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Production and properties of keratinolytic proteases from three novel Gram-negative feather-degrading bacteria isolated from Brazilian soils

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Abstract The keratinolytic potential and protease properties of three novel Gram-negative feather-degrading bacteria isolated from Brazilian soils was described. *Aeromonas hydrophila* K12, *Chryseobacterium indologenes* A22 and *Serratia marcescens* P3 were able to degrade feather meal, producing high amounts of soluble proteins and forming thiol groups. The proteases of strains K12, A22 and P3 had optimal pH of 8.0, 7.5 and 6.0, respectively; this last is an uncommon feature for bacterial keratinases. The optimal temperature was in the range 45–55°C. All three proteases were active towards azokeratin and were inhibited by EDTA, suggesting that they are keratinolytic metalloproteases. The proteolytic activity of K12 was stimulated by organic solvents and the detergent SDS, suggesting its potential application for detergent formulations and peptide synthesis. Strains A22, K12 and P3 have great potential for use in biotechnological processes involving hydrolysis of keratinous byproducts.

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Introduction

Feathers are keratin-rich wastes largely produced by poultry industries. An estimated 5 million tons are produced annually as a waste stream from the production of chicken meat (Poole et al. 2009). Keratin is a protein difficult to degrade as the polypeptide is densely packed and strongly stabilized by several hydrogen bonds and hydrophobic interactions, in addition to several disulfide bonds (Brandelli 2008). Microorganisms are the most responsible for keratin recycling, playing an important role to avoid keratin accumulation in nature. Much work has been done on the bioconversion of feather residues by keratin-degrading microorganisms. These microbial strains produce specific proteases, named keratinases, capable of hydrolyzing keratin. Many promising applications have been given to microbial keratinases, including the bioconversion of keratinous wastes into feed and fertilizers, thus converting huge amounts of byproducts into added-value products (Gupta and Ramnani 2006; Brandelli et al. 2010).

Keratinolytic microorganisms have been frequently isolated from soils where keratinous materials are deposited (Kaul and Sumbali 1997; Riffel and Brandelli 2006). Among fungi, many keratinases

have been described among dermathophytes isolated from human and animal injuries. Among bacteria, keratinolytic activity has been widely documented for strains of the Gram-positive genera *Bacillus* and *Streptomyces* (Bressolier et al. 1999; Daroit et al. 2009). Nevertheless, some studies show that the diversity of bacteria with this ability must be higher. Lucas et al. (2003), investigating the diversity of keratinolytic bacteria among isolates from the soil environment under temperate climate, revealed that strains of the Gram-negative *Proteobacteria* and *Cytophaga-Flavobacterium* (CFB) group are predominant.

The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao et al. 1998; Gupta et al. 2002). However, the technological application of enzymes is sometimes not feasible for the highly demanding industrial conditions. Thus, attention has been given to the isolation of novel proteases from new microbial sources, surveying the biotechnological potential of biodiversity (Giongo et al. 2007). In a previous work, keratinolytic bacteria were isolated from soils of two important Brazilian biomes, the Atlantic Forest and the Amazon Forest (Bach et al. 2011). Considering that relatively few information is available on keratinases of Gram-negative bacteria, three novel feather-degrading strains of *Aeromonas hydrophila, Chryseobacterium indologenes* and *Serratia marcescens* were selected for characterization. The aim of this study was to investigate the keratinolytic potential of these bacteria and to characterize their keratinolytic proteases.

Materials and methods

Microorganisms, media and culture conditions

Gram-negative bacteria have been isolated from Brazilian native soils and selected for their proteolytic potential through halo formation on skimmed milk agar plates and feather degrading ability. Sequencing of the 16S rRNA gene fragment has been previously carried out and the strains were identified as *A. hydrophila* K12, *C. indologenes* A22 and *S. marcescens* P3 (Bach et al. 2011).

Isolates were maintained and subcultured on feather meal agar (FMA), which contained 10 g l^{-1} feather meal, 0.5 g l^{-1} NaCl, 0.3 g l^{-1} K_2HPO_4, 0.4 g l^{-1} KH_2PO_4 and 15 g l^{-1} of agar. Feather meal broth (FMB) was prepared in the same manner as FMA, except for the addition of agar. All cultivation media were autoclaved at 121°C, 105 kPa for 15 min.

Erlenmeyer flasks containing 50 ml of FMB were inoculated with each isolate and incubated at 30°C in an orbital shaker (125 rpm). After 24 h, 5% (v/v) inoculums (10^8 CFU ml^{-1}) were added to 150 ml of FMB and incubated on the same conditions. Samples were taken from each of duplicate cultivations every 4 h to evaluate growth, pH, soluble protein production, enzyme activity and formation of thiol groups. Duplicate assays were performed for each parameter.

