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ORIGINAL PAPER

Alkaline protease from *Bacillus sp.* isolated from coffee bean grown on cheese whey

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Abstract Two strains of *Bacillus*, one from a culture collection (*B. subtilis* ATCC 6633) and a wild type (*Bacillus sp.* UFLA 817CF) isolated during coffee fermentation in the south of Minas Gerais, Brazil, were evaluated in relation to secretion of alkaline proteases. The strains were grown on nutrient broth, nutrient broth with sodium caseinate and nutrient broth with three different concentrations of cheese whey powder for 72 h. Samples were collected at 24-h intervals to evaluate the proteolytic activity, protein content and cell population. Maximum protease activity was observed after 24-h growth for both the microorganisms, a period that coincided with the end of the exponential phase. The specific activity values were, respectively, 839.8 U/mg for *B. subtilis* ATCC 6633 and 975.9 U/mg for *Bacillus sp.* UFLA 817CF. The 60% saturation presented the best results for specific protease activity in all the growth culture media tested with *B. sp.* UFLA 817CF. *Bacillus sp.* UFLA 817CF showed highest enzymatic activity at pH 9.0 and 40°C in the three culture media tested. The protease obtained from culture of the wild *Bacillus* strain presented stability at pH 7.0 and considerable heat stability at 40°C and 50°C, and could be an alternative for the industry to utilize cheese whey to produce proteolytic enzymes.

Keywords Alkaline protease · *Bacillus* · Proteolytic enzymes · Cheese whey powder · Microbial enzyme

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Introduction

Proteolytic enzymes can be secreted across the cytoplasmic membrane and cell wall. These extracellular enzymes are synthesized by diverse groups of microorganisms, including fungi, yeasts and bacteria. Among extracellular alkaline proteases, those from *Bacillus* species have wide use and importance in several industrial sectors, such as the food (dairy, Razak et al. (1994); obtaining of protein hydrolysates, Carreira et al. (2004) and Soares et al. (2007)), leather (Takami et al. 1992; Giongo et al. 2007), detergent sectors (Ito et al. 1998; Hadj-Ali et al. 2007) and in the synthesis of biologically active peptides (Kumar and Bhalla 2005). The genus *Bacillus* is one of the most important extracellular protease producers (Harwood 1992; Sarvas et al. 2004). About 75% of world sales for industrial enzymes application are hydrolytic enzymes, of which proteolytic enzymes correspond to 60%. Alkaline proteases account for approximately 25% of the world enzyme market. The majority of the commercially available alkaline proteases are derived from *Bacillus* strains, which are recognized as important sources of this enzyme because their ability to secrete large amounts of proteinases showing high activity and stability. These proteases secreted by

Bacillus sp. show activity over a wide range of pH (7.0–11.0) and temperature (30–60°C) (Horikoshi 1999; Joo et al. 2002; Gupta et al. 2002a).

Natural and spontaneous fermentation is a rich source for new microorganisms with potential industrial or commercial value. The genus *Bacillus* is a taxon with broad distribution in diverse environments and has been the subject of attention for application of its members in biotechnology. These bacteria are relatively easy to isolate and they are also able to grow in both complex and synthetic media (Johnvesly and Naik 2001). It is known that the amount of enzyme produced varies greatly with strain and growth conditions, in which the cultivation medium is an important parameter. In order to obtain high yields of protease it is necessary to plan and test media and cultivation conditions for wild strains (Anwar and Saleemuddin 1998). Due to the potential uses of proteases, there is a need for the search of new strains of bacteria that produce proteolytic enzymes with novel properties and the development of low cost media. According to Hinman (1994), almost 40% of costs for the enzyme production are due to the cost of growth substrate. The use of complex medium for protease production by *Bacillus* has been reported in many scientific works. Such medium can be derived from industrial effluents or alternative sources as substrate for growth of *Bacillus*, including nug meal (Gessesse 1997), shrimp and crab shell powder (Yang et al. 2000), fish flour (Ellouz et al. 2001), amaranth seed meal (Pastor et al. 2001), soybean meal (Joo et al. 2002; Joo and Chang 2005), chicken feather (Gessesse et al. 2003) and arrowroot (Kumar and Parrack 2003). Cheese whey is a byproduct obtained from cheese manufacture, as supernatant from the precipitation of the casein in milk. It is a complex substrate, rich in proteins and carbohydrates (lactose) and contains considerable concentrations of vitamins and minerals. Cheese whey corresponds to about 90% of the milk volume, containing 20% of the soluble proteins and 50% of the other nutrients (Siso 1996). In Brazil cheese whey is still an effluent of concern. It is estimated that only 15% of the cheese whey produced annually, about 70.000 tons, is used in other industrial sectors (Capitani et al. 2005). Cheese whey has been used for several purposes, mainly in the food industry (Wit 1998). Some researchers have used cheese whey as substrate for microorganism growth for biotechnology purposes to obtain yeast cells, enzymes and polysaccharides (Champagne et al. 1990; Kawahara and Obata 1998; Kumar et al. 1999; Ghaly et al. 2003), and this is an alternative for its use. The objective of this study was to investigate the production of extracellular alkaline protease from *Bacillus sp.* UFLA 817CF isolated from coffee beans with significant protease activity, grown in culture medium with different amounts of cheese whey powder added as enzymatic inducer and the partial characterization of the enzyme.

