DOI: 10.4025/actasciagron.v33i3.6734

Proteolytic activities of bacteria, yeasts and filamentous fungi isolated from coffee fruit (*Coffea arabica* L.)

Mirian Pereira Rodarte[1], Disney Ribeiro Dias[2], Danielle Marques Vilela[3] and Rosane Freitas Schwan[3*]

*[1]Faculdade de Farmácia e Bioquímica, Universidade Federal de Juiz de Fora, Juiz de Fora, Minas Gerais, Brazil. [2]Departamento de Ciência dos Alimentos, Universidade Federal de Lavras, Lavras, Minas Gerais, Brazil. [3]Departamento de Biologia, Setor de Microbiologia, Universidade Federal de Lavras, Cx. Postal 3037, 37200-000, Lavras, Minas Gerais, Brazil. *Author for correspondence. E-mail: rschwan@dbi.ufla.br*

ABSTRACT. One hundred forty-four microorganisms previously isolated from coffee fruit (*Coffea arabica*) were grown on casein agar to evaluate their proteolytic activities. Fifty percent of filamentous fungi, 52.5% of bacteria and 2.6% of yeasts were able to secrete proteases. Positive isolates were further examined in liquid culture for their protease activities by hydrolysis of casein at different pH values (5.0, 7.0 and 9.0) at 30 ºC. *Bacillus megaterium*, *B. subtilis*, *Enterobacter agglomerans*, *Kurthia* sp, *Pseudomonas paucimobilis* and *Tatumella ptyseos* demonstrated the highest proteolytic activities at pH 9.0. One yeast isolate, *Citeromyces matritensis*, had a proteolytic activity of 2.40 U at pH 5.0. *Aspergillus dimorphicus*, *A. ochraceus*, *Fusarium moniliforme*, *F. solani*, *Penicillium fellutanum* and *P. waksmanii* showed the highest activities. Of the bacterial isolates, the highest enzyme activities were observed in *B. subtilis* 333 (27.1 U), *Tatumella ptyseos* (27.0 U) and *B. megaterium* 817 (26.2 U). Of the filamentous fungi, *Aspergillus ochraceus* (48.7 U), *Fusarium moniliforme* 221 (37.5 U) and *F. solani* 359 (37.4 U) had the highest activities at pH 9.0.

Keywords: microbial enzyme, protease, coffee fermentation.

RESUMO. Atividade proteolítica de bactérias, leveduras e fungos filamentosos presentes em grãos de café (*Coffea arabica* L.). Este trabalho teve por objetivos avaliar a capacidade de secreção de proteases extracelulares por 144 microrganismos, previamente isolados de grãos de café (*Coffea arabica*) durante fermentação por via seca, e determinar a atividade das enzimas produzidas. Os microrganismos foram cultivados em ágar-caseína para avaliação da produção de enzimas proteolíticas. Dos 40 isolados de bactéria presentes na amostra, 52,5% apresentaram resultado positivo para o teste. Considerando os 66 isolados de fungos filamentosos, 50% foram capazes de secretar proteases, enquanto que dos 38 isolados de leveduras, apenas 2,6% conseguiram promover a hidrólise da caseína do meio. Os isolados que apresentaram capacidade de secreção de proteases foram, posteriormente, cultivados em meio líquido para a determinação da atividade enzimática em diferentes valores de pH (5,0, 7,0 e 9,0) a 30 ºC. Os isolados *Aspergillus ochraceus* (48,7 U), *Fusarium moniliforme* 221 (37,5 U) e *F. solani* 359 (37,4 U) apresentaram os melhores resultados de atividade enzimática, o que foi verificado em pH 9,0. Também neste pH os isolados *Bacillus subtilis* 333 (27,1 U), *Tatumella ptyseos* (27,0 U) e *B. megaterium* 817 (26,2 U) apresentaram maior valor de atividade. A levedura *Citeromyces matritensis* apresentou atividade em pH 5,0 (2.40 U).

Palavras-chave: enzimas microbianas, protease, fermentação de café.

Introduction

Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, facile culture conditions and ease of cell manipulation. Among microbial enzymes, proteases are the most important for the industry, and constitute approximately 60% of the total industrial enzyme market. These enzymes are used for food processing, pharmaceuticals, leather processing, silver retrieval in the x-ray film industry, industrial waste treatment and as detergent additives (DIAS et al., 2010; HAKI; RAKSHIT, 2003; SUMANTHA et al., 2006).

The increasing industrial use of enzymes has led to the need for more specific proteases that can act on some substrates while not interfering with others, and that have defined characteristics for the processes in which they will be used (DIAS et al. 2008; GUPTA et al., 2002). Proteases are complex enzymes that differ from each other in properties such as substrate specificity, active site and mechanism of action (RAO et al., 1998). Several factors can affect the stability of microbial proteases

in industrial processes, including microorganism strain, production system, purification process, temperature, pH and substrate (KOKA; WEIMER, 2000; STONER et al., 2004).

Microbial proteases are obtained through fermentative processes (POZA et al., 2001; RAO et al., 1998). Diverse microorganisms have been investigated in an effort to obtain new isolates that are good protease producers, and in order to increase productivity and enzyme stability (BEG; GUPTA, 2003). The search for new species is stimulated by protease-producing companies looking for species with particular characteristics that can vary with the growth substrate.

