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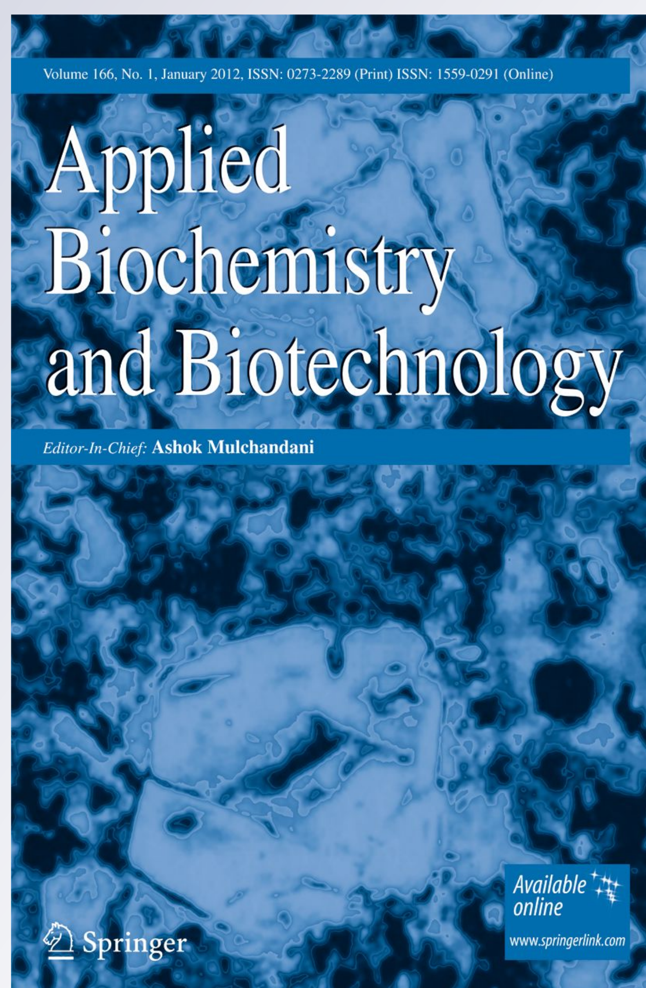

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Biodegradation of a Keratin Waste and the Concomitant Production of Detergent Stable Serine Proteases from *Paecilomyces lilacinus*

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Abstract *Paecilomyces lilacinus* (LPS 876) efficiently degraded keratin in chicken feather during submerged cultivation producing extracellular proteases. Characterization of crude protease activity was done including its compatibility in commercial detergents. Optimum pH and temperature were 10.0 and 60 °C, respectively. Protease activity was enhanced by Ca^{2+} but was strongly inhibited by PMSF and by Hg^{2+} suggesting the presence of thiol-dependent serine proteases. The crude protease showed extreme stability toward non-ionic (Tween 20, Tween 85, and Triton X-100) and anionic (SDS) surfactants, and relative stability toward oxidizing agent (H_2O_2 and sodium perborate). In addition, it showed excellent stability and compatibility with various solid and liquid commercial detergents from 30 to 50 °C. The enzyme preparation retained more than 95% of its initial activity with solid detergents (Ariel™ and Drive™) and 97% of its original activity with a liquid detergent (Ace™) after pre-incubation at 40 °C. The protective effect of polyols (propylene glycol, PEG 4000, and glycerol) on the heat inactivation was also examined and the best results were obtained with glycerol from 50 to 60 °C. Considering its promising properties, *P. lilacinus* enzymatic preparation may be considered as a candidate for use in biotechnological processes (i.e., as detergent additive) and in the processing of keratinous wastes.

Keywords *Paecilomyces lilacinus* · Alkaline serine proteases · Keratinolytic activity · Chicken feather · Detergent stable

Introduction

Proteases make up more than 60% of the total worldwide sale of industrial enzymes, finding application in food, detergent, and leather processing. Among these different proteases, keratinases constitute a group of enzymes capable of disrupting the highly stable keratin

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structure consisting of disulfide, hydrogen, and hydrophobic bonds in the form of α-helices and β-sheets [1].

Argentina's economy has traditionally been based on agriculture and related industries. Livestock (cattle, sheep, and poultry) and grains have long been the bulwark of its wealth; its cattle herds are among the world's finest. There are more than 50 million livestock which generate large amounts of waste including insoluble keratin-containing animal material such as feather, hair, wool, nails or claws, hooves, horns, and beaks. Around 580 million chickens, with an individual body weight of 2 kg, are processed per year in Argentina. Considering the fact that feather constitute nearly 8.5% of the total weight of a chicken, the amount of keratinous solid waste generated would be approximately 98.6 million kilograms per year. The same estimations for India and USA are around 140 million and 1.044 billion, respectively [2, 3].

Thus, there is quite a significant amount of this low-cost resource available for the biotransformation into value-added products, especially in the form of proteolytic enzymes. Out of the vast pool of enzymes, proteolytic enzymes from microorganism are the most widely exploited enzymes in the detergent industries [4–6]. Over the past 30 years, the importance of proteases in detergents has changed from being minor additives to being key ingredients. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently used enzyme-based detergents [7]. Proteolytic enzymes incorporated into detergent formulations must have some particular characteristics: they should be active and stable at alkaline pH values and at relatively high temperatures (40–50 °C and even above) and compatible with other detergent components like surfactants, perfumes, bleaches, oxidizing and sequestering agents, etc. [8]. In general, the majority of commercially available enzymes are not stable in the presence of bleaching/oxidizing agents. Hence, the latest trend in enzyme-based detergents is the use of recombinant DNA technology to produce bioengineered enzymes with better stability [9–11].