Materials and methods

Growth of microorganisms and enzyme production

The two strains of bacilli tested were *Bacillus subtilis* ATCC 6633, acquired from the Tropical Culture Collection at the André Tosello Foundation, Campinas, Brazil, and *Bacillus* sp. UFLA 817CF, isolated from coffee beans (Silva et al. 2000) that is part of the microorganism collection at the Microbial Physiology Laboratory of the Biology Department at the Federal University of Lavras, Minas Gerais, Brazil. The pure cultures were kept in nutrient agar at 4°C. For inoculation into the media to be tested, the microorganisms were previously incubated in nutrient broth for 24 h at 28°C. Then 3 ml of this culture with 10^8 c.f.u./ml population were inoculated into 500-ml Erlenmeyer flasks containing 300 ml of the culture medium to produce proteolytic enzymes. The flasks were incubated in an orbital incubator at 28°C and 150 rev/min. The culture media used were NB (nutrient broth, Difco), NBC (NB plus 0.01% w/v sodium caseinate, Sigma-Aldrich), NBW_1 (NB plus 0.01% w/v cheese whey powder, Prolacteos Dairy Industry, Contagem, MG, Brazil), NBW_2 (NB plus 0.1% w/v cheese whey powder) and NBW_3 (NB plus 1% w/v cheese whey powder), with initial pH 7.0. Samples of 10 ml were collected at 24, 48 and 72 h to determine the total proteins and proteolytic activity (supernatant obtained from centrifuging the sample at 6,000×*g* a 4°C for 15 min). The determinations were made in triplicate, and the results presented as the mean obtained. The Scott-Knott test was used with 5% significance to assess statistical differences in the protease production in the different culture media tested.

Protein quantification

Protein concentrations in the supernatant previously cited were measured spectrophotometrically at 595 nm by the Bradford dye-binding method, using bovine serum albumin (BSA, Merck, Germany) as a standard and Bradford reagent from Sigma-Aldrich.

Protease assay

The alkaline proteolytic activity was determined by hydrolysis of casein. The culture broth was harvested by centrifugation (Sigma AK-15) at 6,000×*g* and 4°C for 15 min. Aliquots of 500 µl of 0.5% (w/v) casein solution (Sigma-Aldrich) in Tris-HCl buffer (50 mM, pH 9.0) was

mixed with 250 µl of diluted supernatant and hydrolysed under 37°C, pH 9.0 for 30 min. The reaction was stopped by adding 500 µl of 10 % (w/v) trichloroacetic acid solution (Merck) and the mixture was centrifuged at 15000×*g* for 15 min at 4°C, and absorbance of the supernatant was measured at 275 *η*m with a UV–VIS spectrophotometer (Shimadzu UV-1601 PC). One unit protease (U/ml) activity was defined as the activity that liberates 1 µg of tyrosine per minute (µg Tyr × ml^{-1} min^{-1}) under described conditions (Çalik et al. 2002; Kumar 2002).

Enzyme precipitation

The supernatant from the crude extracts obtained from the different culture media tested were precipitated with ammonium sulfate at concentrations of 40, 60 and 80% saturation (Scopes 1994). The tests were carried out in quadruplicate. After adding ammonium sulfate, the sample was carefully homogenized and chilled at 4°C for 2 h, before centrifuging at 6,000×*g* for 15 min at 4°C. The precipitate was resuspended in a four volumes of 50 mM Tris-HCl buffer, pH 9.0, supplemented with 5 mM $CaCl_2$ and transferred to dialysis membranes (cut-off 18 kDa). The membranes were immersed in 50 volumes of the same buffer and dialysis occurred for 24 h at 4°C, with buffer solution renovation every 8 h. Enzymatic activity, stability at different pH values and temperature and electrophoresis in polyacrylamide gel tests were carried out on the recovered precipitates.

Effect of temperature and pH on enzymatic activity

The enzymatic fractions obtained from saturation with ammonium sulfate at 60%, in the NB, NBC, NBW_1 culture media, were incubated as different temperatures (30, 40, 50, 60 and 70°C) and pH, using the citrate 100 mM (3.0 and 5.0), phosphate 50 mM (7.0), Tris-HCl 50 mM (9.0) and Glycine-NaOH 100 mM (11.0) buffers to assess the enzymatic activity. The optimum temperature was determined by verifying the protease activity on casein at pH 9.0.