Coffee fruit pulp and mucilage consist primarily of water (76%), protein (10%), fiber (21%) and minerals (8%). The remaining 4% is composed of different types of soluble and insoluble matter (pectin, tannins, reducing and non-reducing sugars, caffeine, chlorogenic and caffeic acid, cellulose, hemicellulose, lignin and amino acids) (SILVA et al., 2000). Coffee pulp and mucilage are natural substrates for the growth of microorganisms. Yeast, bacteria and fungi have been implicated in the processing of coffee fruits. The microbial consortium involved in coffee fermentation has been shown to be able to degrade the components of pulp and mucilage and to induce the biochemical transformations necessary for natural and normal fermentation (SILVA et al., 2000, 2008). Many microorganisms have been isolated from coffee fruit during natural or dry fermentation comprising 44 genera and 64 different species, as reported by Silva et al. (2008).

This paper describes the protease screening of 144 isolates of bacteria, yeasts and filamentous fungi isolated from coffee fruit. The secreted proteolytic activities by selected microorganisms were quantified and characterized at different pH values.

Material and methods

Microorganisms isolation and growth conditions

Bacteria, yeast and filamentous fungi isolates belonging to the Culture Collection of the Laboratory of Microbial Physiology at DBI/UFLA, Lavras, Minas Gerais State, Brazil, which were previously isolated from coffee fruit (*Coffea arabica* L. var. Acaiá) during the fermentation process (SILVA et al., 2008), were screened for proteolytic activity. One hundred forty four isolates were evaluated: 40 bacteria, 38 yeasts and 66 filamentous fungi. The identities of the microbial species are shown on Tables 1-3. Bacterial strains were maintained on nutrient broth (3% meat extract and 5% bacteriological peptone), yeasts on YPD (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) and filamentous fungi on MEA (2% malt extract, 2% glucose and 1% peptone) at -80ºC.

Screening for proteolytic activity

Proteolytic activity was detected by casein hydrolysis on agar plates containing YNB (DIFCO) medium supplemented with 0.5% of casein, 0.5% of glucose, and 2% of agar (w v^{-1}), pH 7.0 (LARSEN et al., 1998). The plates were incubated at 28ºC for 7-8 days. Enzyme activity was indicated by the formation of a clear zone around colonies after precipitation with 1 M HCl solution. A commercial protease solution (Sigma P-4032) at 0.001% (w v^{-1}) was used as the positive control.

Protease production

Bacteria: The culture medium used for bacterial enzyme production was nutrient broth containing 0.01% (w v^{-1}) sodium caseinate (NBC). A volume of 100 mL of NBC was dispensed in Erlenmeyer flasks, which were inoculated with 1.0 mg dry-weight equivalent of organisms from a 24 hours starter culture (100 mL of medium inoculated with part of a single colony and incubated at 28ºC on an orbital shaker at 200 rpm). Cultures were incubated at 28 or 37ºC for 24 hours.

Yeasts: For protease production, YCB (DIFCO) medium supplemented with 0.01% (w v^{-1}) sodium caseinate and 0.1% (w v^{-1}) glucose was used. An Erlenmeyer flask containing 100 mL of culture medium was inoculated with 2.5 mL of culture containing 10^8 cells mL^{-1}. Flasks were incubated under agitation at 120 rpm at 28ºC for 48 hours.

Cultures of bacteria and yeasts were sampled at intervals, and growth (dry weight mL^{-1}) was determined from OD measurements at 600 nm against an appropriate calibration curve. After pelleting the cells by centrifugation at 5000 rpm for 10 min. at 4ºC, samples of the supernatant were used for the determination of proteolytic activity.

Filamentous fungi: The culture medium used for protease production was mineral medium containing (g L^{-1}): $MgSO_4$, 0.52; KCl, 0.52; KH_2PO_4, 1.52; $FeSO_4$ $7H_2O$, 0.01; $ZnSO_4$ $7H_2O$, 0.01; sodium caseinate, 5. A suspension (1 mL) containing 10^8 spores mL^{-1} from 8-day-old colonies was inoculated into Erlenmeyer flasks containing 100 mL of culture medium. Flasks were kept at 28ºC, under agitation at 150 rpm, for 5 days. The mycelial mass was obtained by filtering the contents of each flask and then drying the mass at 60ºC until it reached a constant weight. The flasks were sampled at intervals and the culture supernatant

used as the enzymatic source. All microbial cultivation was performed in triplicate for each selected isolate.

Enzyme activity assay

A proteolytic activity assay, using casein as the substrate, was performed according to described by Ramakrishna and Pandit (1988) with some modifications. Enzyme activity was determined by incubating 250 μL of the culture supernatant with 500 μL 1% (w v^{-1}) casein sodium salt (Sigma 8654) in 50 mM buffer (pH 5.0, 7.0 and 9.0) for two hours at 30°C. The reaction was stopped by the addition of 375 μL 20% (w v^{-1}) trichloroacetic acid. The tubes were placed in an ice bath for 30 minutes and then centrifuged at 5000 x g for 15 min. at 4°C. Proteolytic activity was determined by the absorbance reading of the supernatant at 280 nm versus an appropriate blank. One unit (U) of enzyme activity was defined as the amount of enzyme that, under the assay conditions described, gives rise to an increase of 0.1 unit of absorbance (280 nm) in 60 min. at 30°C (TREMACOLDI; CARMONA, 2005).

Protease activity at different pH values

Protease activity was assayed using different pH values of the substrate solution (5.0, 7.0 and 9.0). The substrate was prepared in three different 50 mM buffers: sodium citrate (pH 5.0), sodium phosphate (pH 7.0) and Tris-HCl (pH 9.0).

Statistical analyses

Analysis of variance (ANOVA) and Tukey tests ($p < 0.05$) of three replicates were done using STATISTICA software version 6.

Results and discussion

Screening for protease activity

One hundred forty four microorganisms were evaluated for their potential to produce and secrete proteases. Of these isolates, 21 (52.5%) out of 40 bacteria strains, one yeast (2.6%) isolate (*Citeromyces matritensis*) out of 38 strains and 33 (50%) out of 66 strains of filamentous fungi were positive for protease secretion when grown on casein agar.