Looking into depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties suitable for commercial exploitation. Within this context, the purposes of the present study were to characterize the extracellular protease activity produced by *Paecilomyces lilacinus* strain LPS #876 grown using feather (waste of chicken processing industry) as substrate, to study an efficient stabilization method toward heat inactivation, and finally to investigate its compatibility with various commercial liquid and solid detergents.

Materials and Methods

Microorganism, Media, and Culture Conditions for Enzyme Production

P. lilacinus (Thom) Samson LPS #876 was used. This is a non-pathogenic fungal strain, locally isolated from alkaline forest soils, which was deposited at the Spegazzini Institute fungal culture collection (La Plata National University, Argentina). It was maintained in a potato dextrose agar (Britania, Argentina) medium. The medium used for enzyme production had the following composition (g/l): chicken feathers, 10; NaH_2PO_4, 0.496; K_2HPO_4, 2.486; $FeCl_3 \cdot 6H_2O$, 0.016; $ZnCl_2$, 0.013; $MgCl_2$, 0.010; and $CaCl_2$, 0.11; pH 7.0 [12].

Chicken feathers were supplied by a local slaughterhouse. They were washed thoroughly with 0.1% (w/v) sodium dodecyl sulfate (SDS) to remove surface contaminants and then with 1:1 (v/v) methanol and water with shaking for 18 h at 28 °C. Then they were rinsed with distilled water and finally with 95% ethanol, and air dried [13].

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Cultures were performed in 500-ml Erlenmeyer flasks containing 100 ml of medium inoculated with 2×10^6 conidia/ml, on a rotary shaker (200 rpm), for 70 h at 28 °C. The culture was centrifuged at $3{,}000 \times g$ for 15 min at 4 °C and the supernatant was kept frozen (−20 °C) until used as crude extract (CE).

Assay of Protease Activity

Protease activity was measured by the method of Liggieri et al. [14]. Azocasein, used as substrate, was synthesized according to Charney and Tomarelli et al. [15]. A suitable diluted aliquot of the CE (0.1 ml) was mixed with 0.250 ml of azocasein (1.0% w/v in 100 mM Tris–HCl buffer, pH 9.0) and incubated for 30 min at 37 °C. The reaction was stopped by the addition of 1.0 ml of trichloroacetic acid (10% w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at $3{,}000 \times g$ for 10 min. One milliliter of NaOH (1 N) was added to 1 ml of the supernatant and absorbance was measured at 440 nm. Measurement was made in triplicate and a blank using heat-inactivated CE was also performed.

Protease activity was expressed as azocasein unit (U_C), which was defined as the amount of enzyme that, under test conditions, causes an increase of 0.1 in the absorbance at 440 nm per minute.

Effect of pH on Enzyme Activity and Stability

The optimum pH of the protease activity was studied over a range of 6.0–13.0 (azocasein is insoluble at pH <6.0) whereas its pH stability was studied over a range of 4.0–13.0. For the measurement of pH stability, the CE was incubated for 1 h at 37 °C, and residual protease activity was determined under standard assay conditions. In both cases, a mixture of buffers (glycine, MES, and Tris–HCl, 20 mM each) adjusted to the required pH was used.

Effect of Temperature on Enzyme Activity and Stability

To investigate the effect of temperature, the protease activity was tested at different temperatures between 18 and 70 °C for 30 min at pH 9.0. The thermostability was examined by incubating the CE at different temperatures for 180 min. Aliquots were withdrawn at desired time intervals, and the remaining protease activity was measured under standard assay conditions. The non-heated crude protease was taken as 100%.

The protective effect of $CaCl_2$ (5 mM), propylene glycol (10% v/v), glycerol (10% v/v), and polyethylene glycol 4000 (PEG 4000, 10% w/v) on heat inactivation was also studied. The CE was incubated at 50–60 °C with and without the chemicals mentioned above, and residual protease activity was measured at regular intervals under standard assay conditions.

Effect of Inhibitors and Metal Ions on Protease Stability

The effect of different enzyme inhibitors on protease activity was studied using phenylmethylsulfonyl fluoride (PMSF, 2 mM), iodoacetate (10 mM), ethylenediaminetetraacetate (EDTA, 5 mM), 1,10-phenantroline (1 mM), and pepstatin A (100 μg/ml). An aliquot of CE was pre-incubated with the corresponding inhibitor for 1 h at room temperature (20 °C). The remaining protease activity was determined and expressed as percentage of residual activity relative to a control (100%) without any inhibitor.

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The effect of different metal ions (at a concentration of 1 mM) on protease activity was investigated by adding divalent metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, and Hg^{2+}) to an aliquot of CE and pre-incubating the mixture for 1 h at room temperature. The remaining protease activity was determined and expressed as a percentage of residual activity relative to a control (100%) without any metal ion.