Bacterial growth determination

Aliquots of the culture medium were serially diluted in saline solution (8.75 g l^{-1} NaCl), homogenized, and 20 μl of appropriate dilution were plated by drop culture method, on Tryptic Soy Agar (TSA, Mast Diagnostics, Merseyside, UK). Plates were incubated for 24 h at 30°C and counts were performed (Sangali and Brandelli 2000). Each experiment was carried out twice, with duplicate counts.

Determination of soluble protein

Samples of the culture broth were centrifuged (13,000×*g* for 7 min) and supernatants were utilized for determining the soluble protein concentration by the method of Lowry et al. (1951). Absorbance at 750 nm was measured with bovine serum albumin as a standard.

Enzyme assays

Proteolytic activity was assayed in culture supernatants using azocasein as substrate (Daroit et al. 2009). Briefly, 100 μl Tris–HCl buffer (0.1 M, pH 7.0) and 300 μl of 10 mg ml^{-1} azocasein (in the same Tris–HCl buffer) were added to 100 μl of crude enzyme and incubated in a water bath at 37°C for 30 min. The reaction was stopped with 600 μl of 10% (w/v) trichloroacetic acid (TCA) and centrifuged (10,000×*g* for 5 min). Then, 800 μl of the supernatant was added to 200 μl of 1.8 M NaOH and the

absorbance was measured at 420 nm. One unit (U) of protease activity was defined as the amount of enzyme that caused an increase of 0.1 absorbance unit at the defined assay conditions. A blank was prepared for each duplicate, stopping the reaction with TCA prior to incubation. Keratinolytic activity was also assayed by a similar protocol utilizing azokeratin as substrate. Azokeratin was synthesized as described elsewhere (Riffel et al. 2003a).

Determination of thiol formation

Free thiol groups were determined as described elsewhere (Sangali and Brandelli 2000), in culture supernatants. To 1 ml of sample were added 0.2 ml of NH_4OH, 1 ml of 0.5 g l^{-1} NaCN, and 1 ml of distilled water. The mixture was incubated for 20 min at 25°C, and then 0.2 ml of 0.5 g l^{-1} sodium nitroprusside were added. Absorbance at 530 nm was measured within 2 min.

Determination of pH and temperature optima for the crude proteases

To determine the effects of pH and temperature on the activity of crude proteases from strains K12, A22 and P3, culture supernatants (corresponding to the peak of protease production by each strain during cultivation) were used. Enzyme assays using azocasein as the substrate were carried out. The optimal pH was determinate over a range of 5.0–10.0 with increments of 0.5 pH units. Citrate buffer was used for pH between 5.0 and 6.5 and Tris–HCl buffer for pH between 7.0 and 10.0. The assay was carried out as described above using different buffers in the mixture. The activity of the fraction at the pH with the highest activity was taken as 100%.

The optimal temperature was determined over a range of 30–80°C, using the pH of highest activity in a similar assay as described above, except for the incubation of the mixture in different temperatures. The temperature in which the highest activity was observed was taken as 100%. All experiments were done in duplicates with blanks for each condition.

Zymogram analysis

Zymography was based on a previously described method (Garcia-Carreno et al. 1993), with minor modifications (Hmidet et al. 2008). Crude proteases (culture supernatants) were submitted to a modified SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In this protocol the samples were not heated before electrophoresis at room temperature on 12% polyacrylamide gels. After electrophoresis, gels were washed twice, for 30 min, with 0.1 M Tris–HCl buffer containing 2.5% (v/v) Triton X-100, and then with same buffer without detergent for 60 min. Gels of samples from strains P3, A22, and K12 were then submerged in a casein solution (1%, w/v) prepared with 0.1 M citrate buffer (pH 6.0), 0.1 M Tris–HCl buffer (pH 7.0), or 0.1 M Tris–HCl buffer (pH 8.0), respectively, and incubated overnight at room temperature. Finally, the gels were stained with Coomassie Brilliant Blue R-250 and then destained. Protease bands appeared as clear zones on a blue background. Three independent gels for each sample were analyzed.

Effects of chemicals on protease activity

The azocasein assay described above was carried out with various chemicals, which were added to the buffer. The salts tested (Table 1) were added to reach a concentration of 10 mM. The protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and EDTA, the detergents SDS and Triton X-100, the organic solvents dimethyl sulfoxide (DMSO), isopropanol and acetonitrile, and the reducing agents 2-mercaptoethanol and dithiothreitol (DTT) were used in the final concentrations listed in Table 2. Appropriate blanks were prepared to each enzyme-chemical solution. A control tube was carried out with no chemicals added to the buffer and its activity was considered as 100%. The enzyme activity in presence of each chemical was expressed as percentage relative activity of the control assay (without chemicals).