Four different species of *Bacillus* (*B. subtilis*, *B. macerans*, *B. megaterium* and *B. polymyxa*) were selected for quantitative evaluation of protease activity. Other *Bacillus* species (*B. cereus* and *B. fastidiosus*) did not show positive results in the qualitative test (Table 1). The genus *Bacillus* is known for its production of extracellular proteases (BEG; GUPTA, 2003). Several *Bacillus* species isolated from many different environments have been exploited for their alkaline proteases (GUPTA et al., 2002; JOO; CHANG, 2005; KUMAR, 2002).

Two species of *Pseudomonas* (*P. paucimobilis* and *P. putrefaciens*) were also capable of secreting proteases (Table 1). The genus *Pseudomonas* is well known for its production of both alkaline and acid proteases (KOKA; WEIMER, 2000). Oh et al. (2000) reported protease production in *P. aeruginosa* that was active in the pH 7.0-9.0 range, with optimum activity at pH 8.0. Koka and Weimer (2000) reported metalloprotease production in *P. fluorescens*, with an optimum activity at pH 5.0 and incubation temperature of 35°C.

Table 1. Casein hydrolysis by bacterial isolates from coffee fruit.

Species	Isolate	Casein hydrolysis
Acinetobacter sp.	378	+++
	500	++
Arthrobacter sp.	407, 408	-
Bacillus cereus	318, 338, 421	-
Bacillus fastidiosus	1024	-
Bacillus macerans	376	+
Bacillus subtilis	333, 351	+++
Bacillus megaterium	749, 817	+++
	610	-
Bacillus polymyxa	345	++
	379	-
Cedecea lapagei	1082	-
Enterobacter aerogenes	1068	++
Enterobacter agglomerans	1037	+++
	512	-
Enterobacter sakazakii	543	-
Enterobacter cloacae	558	-
Klebsiella oxytoca	317	-
Klebsiella ozanae	322	-
Kurthia sp.	1044, 1095	+++
	1080	++
Proteus mirabilis	1103	-
Providencia rettigeri	1086	++
Pseudomonas paucimobilis	536, 1046	++
Pseudomonas putrefaciens	873	-
Serratia plymutica	719, 816	+++
Serratia rubidea	875, 1047, 1058	-
Tatumella ptyseos	804, 1093	+++
	699	+

-Absence of clear zone around the colony: non-caseinolytic strain. + Presence of clear zone around the colony: caseinolytic strain. Diameter (cm) of proteolysis: +++ (0.8–1); ++ (0.5–0.8); + (0.1–0.5); – (0).

While the genus *Serratia* has not been considered a typical protease producer, Longo et al. (1999) found high levels of protease production in a *Serratia marcescens* isolate when compared to a *B. subtilis* isolate. The two *S. plymutica* isolates whose proteolytic capacities were evaluated in this study showed positive results, but no enzymatic activity was observed in any of the *S. rubidea* isolates (Table 1).

Of 38 yeast isolates, only *Cyteromyces matritensis* showed a small zone of hydrolysis around the yeast colony (Table 2). Extracellular proteases secreted by yeasts have been investigated for industrial use due to the organisms fast growth and ability to grow in diverse substrates (BRAGA et al., 1998; POZA et al., 2001).

However, it has been reported that high proteolytic activity is relatively rare in yeasts. *Kluyveromyces* and *Candida* species have been shown to produce alkaline proteases (KOELSCH et al., 2000; POZA et al., 2001).

The protease secretion of human pathogenic yeasts such as *Candida albicans* have also been studied due to their importance for pathogenicity, and for the development of new candidiasis drugs (KOELSCH et al., 2000). In addition to the extracellular proteases used for industrial purposes, the intracellular proteases produced by yeasts have also been studied due to their metabolic importance. Bolumar et al. (2005) purified and determined the biochemical properties of an intracellular protease of *Debaryomyces hansenii*. This protease was found to be important for nitrogen metabolism that interferes in the physiology and adaptation of this yeast in the production of fermented food.

Table 2. Casein hydrolysis by yeasts isolated from coffee fruit.

Species	Isolate	Casein hydrolysis
Candida sp.	441a, 734, 855	-
Candida fermentati	383, 390, 398	-
Candida membranifaciens	474	-
Candida saitoana	369, 476	-
Cyteromyces matritensis	567	++
Debaryomyces hansenii	640, 641, 642	-
Debaryomyces polymorphus	384, 385, 650	-
Dekkera bruxellensis	955b	-
Pichia anomala	507, 508, 702	-
Pichia burtonii	553, 605	-
Pichia guilliermondii	381, 397, 493	-
Pichia jadinii	933	-
Pichia holstii	441b, 957b	-
Pichia sydowiorum	732, 759	-
Saccharomyces cerevisiae	856	-
Saccharomyces kluyveri	567, 768	-
Stephanoascus smithiae	707, 733, 737	-
Zygoascus hellenicus	366, 368	-

-Absence of clear zone around the colony: non-caseinolytic strain. + Presence of clear zone around the colony: caseinolytic strain. Diameter (cm) of proteolysis: +++ (0.8–1); ++ (0.5–0.8); + (0.1–0.5); – (0).

The small numbers of caseinolytic yeasts obtained from this screen was similar to the results reported by Poza et al. (2001), in which the majority of yeasts tested showed little to no extracellular protease activity. However, there is always the possibility of finding isolates with new characteristics that could be useful for biotechnology applications (POZA et al, 2001).