Effect of Surfactants and Oxidizing Agents on Protease Stability

The suitability of the CE of *P. lilacinus* as a detergent additive was determined by testing the protease stability towards some surfactants (SDS, Triton X-100, Tween 20, and Tween 85) and oxidizing agents (H_2O_2 and sodium perborate). An aliquot of the CE was incubated with different concentrations of these additives for 1 h at various temperatures (18 to 40 °C). The remaining protease activity was determined under standard conditions and expressed as percentage of residual activity relative to a control (100%) without any additive.

Detergent Compatibility

The compatibility of protease activity in CE with commercial solid and liquid laundry detergents (locally available) was also studied. The solid detergents tested were Drive, Skip and Ala matic (Unilever), and Ariel (Procter & Gamble), and the liquid one was Ace (Procter & Gamble).

Solid detergents were diluted in tap water to give a final concentration of 7 mg/ml and liquid detergent was diluted 100-fold to simulate washing conditions [16]. The endogenous enzymes contained in laundry detergents were inactivated by heating the diluted detergents for 1 h at 65 °C prior to the addition of an aliquot of CE. The corresponding reaction mixtures were incubated for 1 h at different temperatures (30–55 °C), and the remaining activities were determined under standard conditions. The enzyme activity of a control, incubated under similar conditions without detergent, was taken as 100%.

Statistical Analyses

Results are represented as mean ± SD of at least three replicates. The analysis of variance was carried out using ANOVA. Pearson's correlation coefficient were considered significant if $p < 0.05$.

Results

Enzyme Production

P. lilacinus LPS #876 grew and produced alkaline proteases in liquid cultures containing chicken feathers as the sole source of carbon and nitrogen supplemented with a mixture of mineral salts. Maximum protease activity (1.53 U_C/ml) was observed at 70 h of cultivation with the concomitant complete degradation of feathers including feather's barbules and rachis (Fig. 1). The synthesis of extracellular proteases was associated with the increment in soluble protein in culture broth. A continuous increase in pH values was also observed (data not shown). This change in pH values has been pointed out as an important indicator of the keratinolytic potential in microorganisms because of the high level of deamination, with

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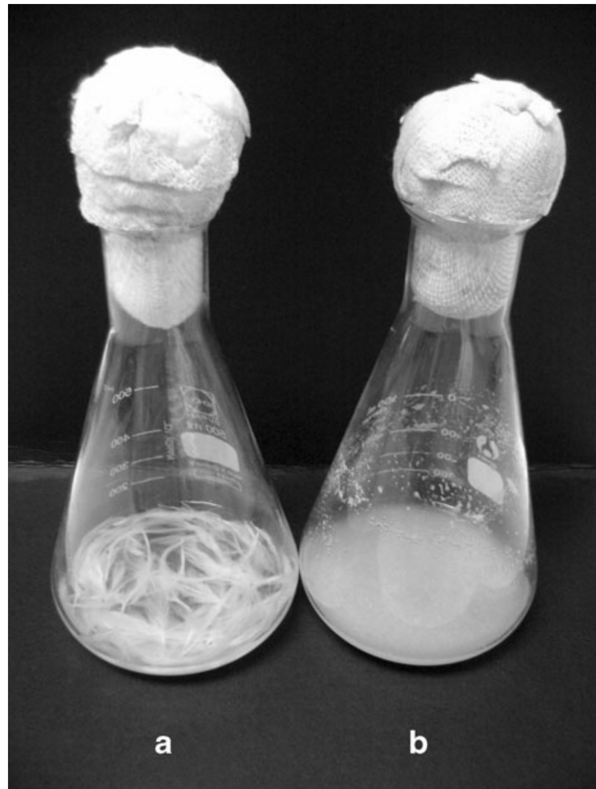

Fig. 1 Degradation of chicken feather by *P. lilacinus* LPS #876. Control flask (*left*) shows intact feathers, while experimental flask (*right*) shows complete hydrolysis of feathers after 70 h of cultivation at 28 °C, 200 rpm

the concomitant ammonium accumulation in culture medium [17]. A supernatant of 70-h culture was used as CE for enzyme characterization.

Effect of pH on Enzyme Activity and Stability

The effect of pH on protease activity was determined over a pH range of 6.0–13.0. The CE was highly active in a broad range of pH (7.0–13.0) with an optimum pH of 10.0. At pH 13.0 the relative activity was about 70 % (Fig. 2a).

The pH stability profile showed that the CE is highly stable at a pH range between 5.0 and 13.0, maintaining almost 100% of its original activity after 1 h of incubation at 37 °C. At pH 4.0, it retained about 90% of its initial activity (Fig. 2b).

Effect of Temperature on the Activity and Stability of the CE

The relative activity at various temperatures of *P. lilacinus* CE was tested. It was active at temperatures from 30 to 70 °C with an optimum at 60 °C (Fig. 3a).

Figure 3b represents the effect of temperature on enzyme stability. The results indicated that it was completely stable at temperatures up to 40 °C, but was inactivated at higher temperatures. At 30 and 40 °C, the CE remained fully active even after 3 h of incubation, indicating that it might be used under mild heating conditions. At 50 and 55 °C, it retained 62% and 23% of its initial activity after 1 h of incubation, respectively, and 19% after 1 h of incubation at 60 °C.