Statistical analysis

Results were expressed as means ± SD and the *t* test was carried out to assess significant differences between means at a 95% confidence level ($P < 0.05$).

Results and discussion

Aeromonas hydrophila K12, *Chryseobacterium indologenes* A22 and *Serratia marcescens* P3 showed

Table 1 Effect of salts on the activity of crude proteases from *Aeromonas hydrophila* K12, *Chryseobacterium indologenes* A22 and *Serratia marcescens* P3

Salt (10 mM)	Relative activity (%)[a]		
	K12	A22	P3
Control	100 ± 2.3	100 ± 2.0	100 ± 1.6
NaCl	130 ± 8.1	52 ± 0.5	118 ± 0.8
$CaCl_2$	92 ± 7.5[b]	108 ± 13.5[b]	114 ± 1.8
$CoCl_2$	64 ± 8.1	0 ± 0	135 ± 0.14
$HgCl_2$	1 ± 1.4	9 ± 0.5	0 ± 0
$MnCl_2$	94 ± 7.5[b]	66 ± 11.6	119 ± 1.9
$CuSO_4$	8 ± 4.0	23 ± 1.4	105 ± 0.5[b]
$FeSO_4$	0 ± 0	0 ± 0	125 ± 1.6
$MgSO_4$	151 ± 21	56 ± 0.5	116 ± 0.3
$ZnSO_4$	39 ± 4.08	53 ± 8.7	124 ± 0.7

[a] Values are means of two independent experiments

[b] Not significantly different from control at a 95% confidence level ($t_{tab;0.05;3}$) = 3.18

feather-degrading activity, causing a partial disintegration of chicken feathers (Fig. 1). Therefore, no complete dissolution was observed as for *Chryseobacterium* sp. kr6 and other bacteria, which completely degraded native feathers in 48–72 h (Riffel et al. 2003b; Daroit et al. 2009; Fakhfakh et al. 2009; Fakhfakh-Zouari et al. 2010). Whole feather fragmentation seems to be highly variable among keratin-degrading microorganisms. In keratinolytic bacteria isolated from soil, for instance, the percentage of remaining feathers was usually within 20–90% after a 3-week cultivation period (Lucas et al. 2003).

Protease production was investigated during bacterial growth in FMB. Growth curves are shown in Fig. 2A. All three bacteria showed maximum protease production during the late exponential or stationary growth phases (Fig. 2B): *A. hydrophila* K12 after 12 h, *C. indologenes* A22 after 48 h, and *S. marcescens* P3 after 36 h of cultivation. Microbial keratinases are predominantly extracellular enzymes, whose production is usually induced by keratinous growth substrates (Gupta and Ramnani 2006). The production of extracellular proteases during the stationary phase of growth is characteristic of many bacterial species (Gupta et al. 2002). The peak of proteolytic activity appeared to be followed by an increase of

Table 2 Effect of chemicals on the activity of crude proteases from *Aeromonas hydrophila* K12, *Chryseobacterium indologenes* A22, and *Serratia marcescens* P3

Chemical	Concentration	Relative activity (%)[a]		
		K12	A22	P3
Control	–	100 ± 2.3	100 ± 2.0	100 ± 1.6
PMSF	5 mM	111 ± 3.1	126 ± 16.8[b]	118 ± 4.9
EDTA	5 mM	14 ± 14.6	24 ± 23.5	46 ± 6.0
SDS	10 mM	175 ± 11.5	83 ± 3.4	76 ± 2.8
Triton X-100	0.1% (v/v)	0 ± 0	10 ± 4.0	77 ± 5.1
	0.5% (v/v)	11 ± 1.3	22 ± 10.5	76 ± 3.4
DMSO	1% (v/v)	323 ± 46.2	46 ± 17.0	97 ± 2.6
	5% (v/v)	290 ± 34.0	83 ± 49.8[b]	74 ± 1.6
Isopropanol	1% (v/v)	342 ± 36.0	110 ± 10.6[b]	91 ± 0
	5% (v/v)	209 ± 14.3	36 ± 13.0	60 ± 1.7
Acetonitrile	1% (v/v)	300 ± 46.9	82 ± 26.1[b]	82 ± 1.5
	5% (v/v)	186 ± 60.6[b]	129 ± 19.4[b]	50 ± 2.6
2-Mercaptoethanol	0.1% (v/v)	158 ± 18.3	46 ± 1.9	49 ± 4.8
	0.5% (v/v)	50 ± 25.8	68 ± 30.0[b]	22 ± 2.0
DTT	0.1% (v/v)	43 ± 25.8	0 ± 0	24 ± 1.1
	0.5% (v/v)	25 ± 21.7	0 ± 0	8 ± 2.5