Bacterial proteases are widely used in industry; however, they demand heavy investments in filtration methodology in order to clear the microorganisms from enzymatic preparations (DIAS et al., 2008). Proteases originating from fungi offer the advantage that the mycelium is easily removed by filtration (BARATA et al., 2002; GERMANO et al., 2003). Among the filamentous fungi isolates, 33 (50%) of the 66 analyzed for casein hydrolysis showed a clear zone around the colonies, characterizing them as proteolytic. *Aspergillus dimorphicus*, *A. ochraceus*, *Fusarium illudens*, *F. lateritium*, *F. solani*, *Paecilomyces* sp., *Penicillium aurantiogriseum* Dierckx, *P. brevicompactum*, *P. citrinum*, *P. chrysogenum*, *P. corylophilum*, *P. crustosum*, *P. expansum*, *P. fellutanum*, *P. implicatum*, *P. roqueforti*, *P. solitum* and *P. waksmanii* showed proteolytic activities (Table 3). According to our data, protease secretion is specific to each fungal isolate and not for related species, as previously discussed in this paper. Efficient protease expression has been described for *Aspergillus* (ABRUNHOSA et al., 2006; KITANO et al., 2002), *Fusarium* (BARATA et al., 2002) and *Penicillium* (GERMANO et al., 2003).

Table 3. Casein hydrolysis by filamentous fungi isolates from coffee fruit.

Species	Isolate	Casein hydrolysis
Aspergillus dimorphicus	670, 671	+++
Aspergillus flavus	516, 517	-
Aspergillus foetidus	663	-
Aspergillus niger	528	-
Aspergillus sydowii	557	-
Aspergillus ochraceus	418	+++
Aspergillus viridicatum	647	-
Fusarium concolor	377, 625	-
Fusarium equiseti	379	-
Fusarium illudens	358	+++
Fusarium lateritium	224	+
	540, 711	-
Fusarium moniliforme	221	+++
	223	-
Fusarium nivale	222	-
Fusarium solani	237, 359	+++
	176	-
Fusarium stilboides	371	-
Fusarium trincictum	480	-
Fusarium xylaroides	369, 370, 490	-
Paecilomyces sp.	321	+
	258	-
Penicillium aurantiogriseum	439, 526	+
Penicillium brevicompactum	243	+++
	479	++
	343	-
Penicillium citrinum	391, 723	+++
	725	++
Penicillium chrysogenum	251, 351, 578	+
Penicillium corylophilum	584	++
	319, 325	-
Penicillium crustosum	261, 388	+
	664	-
Penicillium expansum	356	+
Penicillium fellutanum	309	+++
Penicillium funiculosum	218, 219	-
Penicillium implicatum	660	++
Penicillium janthinellum	345	-
Penicillium minioluteum	215, 216, 256	-
Penicillium purpurogenum	284, 585	-
Penicillium roqueforti	393, 397, 425	+
Penicillium solitum	324, 482, 637	++
Penicillium viridicatum	647	-
Penicillium waksmanii	449, 668	+++

-Absence of clear zone around the colony: non-caseinolytic strain. + Presence of clear zone around the colony: caseinolytic strain. Diameter (cm) of proteolysis: +++ (0.8–1); ++ (0.5–0.8); + (0.1–0.5); – (0).

Quantitative determination of protease activity versus pH

Proteolytic activity in the culture supernatant, which was considered a crude enzymatic extract, was determined after cultivation. Determination of enzymatic activity was performed after incubation at 30ºC at three different pH values (5.0, 7.0 and 9.0).

Of the bacteria tested *Bacillus subtilis* UFLA333, *Tatumella ptyseos* UFLA1093, *B. megaterium* UFLA817, *E. agglomerans* UFLA1037, *P. paucimobilis* UFLA1046 and *Kurthia* sp. UFLA1095 showed high enzymatic activities at different pH values. It is clear that pH is an important variable for protease activity and that this value is specific to each isolate.

The highest proteolytic activity at pH 5.0 was exhibited by *Pseudomonas paucimobilis* UFLA1046 crude extract (26.50 U). *Enterobacter agglomerans*, *Bacillus polymyxa* and *Tatumella ptyseos* 1093 showed activities of 19.51 U, 16.44 U, and 16.34 U, respectively. These values did not differ statistically based on a Tukey test at 5% (Table 4). *P. paucimobilis* UFLA1046 showed the highest acidic proteolytic activity of all microbial isolates. The variation in enzymatic activity produced by *P. paucimobilis* UFLA1046 at the three pH values is found in Table 4. Isolate 1046 showed similar enzyme activities at pH 5.0 and 9.0 (26.50 U and 25.37 U, respectively); however, the proteolytic activity was significantly lower at pH 7.0 (17.02 U) (Table 4). This variation in enzymatic activity according to pH may be due to the production of several proteases by the same isolate (KOKA; WEIMER, 2000). The proteases produced by isolates with enzymatic activity optima at pH 5.0 could be used to coagulate milk proteins for the dairy industry, as debittering agents in cheese and in peptide synthesis (SUMANTHA et al., 2006).

Table 4. Proteolytic activities of bacteria evaluated at three pH values.