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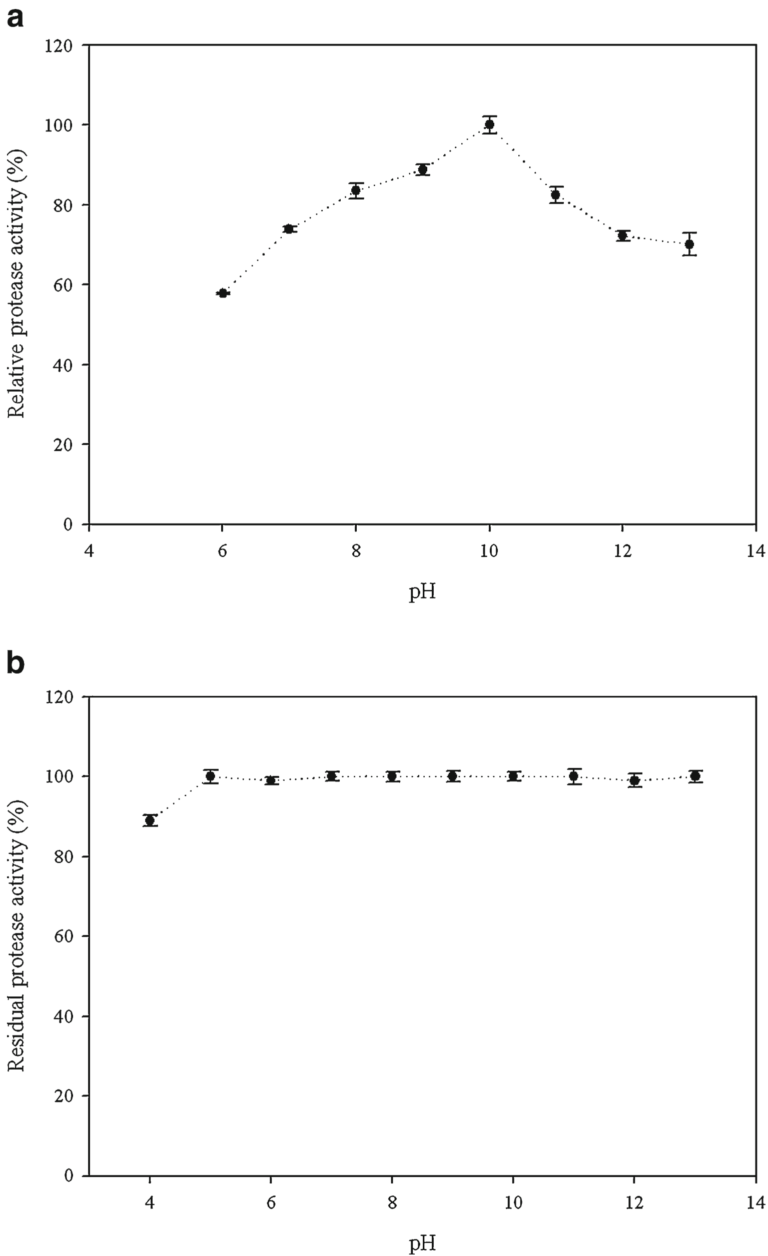

Fig. 2 **a** Effect of pH on protease activity. The maximum activity obtained at pH 10 was considered as 100% activity. Values are means of three independent determinations. *Error bars* (±SD) are shown when larger than the *symbol.* **b** Effect of pH on protease stability. The protease activity before incubation was taken as 100%. Values are means of three independent determinations. *Error bars* (±SD) are shown when larger than the *symbol*

To examine the protective effect of some stabilizers at 50–60 °C, Ca^{2+} (5 mM), propylene glycol (10% v/v), glycerol (10% v/v), and polyethylene glycol 4000 (PEG, 10% w/v) were added. As shown in Fig. 3c, the apparent half-life ($t_{1/2}$) of enzyme