[a] Values are means of two independent experiments

[b] Not significantly different from control at a 95% confidence level ($t_{tab;0.05;3}$) = 3.18

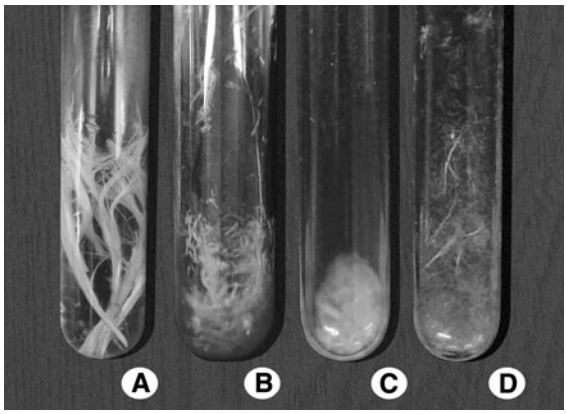

Fig. 1 Feather degradation in control (**A**) and by cultivation of *Aeromonas hydrophila* K12 (**B**), *Chryseobacterium indologenes* A22 (**C**), and *Serratia marcescens* P3 (**D**) on feather broth for 168 h at 30°C

soluble proteins in the culture supernatants (Fig. 2C). The increase in soluble protein concentration in fermentation broth is generally employed as a measurement of keratinous wastes digestion (Szabo et al. 2000). Soluble protein concentration raised and diminished during growth (Fig. 2C), probably due to both the secretion of proteins by the bacterial strains and biomass formation through the utilization of solubilized keratin (Daroit et al. 2009). The results suggest that substrate and metabolite levels in the extracellular environment regulate the secretion of keratinolytic enzymes (Böckle et al. 1995; Riffel et al. 2003b).

The cultivation of the strains on feather meal broth resulted in higher amounts of hydrolysis products (Fig. 2C) when compared to other feather degrading microorganisms, which could be useful for feed production (Sangali and Brandelli 2000; Grazziotin et al. 2007). *S. marcescens* P3, *C. indologenes* A22 and *A. hydrophila* K12 produced 5, 6 and 9 g l^{-1} of soluble protein, respectively, throughout cultivation, whereas *Bacillus* sp. P7, grown on the same medium, produced 4 g l^{-1} (Corrêa et al. 2010). Growth of the Gram-negative *Vibrio* sp. kr2 (Grazziotin et al. 2007) and *Bacillus* sp. P45 (Daroit et al. 2009) on whole feather medium resulted in approximately 2.5 g l^{-1} of soluble protein, and 6.8 g l^{-1} soluble protein was observed during submerged cultivations of *Bacillus* sp. kr16 on whole feathers (Werlang and Brandelli 2005).

Other parameters often associated with keratinolysis by most microorganisms include an increase in alkalinity and thiol groups in the medium (Gupta and Ramnani 2006). The protein degradation releases soluble peptides and amino acids that are subsequently deaminated, releasing ammonium, and thus increasing the pH (Kumar et al. 2008). The resulting increase of pH is typical for microorganisms growing on protein substrates and is an indicative of keratinolytic abilities (Riffel et al. 2003b). In this work, all three bacteria increased the pH of culture medium to 8.0, from a starting pH value of 6.0.

Keratinolysis is not only accomplished by keratinases, but also by disulfide reduction mechanisms, either through enzymes as disulfide reductases, chemical mechanisms (sulfite or thiosulfate), or by a cell-bound redox system (Yamamura et al. 2002; Gupta and Ramnani 2006). The breakdown of disulfide bonds leads to the release of thiol groups and, thus, the concentration of thiol groups tends to increase during cultivation (Riffel et al. 2003b; Kumar et al. 2008), which was not clearly observed in this work. *A. hydrophila* K12 had a peak of 5.6 μg ml^{-1} after 168 h, *C. indologenes* A22 reached 5.0 μg ml^{-1} in 28 h, and the isolate *S. marcescens* P3 produced 3.5 μg ml^{-1} in 120 h of cultivation, all strains showing a high variability of thiol formation. Despite the fact that a pattern could not be observed, thiol groups have been formed, which suggests that keratin degradation by these three isolates is done mainly through proteolysis, and that sulfitolysis mechanisms contribute for keratinolysis to a lesser extent.