Species	Isolate	Proteolytic activity (U)*		
		pH 5.0	pH 7.0	pH 9.0
Acinetobacter sp.	378	10.92 ^{Bd}	12.19 ^{Bc}	21.00 ^{Ab}
	500	1.15 ^{Bh}	5.34 ^{Ad}	7.86 ^{Ac}
Bacillus macerans	376	0.00 ^{Ai}	0.00 ^{Af}	0.73 ^{Aa}
Bacillus megaterium	817	13.94 ^{Bc}	26.22 ^{Aa}	26.18 ^{Aa}
	749	12.90 ^{Bc}	25.30 ^{Aa}	26.08 ^{Aa}
Bacillus subtilis	333	16.40 ^{Bb}	27.19 ^{Aa}	27.12 ^{Aa}
	351	11.56 ^{Bd}	21.11 ^{Ab}	22.17 ^{Ab}
Bacillus polymyxa	345	16.44 ^{Bb}	22.85 ^{Aa}	20.78 ^{Ab}
Enterobacter aerogenes	1068	5.52 ^{Cf}	14.24 ^{Bc}	20.02 ^{Ab}
Enterobacter agglomerans	1037	19.51 ^{Cb}	22.70 ^{Ba}	25.74 ^{Aa}
Kurthia sp.	1044	10.86 ^{Cd}	20.90 ^{Bb}	24.94 ^{Aa}
	1080	7.07 ^{Bc}	18.98 ^{Ab}	19.64 ^{Ab}
	1095	13.02 ^{Bc}	26.67 ^{Aa}	25.98 ^{Aa}
Providencia rettigeri	1086	14.21 ^{Bc}	14.89 ^{Bc}	24.28 ^{Aa}
Pseudomonas paucimobilis	536	5.34 ^{Cf}	14.84 ^{Bc}	20.26 ^{Ab}
	1046	26.50 ^{Aa}	17.02 ^{Bb}	25.37 ^{Aa}
Serratia plymutica	719	11.51 ^{Bd}	23.60 ^{Aa}	22.73 ^{Aa}
	816	6.53 ^{Cf}	19.60 ^{Bb}	24.86 ^{Aa}
Tatumella ptyseos	699	0.31 ^{Ah}	2.15 ^{Ae}	3.14 ^{Ad}
	804	4.12 ^{Cg}	15.51 ^{Bc}	20.52 ^{Ab}
	1093	16.34 ^{Bb}	26.37 ^{Aa}	27.02 ^{Aa}

*Proteolytic unit (U): One unit (U) of enzyme activity was defined as the amount of enzyme that produces an increase of 0.1 unit of absorbance (280 nm) in 60 min. at 30ºC on supernatant. Means followed by the same letters did not differ from one another by the Tukey test ($p < 0.05$). Capital letters compare enzyme activities at different pH values for the same microorganism. Lower case letters compare enzyme activities for each microorganism at the same pH.

Of all the isolates evaluated, the highest proteolytic activities at pH 9.0 were obtained from *B. subtilis* UFLA333 (27.12 U), *Tatumella ptyseos* UFLA1093 (27.02 U) and *Bacillus megaterium* UFLA817 (26.18 U) (Table 4). There were no significant differences in activities between pH 7.0 and 9.0 for these microorganisms; however, there was a significant decrease in enzyme activity at pH 5.0. Proteases produced by *Bacillus* species are by far the most important group of enzymes being industrially exploited. The results presented here are in agreement with the literature, as several *Bacillus* species are known to be good alkaline protease producers and have been widely used in the detergent industry (BEG; GUPTA, 2003; UYAR; BAYSAL, 2004). *Kurthia* sp. UFLA1095 showed a similar enzyme activity to that observed for *B. megaterium* UFLA817. The optimum pH values were 7.0 (26.67 U) and 9.0 (25.98 U), with a decrease in activity at pH 5.0 (13.02 U). The three isolates of *Tatumella ptyseos* showed different protease activities (Table 4). *Tatumella ptyseos* UFLA1093 exhibited an enzymatic activity that was nine times higher than that of the isolate UFLA699. Strain 1093 also showed high levels of protease activity at pH 7.0 and 9.0. This bacterial strain also showed a decrease in proteolytic activity at pH 5.0 (16.34 U), but the loss of activity was lower than those shown by *B. megaterium* and *Kurthia* sp. By evaluating the proteolytic activities of crude extracts of microorganisms at different pH values, we were able to determine a range of proteolytic activity that is specific to each isolate (AZEREDO et al., 2004; GERMANO et al., 2003). *Bacillus subtilis*, *B. megaterium*, *Kurthia* sp. and *Tatumella ptyseos* showed relatively stable enzyme activities when assayed at either pH 9.0 or 7.0 (Table 4). The stability of protease activity in crude extracts at pH 7.0 and 9.0 could be related to neutral or alkaline protease production (POZA et al., 2001). Stable proteolytic activity over a wide range of pH values enables enzymes to be used in several industrial processes (POZA et al., 2001). The optimal pH values of 7.0 and 9.0 indicate that these microorganisms should be studied for protease production in processes that require a neutral or alkaline pH, such as in the detergent industry (GUPTA et al., 2002; ÇALIK et al., 2002).

Concerning the yeasts isolated from coffee beans, *Citeromyces matritensis* was the only one selected for proteolytic activity quantification. This yeast showed proteolytic activity only at pH 5.0 (2.40 U), and its enzymatic activity was low compared to those of the bacterial and filamentous fungi isolates. Braga et al. (1998) studied protease production in different yeast

species at three pH values: 5.0, 7.0, and 9.0. None of them showed activity at pH 9.0, and at pH 7.0 the activity was low. The authors reported that *C. sorboxylosa* (2.6 U) and *Pichia membranaefaciens* (2.8 U) showed proteolytic activity at pH 5.0, similar to the *C. matritensis* activity observed in the present study. Poza et al. (2001) reported a high proteolytic activity secreted by *Candida caseinolytic*, a species isolated from the necrotic tissues of several species of cactus. From these results we hypothize that the varied results obtained from different proteolytic yeasts could be related to the level of acidity of the original substrate.