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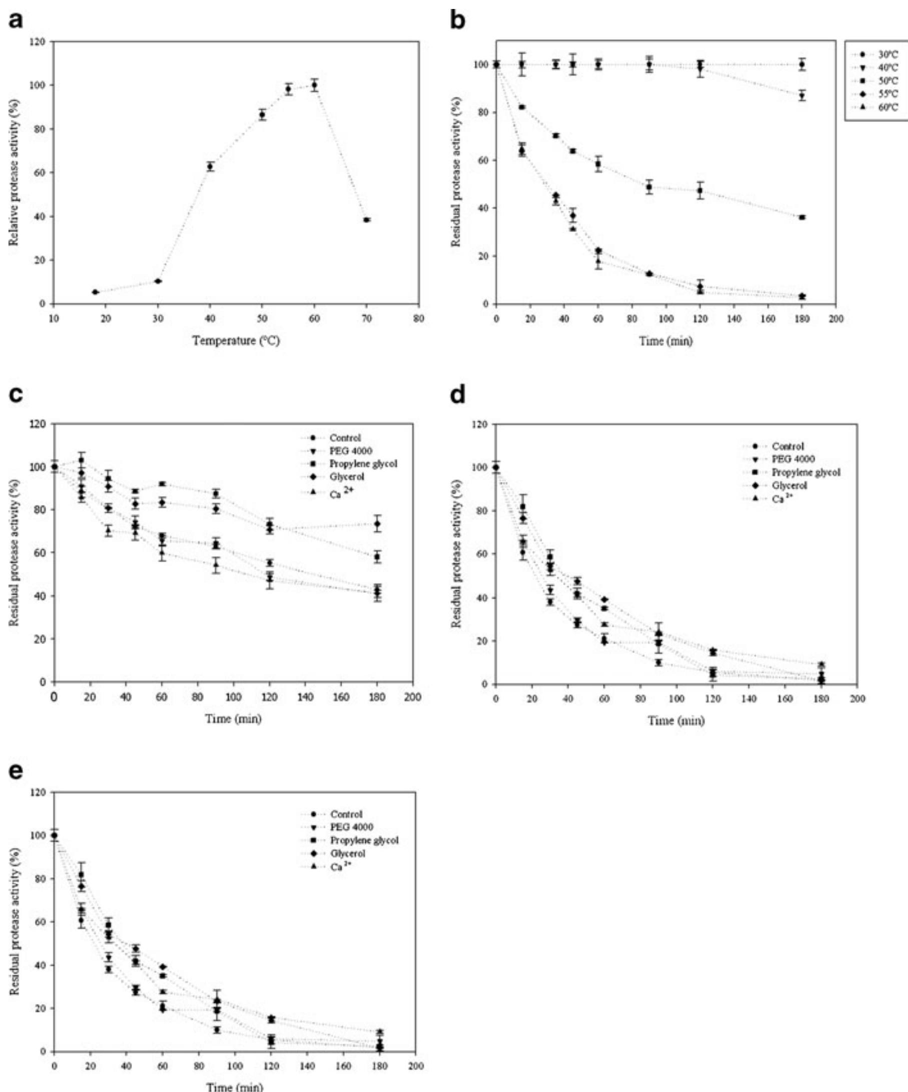

Fig. 3 **a** Effect of temperature on protease activity. The protease activity of the crude extract at 60 °C was taken as 100%. **b** Effect of temperature on protease stability. c Effect of stabilizers on heat inactivation at 50 °C. **d** Effect of stabilizers on heat inactivation at 55 °C. **e** Effect of stabilizers on heat inactivation at 60 °C. For **b**, **c**, **d** and **e** the original activity before pre-incubation was taken as 100%. Values are means of three independent determinations

increased to approximately 290 min at 50 °C by addition of glycerol, while a slight decrease by addition of Ca^{2+} and PEG 4000 was observed when compared to the control containing no stabilizers ($t_{1/2}$=140 min). Similar results for glycerol were found at 55 and 60 °C, but in these cases Ca^{2+} increased its stability as did the others stabilizers also (Fig. 3d, e).

Effects of Inhibitors and Metal Ions on Protease Stability

The effects of chelating agent and group-specific reagents on protease stability are summarized in Table 1. The CE was strongly inhibited after incubation with a serine

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protease inhibitor (PMSF) indicating that the CE contained serine proteases. Incubation with the chelating agent EDTA (5 mM) resulted in a slight inhibition of the protease activity in CE, with 19.90% of its original activity being lost.

The effects of some metal ions, at a concentration of 1 mM, on the protease activity were also studied pre-incubating the CE with the respective cations. It was found that protease activity was enhanced after pre-incubation with Ca^{2+}, while Zn^{2+} and Hg^{2+} inhibited enzyme activity. The inhibition detected after pre-incubation with Hg^{2+} indicates that CE contained not only serine proteases but these serine proteases are thiol-dependent serine proteases.

Effect of Surfactants and Oxidizing Agents on Protease Activity

In order to be effective during washing, a good detergent protease must be compatible and stable with all commonly used detergent compounds such as surfactants, oxidizing agents, and other additives, which might be present in detergent formulation [18, 19]. The CE was incubated during 60 min at room temperature, 30 and 40 °C, in the presence of several additives and then the residual protease activity was assayed under standard conditions. As can be seen in Table 2, protease activity in CE was highly stable in the presence of non-ionic surfactants. It retained 100% of its initial activity in the presence of 5% Triton X-100, 5% Tween 20, and 5% Tween 85 after 1 h of incubation at the three temperatures mentioned above. In the presence of SDS (0.1%, 0.5%, and 1.0% w/v), a strong anionic surfactant, the protease activity in CE exhibited higher stability at high concentration (1.0%) than at low concentrations (0.1–0.5%). In the presence of 0.5% of SDS, the CE retained approximately 68% after 1 h of incubation at 30 °C, while at 40 °C the residual activity was about 62%.