Crude proteases from strains A22 and K12 showed neutral and slightly alkaline characteristics, presenting maximum proteolytic activity at pH 7.0 and 8.0, respectively (Fig. 3A). A22 protease kept at least 60% of its proteolytic activity in pH 6.0 to 9.0, while the crude protease from K12 was active in a narrower pH range, showing no activity at pH 6.0 and reduced activity at pH 8.5. Alkaline proteases have been widely described and studied due to their important applications, especially in the detergent industries (Gupta et al. 2002). Most of the keratinolytic proteases described to date show neutral to alkaline characteristics (Brandelli 2008; Fakhfakh et al. 2009), and some keratinases even show optimal activity at extreme alkalophilic (pH > 12) conditions (Mitsuiki et al. 2004). On the other hand, few bacterial keratinases are observed to be optimally active at acidic pH values (Brandelli et al. 2010); for instance,

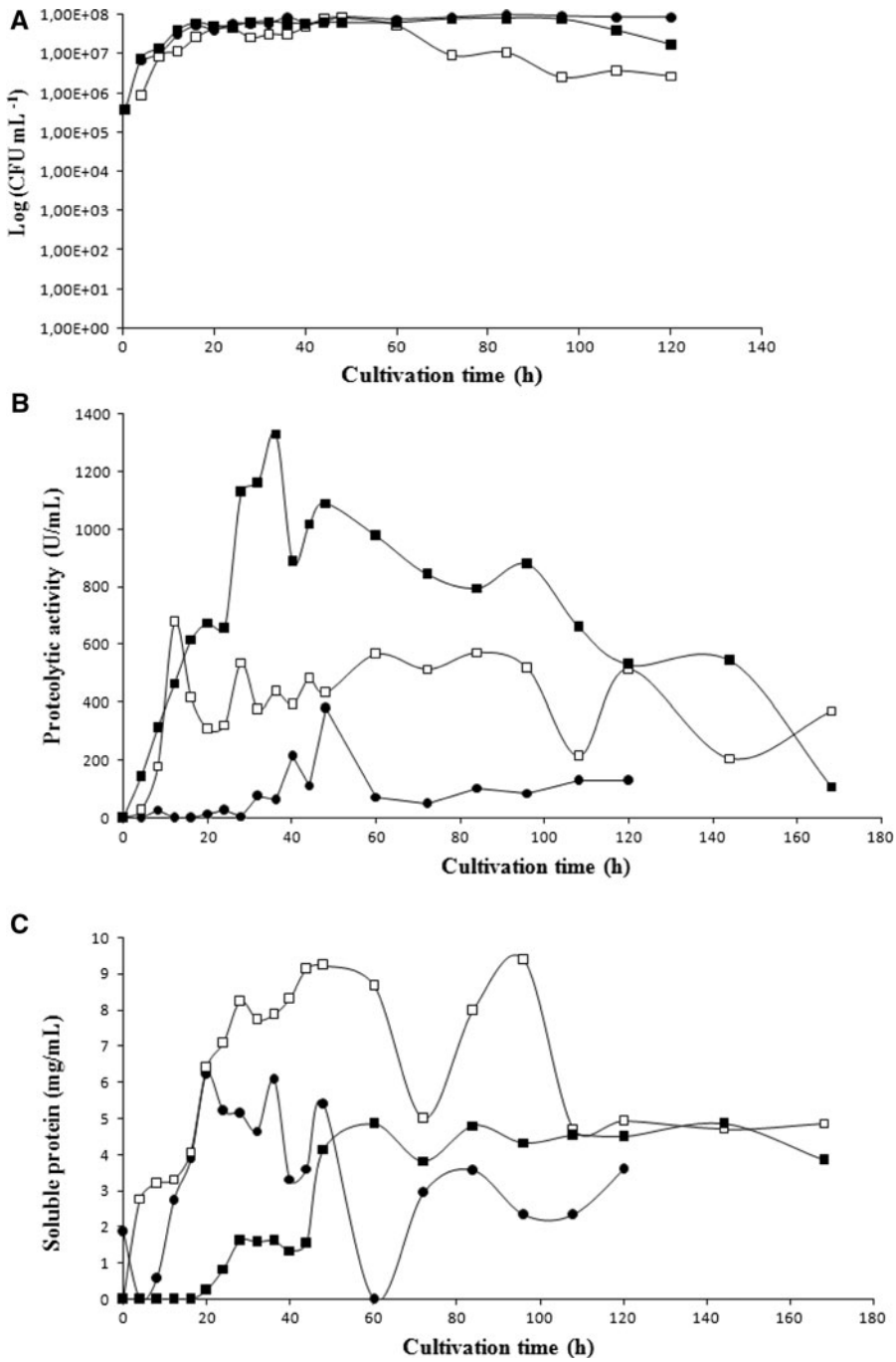

Fig. 2 Growth profile (**A**), protease production (**B**) and soluble protein on culture supernatants (**C**) of three feather-degrading bacteria during cultivation on feather meal broth (FMB) at 30°C, 125 rpm. (*open square*) *Aeromonas hydrophila* K12; (*filled circle*) *Chryseobacterium indologenes* A22; (*filled square*) *Serratia marcescens* P3. Values are the means of duplicate assays from two independent cultivations

a keratinase from *Bacillus subtilis* MTCC (9102) presented maximal activity at pH 6.0 (Balaji et al. 2008). In this sense, P3 protease showed optimal activity at pH values around 6.0–6.5, and maintained at least 40% of its activity at pH values ranging from 5.0 to 8.0. Such character might be interesting for further investigations on potential enzyme applications.