Proteolytic activities at pH 5.0 secreted by filamentous fungi were observed in *Aspergillus dimorphicus* UFLA671, *Penicillium fellutanum* UFLA309 and *Fusarium solani* UFLA359, with 20.35 U, 18.65 U and 18.34 U of activity, respectively (Table 5). *Aspergillus dimorphicus* UFLA671 secreted more proteolytic enzyme at pH 5.0 (20.35 U) than the other fungi. Strain specificity in protease production was also observed within species of filamentous fungi. The two isolates of *F. solani* (UFLA237 and UFLA359) exhibited different enzyme activities at all pH values tested. The enzyme activity of strain UFLA359 was almost 75% higher than that of strain UFLA237 (Table 5) at pH 9.0, and seven times higher at pH 7.0. Different protease secretion profiles among strains belonging to same species were also found for several species of *Penicillium* (*P. brevicompactum*, *P. citrinum*, *P. solitum* and *P. waksmanii*) (Table 5). For almost all isolates, there was a gradual increase in proteolytic activity with the increase in pH to 7.0 and 9.0 (Table 5). *Fusarium solani* 359 was the best protease producer at pH 7.0 (29.11 U).

The highest proteolytic activities at pH 9.0 were obtained from *Aspergillus ochraceus* UFLA418, *Fusarium moniliforme* UFLA221 and *Fusarium solani* UFLA359, with activities of 48.75 U, 37.51 U and 37.40 U, respectively. No significant activity was detected in *Paecilomyces* at any of the pH values evaluated (Table 5). Fungal proteolytic activity during coffee fermentation could aid in the removal of ochratoxin A. Abrunhosa et al. (2006) described the characterization of a metalloproteinase secreted by a strain of *Aspergillus niger* that had a strong hydrolytic activity at pH 7.5 and removed ochratoxin A from cereals. Alkaline proteases from other *Aspergillus* species have also been reported: from *A. clavatus* by Tremacoldi and Carmona (2005) and from *A. terreus* by Wu et al. (2006).

Thus, pH is an important variable for protease activity that is isolate-specific in filamentous fungi, just as it is in bacteria. In all cases, the optimum pH for proteolytic activity was 9.0, and there was a direct relationship between the decrease in proteolytic activity and of pH values. This instability at lower pH values suggests that these proteases will have higher catalytic activities in industrial processes for which the pH is around 9.0. Our data demonstrate that, regardless of any characteristics in common, protease activities are isolate-specific and therefore depend on the strain used (KOKA; WEIMER, 2000).

Table 5. Proteolytic activities of filamentous fungi evaluated at three pH values.

Species	Isolate	Proteolytic activity (U)*		
		pH 5.0	pH 7.0	pH 9.0
Aspergillus dimorphicus	670	10.58 ^{Bd}	20.90 ^{Ab}	21.18 ^{Ac}
	671	20.35 ^{Ca}	23.53 ^{Bb}	31.74 ^{Ab}
Aspergillus ochraceus	418	9.39 ^{Cd}	13.82 ^{Bd}	48.75 ^{Aa}
Fusarium illudens	358	8.74 ^{Cd}	16.51 ^{Bc}	27.59 ^{Ab}
Fusarium lateritium	224	0.52 ^{Ah}	0.11 ^{Ai}	0.00 ^{Ag}
Fusarium moniliforme	221	11.45 ^{Cc}	25.54 ^{Bb}	37.51 ^{Aa}
Fusarium solani	237	4.09 ^{Bf}	4.09 ^{Bg}	21.48 ^{Ac}
	359	18.34 ^{Cb}	29.11 ^{Ba}	37.40 ^{Aa}
Paecilomyces sp.	321	8.18 ^{Cd}	15.38 ^{Bc}	19.46 ^{Ad}
Penicillium aurantiogriseum	526	0.58 ^{Bh}	7.03 ^{Af}	0.41 ^{Bg}
	439	1.17 ^{Ag}	1.39 ^{Ah}	1.56 ^{Af}
Penicillium brevicompactum	243	13.44 ^{Cc}	20.53 ^{Bb}	27.36 ^{Ab}
	479	0.19 ^{Ah}	1.43 ^{Ah}	0.00 ^{Ag}
Penicillium citrinum	391	8.31 ^{Bd}	19.74 ^{Ac}	20.36 ^{Ac}
	723	10.89 ^{Cd}	20.72 ^{Bb}	29.91 ^{Ab}
	725	6.43 ^{Ce}	8.15 ^{Ae}	7.66 ^{Be}
Penicillium chrysogenum	251	1.22 ^{Bg}	1.73 ^{Bh}	4.58 ^{Ae}
	351	3.06 ^{Af}	1.41 ^{Ch}	2.40 ^{Bf}
	578	0.99 ^{Cg}	1.60 ^{Bh}	2.63 ^{Af}
Penicillium corylophilum	584	9.68 ^{Cd}	14.42 ^{Bd}	21.09 ^{Ac}
Penicillium crustosum	261	0.35 ^{Ah}	0.61 ^{Ai}	0.13 ^{Ag}
	388	0.16 ^{Ah}	0.09 ^{Ai}	0.03 ^{Ag}
Penicillium expansum	356	2.55 ^{Bf}	5.12 ^{Ag}	4.78 ^{Ae}
Penicillium fellutanum	309	18.65 ^{Cb}	28.83 ^{Ba}	31.53 ^{Ab}
Penicillium implicatum	660	11.51 ^{Cc}	14.21 ^{Bd}	22.08 ^{Ac}
Penicillium roqueforti	393	0.00 ^{Ah}	0.26 ^{Ai}	0.35 ^{Af}
	397	0.00 ^{Ah}	0.00 ^{Ai}	0.11 ^{Af}
	425	6.02 ^{Ae}	2.74 ^{Bh}	0.65 ^{Cf}
Penicillium solitum	324	10.58 ^{Cd}	20.83 ^{Bb}	31.06 ^{Ab}
	482	1.42 ^{Ag}	0.85 ^{Ai}	1.97 ^{Af}
	637	0.03 ^{Ah}	0.07 ^{Ai}	0.07 ^{Af}
Penicillium waksmanii	449	5.02 ^{Ce}	9.80 ^{Be}	28.62 ^{Ab}
	668	12.45 ^{Cc}	27.63 ^{Ba}	33.85 ^{Ab}