The effect of oxidizing agents onto protease activity in CE was also evaluated (Table 2). In this sense, when the CE was incubated for 1 h at different concentrations of sodium perborate, an enhanced protease activity was observed at room temperature. At 30 °C, a slight inactivation was found, resulting in a 97.6% its residual activity after 1 h of incubation. Even at 40 °C, in the presence of 1.0% of sodium perborate, the CE retained 80% of its original activity. Similar results were detected when H_2O_2 was employed at room temperature and at 30 °C. In these cases, protease activity in CE was enhanced even in the presence of 3% of H_2O_2. At

Table 1 Effects of inhibitors and metal ions on protease activity

Chemicals	Concentration	Residual activity (%)
None	–	100±1.2
PMSF	2 mM	11.8±3.5
Iodoacetate	10 mM	99.0±2.3
EDTA	5 mM	81.1 ±0.9
1,10 Phenanthroline	1 mM	95.0±0.2
Pepstatin A	100 µg/ml	99.0±4.2
Ca^{2+}	1 mM	110±1.0
Zn^{2+}	1 mM	84.0±2.0
Mg^{2+}	1 mM	95.0±3.0
Hg^{2+}	1 mM	15.3±1.0

CE was pre-incubated with inhibitors and metal ions, for 1 h at room temperature, and then the remaining protease activity was determined as a percentage of residual activity relative to control without any additives

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Table 2 Stability of CE protease in presence of surfactants and oxidizing agents

Surfactants/oxidizing agents	Concentration (%)	Residual activity (%)		
		Room temperature	30 °C	40 °C
None	–	100±1.2	100±2.5	100±1.6
Triton X-100	1 (v/v)	100±3.7	100±3.6	94±1.1
	5	100±2.0	100±2.2	100±2.2
Tween 20	1 (v/v)	100±3.5	100±1.5	100±2.3
	5	100±3.7	95±1.9	100±1.5
Tween 85	1 (v/v)	100±2.0	100±0.9	100±2.5
	5	88±0.9	100±3.2	100±3.6
SDS	0.1 (w/v)	100±2.3	97±1.7	83±2.7
	0.5	89±0.7	68±3.7	62±2.6
	1	100±1.4	99±2.1	100±3.9
Sodium perborate	0.2 (w/v)	100±0.5	100±2.9	100±2.7
	0.5	100±0.9	98.5±3.0	85.8±1.4
	1	100±1.9	97.6±2.3	80.0±2.1
H_2O_2	1 (w/v)	100±2.5	100±1.6	79.9±4.0
	2	117.9±0.2	117.8±2.4	53.8±2.9
	3	96.4±0.9	106.1±0.5	32.8±1.4

The CE was incubated with different concentrations of these additives for 1 h at various temperatures (18 to 40 °C), and then the residual protease activities were measured under standard conditions against a control without any additives, which was taken as 100%

40 °C, when the concentration of H_2O_2 increased, there was a decrease in the residual activity. Only 32.8% of its activity remained after 1 h of incubation at 40 °C in the presence of 3% of H_2O_2.

Detergent Compatibility

To check the compatibility of the protease activity in CE with commercial detergents, it was pre-incubated in the presence of various locally available laundry detergents for 1 h at different temperatures. The solid detergents were diluted in tap water to a final concentration of 7 mg/ml (to stimulate washing conditions). Endogenous proteases present in these detergents were inactivated by incubating the diluted detergents at 65 °C for 1 h prior to the addition of CE.

As can be seen in Fig. 4, the CE is extremely stable in the presence of the commercial detergents tested from 30 to 50 °C. It retained more than 85% of its activity in all cases even after 1 h of incubation at 40 °C. For example, in the presence of Ariel, Drive, and Ace, about 95% of the original activity remained after 1 h of incubation at 40 °C. The protease activity in CE also showed excellent stability and compatibility at 50 °C while incubated with most of the detergents tested. It retained about 98.1% and 88.8% of its initial activity in the presence of Ace and Drive, respectively. On the other hand, it showed less stability in the presence of Ariel, Skip, and Ala matic, retaining 68.9%, 64.6%, and 66.5% of its original activity. However, at 55 °C, the CE presented excellent stability with Ace, retaining more than 90% of its original stability whereas in the presence of Drive it also retained about 75.5% of its original activity.

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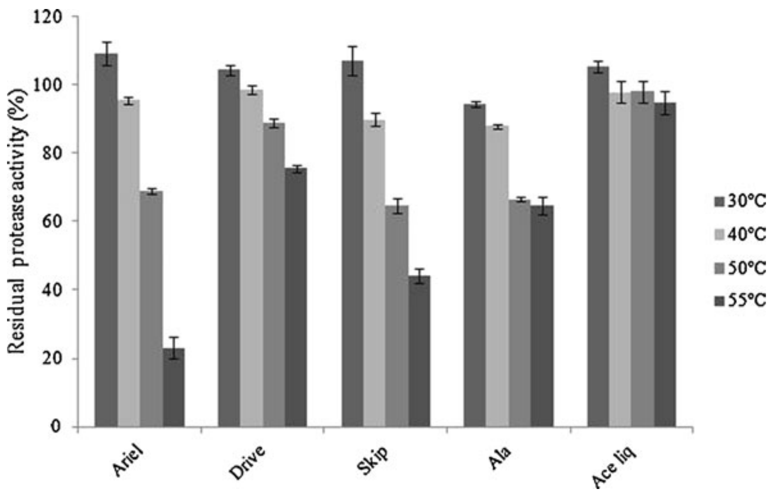

Fig. 4 Stability of the CE in the presence of various commercial solid and liquid detergents. CE was incubated in each detergent mentioned for 1 h at different temperatures (30–55 °C), and the remaining activities were determined under standard conditions. The enzyme activity of a control, incubated under similar conditions without detergent, was taken as 100%

Discussion

In a series of studies on keratinolytic enzymes, our work group has been dealing with a locally isolated fungal strain of *P. lilacinus* able to produce keratinolytic activity when grown under the presence of keratinous materials. As an unexpected result, it was found that the keratinolytic activity produced by this fungus showed interesting characteristics considering its potential application as detergent additive. The work presented here relates the utilization of chicken feathers as carbon and nitrogen source for growth of *P. lilacinus*, the biochemical characterization of protease activity produced, and the investigation of its compatibility with various commercial liquid and solid detergents.