Detection of keratinolytic activity, using the specific substrate azokeratin, was performed at pH values from 6.0 to 8.0. All three crude proteases were active towards azokeratin at the pH tested, with

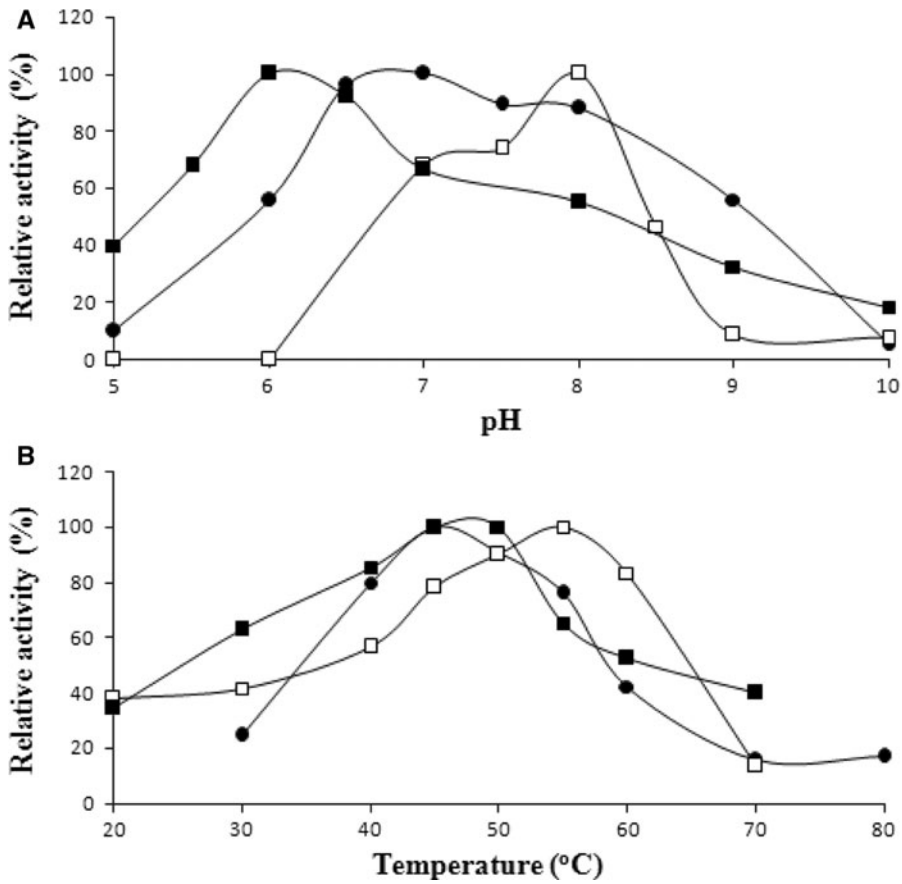

Fig. 3 Effect of pH (**A**) and temperature (**B**) on crude keratinase produced by (*open square*) *Aeromonas hydrophila* K12, (*filled circle*) *Chryseobacterium indologenes* A22, and (*filled square*) *Serratia marcescens* P3 on feather meal broth (FMB). Values are the means of two independent experiments

activity values of 12.16 U ml^{-1} (A22), 12.2 U ml^{-1} (K12) and 27.8 U ml^{-1} (P3) at 50°C. No significant differences were observed among the pH values. The activity of *S. marcescens* P3 was similar to that presented by *Bacillus* sp. P45 (24 U ml^{-1}) (Daroit et al. 2009).

The three enzymes showed maximum activities in the range of 40–60°C (Fig. 3B), which is also well documented for keratinases. The optimum temperature of most keratinases ranges from 30 to 80°C (Gupta and Ramnani 2006), however the temperature optima of 100°C has already been described for thermophilic anaerobic bacteria (Nam et al. 2002). A keratinolytic protease from *Bacillus pumilus* A1 was reported to be active in a wide temperature range, with optimum around 60°C with keratin, and 65°C with casein (Fakhfakh-Zouari et al. 2010). Keratinase A22 had temperature optima of 45°C, being active in a narrow range of temperatures, while K12 had its optimum at 55°C and kept more than 40% of its activity from 20 to 60°C. P3 keratinase was optimally active at 45–50°C, keeping more than 40% of activity from 20 to 70°C. Such wide ranges of proteolytic activity might be useful for industrial applications, since the enzyme does not require a fine temperature regulation for its activity. Moreover, mesophilic microorganisms and enzymes may be advantageous in industrial processing, requiring lower energy inputs than their thermophilic counterparts (Kumar et al. 2008).

