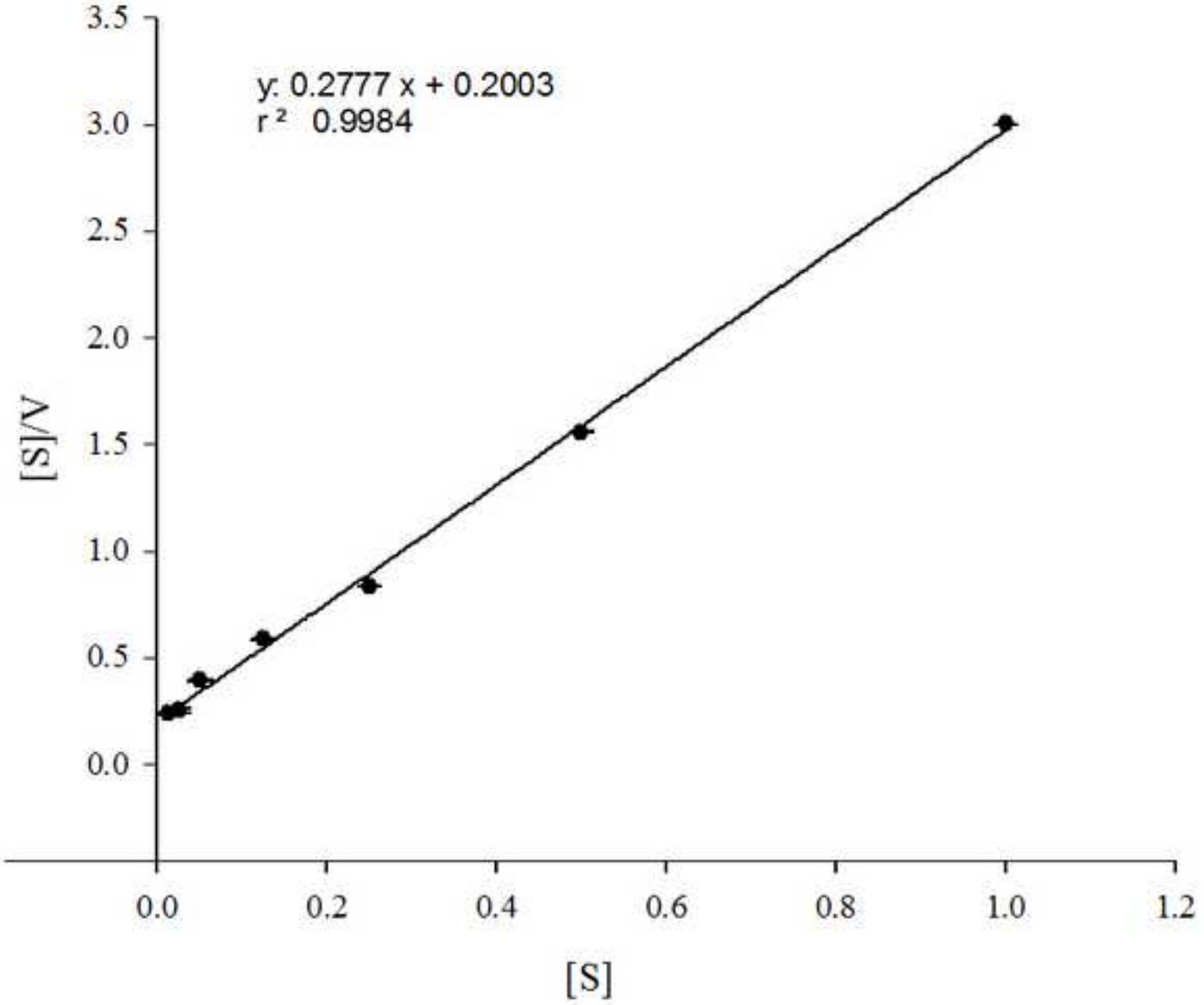

y: 0.2777 x + 0.2003
r² 0.9984
[S]/V
[S]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
0.2
0.4
0.6
0.8
1.0
1.2

Fig. 1 (A) Time course of keratinolytic (▲), proteolytic (●), soluble protein (△) production and pH (○) of *P. lilacinus* in a basal hair medium. Error bars (±S.D.) are shown when larger than the symbol. (B) Scanning electron micrographs of hair waste degradation by *P .lilacinum*. Uninoculated hair fibres after hair-saving unhairing process using sodium sulfite/lime as unhairing agent; (C) degradation of hair fibres by the fungus after 5 days; it can be seen the colonization of *P. lilacinum* on hair surface.

Fig. 2 SDS-PAGE of *P. lilacinus* keratinase. Lane 1: purified keratinase. Lane 2: low molecular weight markers (KDa) Phosphorylase b (97), Albumin (66), Ovalbumin (45), Carbonic anhydrase (30), Trypsin inhibitor (20.1), α-Lactalbumin (14.4).

Fig. 3 Effect of pH on enzyme stability (A) and activity (B). The enzyme activity was measured at 37 ºC for 30 min using azocasein as substrate. Results represent the means of three experiments, and bars indicate ± standard deviation.

Fig. 4 Effect of temperature on enzyme stability. Keratinase was incubated at 40, 50, 60 or 65 ºC up to 180 min, withdrawing samples at different times. Remaining protease activity was measured under standard assay conditions. Results represent the means of three experiments, and bars indicate ± standard deviation.

Fig. 5 Hanes Hultin transformation plot of the purified enzyme using azocasein as susbtrate. *Error bars* correspond to standard deviations from triplicate replicas.