In order to be effective, detergent proteases should be highly active and stable over a range of pH and temperature compatible with washing. Also, they should be effective at low levels (0.4–0.8%) and should also be compatible with various detergent components along with oxidizing and sequestering agents.

Protease activity in CE of *P. lilacinus* culture was highly active in the pH range of 6.0–13.0 with an optimum pH of 10.0. This is a very important characteristic for its eventual use in detergent formulations because the pH of laundry detergents is generally in the range of 9.0–12.0 [8, 20]. These findings are well in accordance with reports of commercially available detergent enzymes such as Alcalase™ produced by *Bacillus licheniformis*, Purafect™, a genetic engineered *Bacillus lentus* protease, and Maxatase™ produced by *B. subtilis* [21]. In addition, protease activity in CE of *P. lilacinus* is very stable over a wide pH range, maintaining 100% of its original activity between 5.0 and 13.0. This stability is higher compared to several proteases reported to be useful as detergent additives, such as *B. licheniformis* NH1 [22], *Bacillus cereus* SV1 [23], and *Bacillus pumilis* A1 [24].

Protease activity increased progressively with temperature and maximum activity was obtained at 60 °C. Nevertheless, it decreased at temperatures higher than 60 °C. This is an

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important characteristic for the use of alkaline proteases as detergent additives considering the possibility of using washing cycles with different preset temperatures. Similar to our results, two important alkaline proteases used in the detergent industry [subtilisin Carlberg, produced by *B. licheniformis*, and subtilisin Novo or Bacterial Protease Nagase (BNP), produced by *Bacillus amyloliquefaciens*] also show optimum temperature at 60 °C [25].

Protease activity in CE produced by *P. lilacinus* was very stable up to 40 °C; it showed the apparent half-life ($t_{1/2}$) of 140, 26, and 24 min at 50, 55, and 60 °C, respectively. To examine the protective effect of some stabilizers at these temperatures, Ca^{2+} (5 mM), propylene glycol (10% v/v), glycerol (10% v/v), and PEG 4000 (10% w/v) were added to the CE. As shown in Fig. 3c, the apparent half-life of protease activity in CE increased by 2-fold and 1.6-fold by the addition of glycerol and propylene glycol, respectively, while a slight decrease was observed by the addition of Ca^{2+} in comparison with the control containing no stabilizer ($t_{1/2}$=140 min). Similar results were observed at 55 and 60 °C, with glycerol (increased $t_{1/2}$ by 1.65-fold and by 2.33-fold, respectively) and with propylene glycol (increased $t_{1/2}$ by 1.6-fold and 2.2-fold, respectively).

It is known that the addition of Ca^{2+} or polyhydric alcohols, such as glycerol and polyethylene glycol, causes an increase in thermal stability of alkaline proteases. Beg and Gupta [21] reported that $t_{1/2}$ of an alkaline protease from *Bacillus mojavensis* was increased by 2.2- and 2.3-fold by adding PEG 6000 and glycerol at 60 °C, respectively. The addition of sorbitol also improved the thermal stability for an alkaline protease from *B. cereus* BG1, which increased the thermal stability by approximately 2-fold at 60 °C [26]. Similar pattern of protection effect was observed in an alkaline protease from *Pseudomonas aeruginosa* MN1 by the addition of glycerol [27]. The increase in thermal stability by addition of polyols is probably due to the reinforcement of the hydrophobic interactions among non-polar amino acids inside the enzyme molecules, and thus increasing their resistance to inactivation.

Among the chemicals tested here, Ca^{2+} caused less stabilization than the other ones; it just increased by 1.4-fold and 1.3-fold the $t_{1/2}$ at 55 and 60 °C, respectively. It is known that Ca^{2+} increases not only enzyme activity but also the thermal stability of alkaline proteases. For example, the $t_{1/2}$ was increased by 2.5-fold at 50 °C, 2.3-fold at 60 °C, and 10-fold at 60 °C by adding Ca^{2+} for alkaline proteases from *Bacillus sphaericus*, *B. mojavensis*, and *Bacillus* sp. GX6638, respectively [21, 28, 29]. The improvement in protease thermal stability in the presence of Ca^{2+} may be explained by the strength of interactions inside protein molecules and by the binding of this metal to the autolysis sites [26].

Protease activity in CE was strongly inhibited by PMSF and a slight inhibition was shown by EDTA (5 mM), with 18% of its original activity being lost. These findings are in accordance with several reports showing that the active structure of serine proteases contains Ca^{2+} binding site(s) and the removal of Ca^{2+} from the strong binding site is associated to a significant reduction in thermal stability [30]. The absence of inhibition in the presence of EDTA is very useful for applications as detergent additive because chelating agents are valuable components of most detergents. Chelating agents function as water softeners and also assist in the removal of stain.