*Proteolytic unit (U): One unit (U) of enzyme activity was defined as the amount of enzyme that produces an increase of 0.1 unit of absorbance (280 nm) in 60 min. at 30°C on supernatant. Means followed by the same letters did not differ from one another by the Tukey test ($p < 0.05$). Capital letters compare enzyme activities at different pH values for the same microorganism. Lower case letters compare enzyme activities for each microorganism at the same pH.

Bacteria and filamentous fungi are known to be good proteases producers (BEG; GUPTA, 2003; WU et al., 2006). In this study, bacteria and filamentous fungi (52.5 and 50%, respectively) performed significantly better than yeasts (2.6%) on the qualitative casein hydrolysis test. Some genera of microorganisms have already been studied for proteolytic activity, such as *Bacillus*, *Pseudomonas*, *Penicillium*, *Aspergillus* and *Fusarium* (BARATA et al., 2002; KITANO et al., 2002; UYAR; BAYSAL, 2004).

Coffee fruit hosts a great diversity of microbial species (SILVA et al., 2000, 2008). The chemical composition and structure of the coffee fruit change

during the fermentation process. As a result, microorganisms present on these fruits must be able to develop in substrates in which conditions, such as pH and nutrient availability, are frequently changing (MALTA; CHAGAS, 2009; SILVA et al., 2008).

Conclusion

The yeasts, bacteria and filamentous fungi used in this study were previously isolated from coffee fruit and grains during the process of drying and storage. Bacteria isolates, mainly *Bacillus* species presented higher concentrations of proteases activity at both pH values 7.0 and 9.0. Of 38 yeast isolates, only *Cyteromyces matritensis* showed enzyme activity. *Aspergillus dimorphicus* UFLA671, *Penicillium fellutanum* UFLA309 and *Fusarium solani* UFLA359 were able to secret proteases at pH value 5.0. Thus, pH is an important variable for protease activity that is isolate-specific in filamentous fungi, just as it is in bacteria. Our data demonstrate that, regardless of any characteristics in common, protease activities are isolate-specific and therefore depend on the strain used.

Acknowledgements

The authors are grateful to CNPq and Fapemig for financial support and to Capes for a scholarship to the first author.

References

ABRUNHOSA, L.; SANTOS, L.; VENÂNCIO, A. Degradation of Ochratoxin A by proteases and by a crude enzyme of *Aspergillus niger*. **Food Biotechnology**, v. 20, n. 3, p. 231-242, 2006.

AZEREDO, L. A. I.; FREIRE, D. M. G.; SOARES, R. M. A.; LEITE, S. G. F.; COELHO, R. R. R. Production and partial characterization of thermophilic proteases from *Streptomyces sp* isolated from Brazilian cerrado soil. **Enzyme and Microbial Technology**, v. 34, n. 3-4, p. 354-358, 2004.

BARATA, R. A.; ANDRADE, M. H. G.; RODRIGUES, R. D.; CASTRO, I. M. Purification and characterization of an extracellular trypsina-like protease of *Fusarium oxysporum* var. lini. **Journal of Bioscience and Bioengineering**, v. 94, n. 4, p. 304-308, 2002.

BEG, Q. K.; GUPTA, R. Purification and characterization of an oxidation-stable, tiol-dependent serine alkaline protease form *Bacillus mojavensis*. **Enzyme and Microbial Technology**, v. 32, n. 2, p. 294-304, 2003.

BOLUMAR, T.; SANZ, Y.; ARISTOY, M.; CONCEPTION, A.; TOLDRÁ, F. Protease B from *Debaryomyces hansenii*: purification and biochemical properties. **International Journal of Food Microbiology**, v. 98, n. 2, p. 167-177, 2005.

BRAGA, A. A.; MORAIS, P. B.; LINARDI, V. R. Screening of yeast from Brazilian Amazon Rain Forest for extracellular proteinases production. **Systematic and Applied Microbiology**, v. 21, n. 3, p. 353-359, 1998.

ÇALIK, P.; BILIR, E.; ÇALIK, G.; OZDAMAR, T. H. Influence of pH conditions on metabolic regulations in serine alkaline protease production by *Bacillus licheniformis*. **Enzyme and Microbial Technology**, v. 31, n. 5, p. 685-697, 2002.

DIAS, D. R.; ABREU, C. M. P.; SILVESTRE, M. P. C; SCHWAN, R. F. In vitro protein digestibility of enzymatically pre-treated bean (*Phaseolus vulgaris* L.) flour using commercial protease and *Bacillus* sp. protease. **Ciência e Tecnologia de Alimentos**, v. 30, n. 1, p. 94-99, 2010.

DIAS, D. R.; VILELA, D. M.; SILVESTRE, M. P. C.; SCHWAN, R. F. Alkaline protease from *Bacillus* sp. isolated from coffee bean grown on cheese whey. **World Journal of Microbiology and Biotechnology**, v. 24, n. 10, p. 2027-2034, 2008.

GERMANO, S.; PANDEY, A.; OSAKU, C. A.; ROCHA, S. N.; SOCCOL, C. R. Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation. **Enzyme and Microbial Technology**, v. 32, n. 2, p. 246-251, 2003.