Strain A22 produces a feather-degrading protease with some properties similar to other *Chryseobacterium* spp. proteases. An isolate of poultry industry, *Chryseobacterium* sp. kr6 produced a keratinase with an optimal activity at pH 7.5 and 55°C (Riffel et al. 2003b). Three metalloproteases were described for a *C. indologenes* strain TKU014, showing optimal pH of 10.0, pH 7.0–8.0 and pH 8.0–9.0, for proteases P1, P2 and P3, respectively (Wang et al. 2008). Their optimal temperatures were also within the range

commonly described for bacterial keratinases, 30–50, 40 and 40–50°C, respectively. An acidophilic protease has already been described for *C. indologenes* strain Ix9a, presenting optimal activity at pH 6.5 and 50°C (Venter et al. 1999).

Strain P3 also showed a slightly acidophilic feature, which is different from the keratinase already reported for the genus *Serratia*. A serine protease with optimal pH and temperature of 10.0 and 60°C has been described for *Serratia* sp. HPC 1383 (Khardenavis et al. 2009). Thus, *S. marcescens* strain P3 might be producing a novel feather-degrading protease for this genus.

Strain K12 is the first isolate of the genus *Aeromonas* reported to produce a feather-degrading protease. Other metalloproteases had been described in *Aeromonas* spp. For instance, *Aeromonas caviae* Ae6 metalloprotease was optimally active at pH 7.5 (Nakasone et al. 2004), and a gelatinolytic metalloprotease from *A. salmonicida* showed optimal activity at 40°C and pH 7.5 (Arnesen et al. 1995), which are different from that described here for protease K12. The enzyme characteristics of *A. hydrophila* strain K12 resembles that of some *Bacillus* species (Werlang and Brandelli 2005; Corrêa et al. 2010) and is also similar to the Gram-negative *Chryseobacterium* sp. kr6 mentioned above (Riffel et al. 2003b).

By zymogram analyses it was possible to observe a single clear zone for each isolate (Fig. 4), suggesting that a major kertinolytic protease is produced by each strain during growth in feather meal. On contrast, keratinolytic strains of *Streptomyces* and *Bacillus* growing on feather meal showed multiple proteolytic bands (Bressolier et al. 1999; Daroit et al. 2009). The activity of the enzymes was investigated in the presence of different chemicals (Tables 1, 2).

The feather-degrading protease of isolate *A. hydrophila* K12 was strongly inhibited by Hg^{2+}, Cu^{2+} and Fe^{2+}, while Zn^{2+} and Co^{2+} also had an inhibitory effect. A significant increase of 1.3 and 1.5 times on the proteolytic activity was achieved in the presence of Na^{+} and Mg^{2+}, respectively. The detergent SDS stimulated enzyme activity, whereas the protease was unstable in the presence of Triton X-100. Contrarily, SDS is reported to inhibit some microbial keratinases (Riffel et al. 2007; Tatineni et al. 2008). Thus, the positive effect of SDS on the proteolytic activity of strain K12, added to the maintenance of at least 40% activity in temperatures

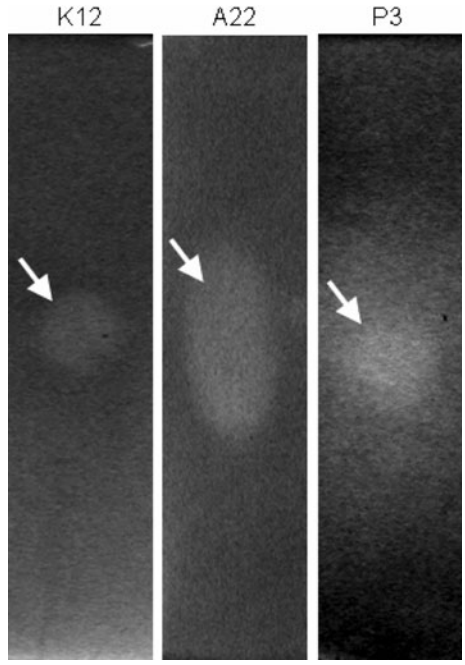

Fig. 4 Casein zymogram analysis of extracellular proteases produced by *Aeromonas hydrophila* K12, *Chryseobacterium indologenes* A22, and *Serratia marcescens* P3 during growth on feather meal broth (FMB). *White arrows* indicate proteolytic bands

such as 20–30°C (Fig. 3B), might suggest its potential use in detergent formulations. Crude protease of strain K12 could be useful for detergent industries due to the current requirement for alkaline proteases active at low washing temperatures, with the aim of maintaining fabric quality and lowering energy demands (Venugopal and Saramma 2006). The organic solvents, in all concentrations, enhanced the residual activity to 323% for DMSO, 342% for isopropanol and 300% for acetonitrile (Table 2).