Among the metal ions tested, the addition of Ca^{2+} increased protease activity by 110% whereas the addition of Zn^{2+} and Hg^{2+} resulted in a reduction of protease activity. This behavior is in accordance with previous studies which showed that serine proteases are inhibited by Cu^{2+}, Mn^{2+}, and Hg^{2+} [23, 24]. Hg^{+2} is recognized as an oxidant agent of thiol group, and the enzyme inhibition by this ion suggests the presence of important –SH group (s) (such as free cysteine) at or near the active(s) site(s) [31]. The strong inactivation by Hg^{2+}

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is typical for proteinases belonging to the thermitase and proteinase K subgroups [32]. This feature makes the proteases, present in CE of *P. lilacinus*, significantly different from the true bacillary subtilisins as well as the serine proteinase from *P. lilacinus* isolated by Bonants et al. [33]. Based on the presence of functionally important sulfhydryl groups, the proteases in the CE resemble proteinase K and bacillary thiol-dependent subtilisins than other fungal serine proteases. Similar results were reported by Kotlova et al. [34] for a thiol-dependent serine proteinase from *P. lilacinus* (Thom) Samson VKM F-3891 isolated from soil samples from burial mound.

SDS is known to be a strong denaturant of proteins including alkaline proteases. It could unfold most proteins through the interaction between the charged head group of SDS and the positively charged amino acid side chains of proteins, and between alkyl chain of SDS and the non-polar parts on the surface as well as in the interior of proteins [35]. The stability towards SDS of protease activity in CE is important to be remarked because SDS stable enzymes have been rarely reported.

Protease activity in CE showed extreme stability towards non-ionic and anionic surfactants (Table 2). In the presence of 0.5% of SDS, the CE retained approximately 68% of the initial activity after 1 h of incubation at 30 °C, while at 40 °C the residual activity was about 62%. This retention of activity was higher than a protease from *Aspergillus clavatus* ES1 which retained about 33% of its activity after 1 h of incubation with 0.5% of SDS [36].

On the other hand, protease activity in CE showed excellent stability toward oxidizing agents, similarly to proteases from *B. licheniformis* NH1 which retained 85% and 80% of its activity after 1 h of incubation at 40 °C with 0.5% H_2O_2 and 0.2% sodium perborate, respectively [22]. In addition, protease activity in CE resulted to be more stable than *B. licheniformis* RP1 proteases which retained 48% of their activities after 1 h of incubation at 40 °C in the presence of 0.2% sodium perborate [8].

Oxidizing agents inactivate proteins oxidatively, and Met is identified as a primary site for oxidative inactivation of enzymes [37]. All subtilisins contain a Met residue next to the catalytic Ser residue so that many of them tend to undergo oxidative inactivation in the presence of an oxidizing agent such as hydrogen peroxide. Thus, many of the available alkaline proteases exhibited low activity and stability towards oxidants which are common ingredients in modern bleach-based detergents. To overcome these shortcomings, several attempts have been made to enhance enzyme stability by protein engineering [10]. That is why it is important to search for enzymes with high stability against surfactants and oxidants for industrial applications.

An ideal detergent enzyme should be stable and active in a detergent solution for a long period of time and should have adequate temperature stability to be effective in a wide range of washing temperatures. Protease activity in CE from *P. lilacinus* retained more than 80% of its activity in the presence of all commercial detergents tested, even after 1 h of incubation at 40 °C, and more than 60% at 50 °C. Singh et al. [38] reported a serine alkaline protease from *Bacillus* sp. SSR1 showing nearly 70–80% of activity in the presence of many detergents at 40 °C. They reported that the addition of an additive like $CaCl_2$ is required for stability above 40 °C of enzyme in detergents. Also, Banerjee et al. [39] and Bhosale et al. [40] reported that a protease enzyme retained high activity in commercial detergents after supplementation of additives such as $CaCl_2$ and glycine. Comparison of our results with those reported by others indicates that protease activity in CE is superior because it remains for a longer period in the presence of laundry detergents at higher temperatures and without any supplementation of additives. In addition, from our results of activity and stability at different temperatures, it can be concluded that protease activity in CE will be more effective at temperatures from 40 to 50 °C for long washing cycles (60 min) and at 55 °C for short

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washing cycles (10–30 min). In cases of Drive and Ace, long washing cycles could be done even at 55 °C because about 75.5% and more than 90% of its original protease activity, respectively, is retained.

Conclusions

P. lilacinus LPS #876 resulted to be a very promising strain for biotechnological applications. This strain was found to produce an extracellular protease activity when grown using chicken feather as the sole source of carbon and nitrogen in a simple culture medium. Alkaline protease activity in the CE obtained was highly stable and active in a wide range of pH and temperatures. It exhibits significant stability towards surfactants and oxidizing agents, showing also excellent stability and compatibility with various commercial detergents. Considering these characteristics, it can be concluded that *P. lilacinus* protease activity is a promissory candidate for industrial applications and, particularly, it may find potential applications in laundry detergents.

Finally, the capacity to degrade chicken feather completely revealed by this strain is a remarkable characteristic considering its potential application for the bioconversion of the large amounts of keratin wastes from poultry processing industry.

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