GUPTA, R.; BEG, Q. K.; LORENZ, P. Bacterial alkaline protease: molecular approaches and industrial applications. **Applied Microbiology and Biotechnology**, v. 59, n. 1, p. 15-32, 2002.

HAKI, G. D.; RAKSHIT, S. K. Developments in industrially important thermostable enzymes: a review. **Bioresource Technology**, v. 89, n. 1, p. 17-34, 2003.

JOO, H. S.; CHANG, C. S. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: optimization and some properties. **Process Biochemistry**, v. 40, n. 3-4, p. 1263-1270, 2005.

KITANO, H.; KATAOKA, K.; FURUKAWA, K.; HARA, S. Specific expression and temperature-dependent expression of the acid protease-encoding gene (pep A) in *Aspergillus oryzae* in solid state culture (rice-koji). **Journal of Bioscience and Bioengineering**, v. 93, n. 6, p. 563-567, 2002.

KOELSCH, G.; TANG, J.; LOY, J. A.; MONOD, M.; JACKSON, K.; FOUNDLING, X. L. Enzymic characteristics of secreted aspartic proteases of *Candida albicans.* **Biochimica et Biophysica Acta (BBA) - Protein Structure and MolecularEnzymology**, v. 1480, n. 1-2, p. 117-131, 2000.

KOKA, R.; WEIMER, B. C. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. **Journal of Applied Microbiology**, v. 89, n. 2, p. 280-288, 2000.

KUMAR, C. G. Purification and characterization of a thermostable alkaline protease from alkalophilic *Bacillus pumilus*. **Letters in Applied Microbiology**, v. 34, n. 1, p. 13-17, 2002.

LARSEN, M. D.; KRISTIANSEN, K. R.; HANSEN, T. K. Characterization of the proteolytic activity of starter cultures of *Penicillium roqueforti* for production of blue veined cheeses. **International Journal of Food Microbiology**, v. 43, n. 3, p. 215-221, 1998.

LONGO, M. A.; NOVELLA, I. S.; GARCIA, L. A.; DIAZ, M. Comparison of *Bacillus subtilis* and *Serratia*

marcescens as proteases producer under operating conditions. **Journal of Bioscience and Bioengineering**, v. 88, n. 1, p. 35-40, 1999.

MALTA, M. R.; CHAGAS, S. J. R. Avaliação de compostos não-voláteis em diferentes cultivares de cafeeiro produzidas na região Sul de Minas Gerais. **Acta Scientiarum. Agronomy**, v. 31, n. 1, p. 57-61, 2009.

OH, Y. S.; SHIH, I. L.; TZENG, Y. M.; WANG, S. L. Protease produced by *Pseudomonas aeruginosa* K-187 and its application in the deproteinization of shrimp and crab shell wastes. **Enzyme and Microbial Technology**, v. 27, n. 1, p. 3-10, 2000.

POZA, M.; MIGUEL, T.; SIERO, C.; VILLA, T. G. Characterization of a broad pH range protease of *Candida caseinolytica*. **Journal of Applied Microbiology**, v. 91, n. 5, p. 916-921, 2001.

RAMAKRISHNA, T.; PANDIT, M. W. Self-association of α-chymotrypsin: effect of amino acids. **Journal of Biosciences**, v. 13, n. 3, p. 215-222, 1988.

RAO, M. B.; TANKSALE, A. M.; GHATGE, M. S.; DESHPANDE, V. V. Molecular and biotechnological aspects of microbial proteases. **Microbiology and Molecular Biology Reviews**, v. 62, n. 3, p. 597-635, 1998.

SILVA, C. F.; SCHWAN, R. F.; DIAS, E. S.; WHEALS, A. E. Microbial diversity during maturation and natural processing of coffee cherries of *Coffea arabica* L in Brazil. **International Journal of Food Microbiology**, v. 60, n. 2-3, p. 251-260, 2000.

SILVA, C. F.; BATISTA, L. R.; ABREU, L. M.; DIAS, E. S.; SCHWAN, R. F. Succession of bacterial and fungal communities during natural coffee (*Coffea arabica*) fermentation. **Food Microbiology**, v. 25, n. 8, p. 951-957, 2008.

STONER, M. R.; DALE, D. A.; GUALFETTI, P. J.; BECKER, T.; MANNING, M. C.; CARPENTER, J. F.; RANDOLPH, T. W. Protease autolysis in heavy-duty liquid detergent formulations: effects of thermodynamic stabilizers and protease inhibitors. **Enzyme and Microbial Technology**, v. 34, n. 2, p. 114-125, 2004.

SUMANTHA, A.; LARROCHE, C.; PANDEY, A. Microbiology and industrial biotechnology of food-grade proteases: a perspective. **Food Technology and Biotechnology**, v. 44, n. 2, p. 211-220, 2006.

TREMACOLDI, C. R.; CARMONA, E. C. Production of extracellular alkaline proteases by *Aspergillus clavatus*. World **Journal of Microbiology and Biotechnology**, v. 21, n. 2, p. 169-172, 2005.

UYAR, F.; BAYSAL, Z. Production and optimization of process parameters for alkaline protease production by a newly isolated *Bacillus* sp. under solid state fermentation. **Process Biochemistry**, v. 39, n. 12, p. 1893-1898, 2004.

WU, T. Y.; MOHAMMAD, A. W.; JAHIM, J. M.; ANUAR, N. Investigations on protease production by a wild-type *Aspergillus terreus* strain using diluted retentate of pre-filtered palm oil mill effluent (POME) as substrate. **Enzyme and Microbial Technology**, v. 39, n. 6, p. 1223-1229, 2006.

Received on March 24, 2009.
Accepted on July 13, 2009.