Chryseobacterium indologenes strain A22 protease was inhibited by Zn^{2+}, Na^{+}, Mn^{2+}, Cu^{2+}, Fe^{2+} and Mg^{2+} while Co^{2+} and Hg^{2+} had a major inhibitory effect (Table 1). These results are similar to *Chryseobacterium* sp. kr6 (Riffel et al. 2003b). When incubated with detergents, strain A22 was slightly inhibited by SDS and inhibited by both concentrations of Triton X-100. Organic solvents also inhibited proteolytic activity (Table 2).

Crude protease from *S. marcescens* strain P3 was totally inhibited only by the ion Hg^{2+}, all other salts resulted in enhancement or in a slight enhancement of proteolytic activity. Contrarily, enzymatic activity of

Serratia sp. strain HPC 1383 was inhibited by Zn^{2+} and slightly inhibited by Co^{2+} and Mn^{2+}, whereas Hg^{2+} had no effect (Khardenavis et al. 2009). The enzyme of strain P3 kept more than 70% of residual activity in the presence of the detergents SDS and Triton X-100. Little inhibition occurred in the presence of 1% (v/v) of organic solvents, while an increase on their concentrations caused a higher inhibitory effect.

Metal ions such as Ca^{2+} and Mg^{2+} could stimulate enzyme activity by acting as salt or ion bridges that stabilize the enzyme in its active conformation and might protect the enzyme against thermal denaturation (Kumar and Takagi 1999; Corrêa et al. 2010). In this study, Ca^{2+} and Mg^{2+} tended to enhance proteolytic activity. The inhibitory effect of Zn^{2+} was observed in strains K12 and A22 and had already been reported for some metalloproteases as a result of bridges formation between zinc monohydroxide ($ZnOH_2$) and catalytic zinc ions at the active site (Riffel et al. 2007). Hg^{2+} has also already been described as a strong enzyme inhibitor, acting as an oxidant agent of thiol groups or even reacting with tryptophan residues and carboxyl groups in amino acids of the enzyme (Daroit et al. 2008). All three enzymes were strongly inhibited by Hg^{2+}, which could be due to presence of important –SH groups at or near the active site.

The stability and/or stimulation of protease activity promoted by organic solvents, especially in the case of strain K12, is a remarkable feature, as such enzyme could be potentially employed in peptide synthesis (Gupta et al. 2002; Wang et al. 2008). Reducing agents are known to enhance keratinolysis through the reduction of disulfide bridges in keratins, allowing a more accessible substrate (Riffel et al. 2003b; Brandelli et al. 2010). In spite of the positive effect of reducing agents on the proteolytic activity of *Chryseobacterium* sp. strain kr6, the three enzymes described here were inhibited by these chemicals. The only positive effect was achieved by 2-mercaptoethanol 0.1% (v/v) on the proteolytic activity of strain K12.

Proteases and, among them, keratinases, are commonly classified through the use of specific substrates and inhibitors (Brandelli et al. 2010). All three proteases described in this work were inhibited by EDTA, a metalloprotease inhibitor, whereas their activity was only slightly affected by PMSF, a serine protease inhibitor (Table 2). The proteolytic activity on the zymogram gel was maintained either with the addition of EDTA or PMSF (data not shown). This might be due to the reversible nature of EDTA inhibition (Riffel et al. 2007) through long hours of incubation, given a false positive result. On the other hand, PMSF inhibition is not reversible and had not inhibited proteolytic activity, corroborating with the azocasein assays. These results suggest that the proteolytic enzymes of *A. hydrophila* K12, *C. indologenes* A22 and *S. marcescens* P3 are metalloproteases (Balaji et al. 2008; Wang et al. 2008).

The increasing demand for new and appropriate biotechnological products for commercial and industrial applications propels the search for novel microbial sources in alternative environments. Most keratinolytic bacteria have been isolated from soils where keratinous materials are deposited (Kaul and Sumbali 1997; Riffel and Brandelli 2006). However, studies in different soil types have shown the isolation of novel keratinolytic microorganisms (Lucas et al. 2003), which may have a biotechnological potential yet to be explored. The novel strains K12, A22 and P3, isolated from Brazilian native soils, may be useful in biotechnological processes involving the biodegradation of keratinous byproducts.

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