To assess the ideal pH, a 0.5% (w/v) casein solution was prepared in the buffers and pH values above and incubated at 37°C to later quantify the enzymatic activity. The heat stability at different pH was verified in the 60% saturation fraction of the NB culture medium in both the bacilli. In the heat stability study, the enzyme was pre-incubated, without adding substrate, at 40, 50 and 60°C for 120 min. Samples were removed to at 30, 60, 90 and 120 min to determine the residual proteolytic activity on casein at pH 9.0. The stability of the enzyme at different pH values was verified by incubating the enzyme, without substrate, at the pH values reported above, for 24 h at 40°C, before determining the residual proteolytic activity at 37°C, pH 9.0 (Kumar 2002; Tremacoldi and Carmona 2005). The electrophoretic profile of the enzymatic fractions precipitated in ammonium sulfate was verified by SDS-PAGE, using methodology according to Laemmli (1970). Separation gel at 12.5% (SDS 10%) was used in Tris-HCl pH 8.8 and 5% gel concentration. The crude extract was previously freeze dried and 25 µl of the treated sample were added to the gel. The electrophoretic run occurred for 4 h with a 20 mA current. Coomassie Brilliant Blue R-250 0.1% (w/v) was used for staining, diluted in a solution of methanol/acetic acid/water (5:1:5, by vol.).

Results and discussion

Proteolytic activity in the crude extract

Two *Bacillus* strains were compared regarding protease production when submitted to growth in five culture media: nutritient broth, as base culture medium, and four different supplementations, using, as protease synthesis inducers, 0.01% sodium caseinate (w/v) and cheese whey powder at concentrations of 0.01%, 0.1% and 1.0% (w/v). The two *Bacillus* strains tested maintained populations varying between 1.1 and 7.2 × 10^7 c.f.u./ml for *Bacillus sp.* UFLA 817CF and between 8.8 and 25.0 × 10^7 c.f.u./ml for the *B. subtilis* ATCC 6633 culture (Fig. 1) during the 72 h that the experiment was carried out in the culture media tested.

The *Bacillus* sp. UFLA 817CF populations decreased in function of time in the NB and NBC medium while in the NBW_1, NBW_2 and NBW_3 media the population increased after 72 h, reaching the maximum growth value of 7.2 × 10^7 c.f.u/ml for the NBW_3 medium (Fig. 1a). Decrease in population as a function of time was also observed in the *B. subtilis* ATCC 6633 in most of the culture media, except for the NBC culture medium, where the maximum population, 17.6 × 10^7 c.f.u./ml, was observed at 48 h (Fig. 1b).

Alkaline protease activity varied with incubation time and the type of culture media in which the *Bacillus* isolates were cultivated (Fig. 1a and 1b). Within the culture media tested, NB was shown to be the best producing source of alkaline protease. This culture medium was reported by Hanson et al. (1964) for enzyme production by *B. subtilis*. Maximum protease activity was observed after 24 h growth for both the microorganisms (Fig. 1a and 1b), a period that coincided with the end of the exponential phase (data not shown). The specific activity values were, respectively, 839.8 U/mg for *B. subtilis* ATCC 6633 and 975.9 U/mg for *B. sp.* UFLA 817CF. Mehrotra et al. (1999) and Patel et al. (2006) studied alkaline protease production by *Bacillus* species isolated from soil and seawater respectively also

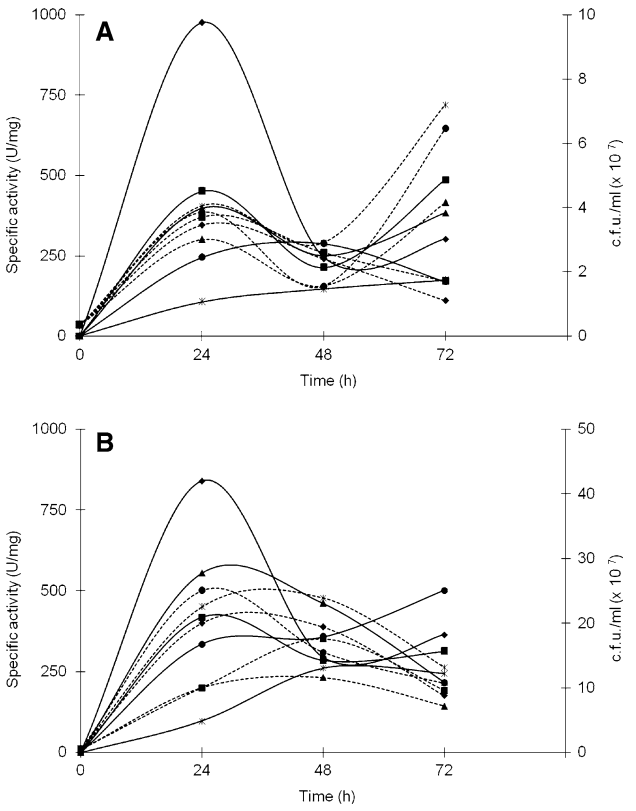

Fig. 1 Specific proteolytic activity (continuous line) and growth (discontinuous line) by *Bacillus sp.* UFLA 817CF (**a**) and *Bacillus subtilis* ATCC 6633 (**b**) cultivated for 72 h in nutrient broth (NB ♦), nutrient broth plus 0.01% (w/v) sodium caseinate (NBC ■), nutrient broth plus 0.01% (w/v) cheese whey powder (NBW_1 ▲), nutrient broth plus 0.1% (w/v) cheese whey powder (NBW_2 ●) and nutrient broth plus 1% (w/v) cheese whey powder (NBW_3 *)

obtained best results after 24 h of cultivation. The enzymatic activity observed in the NB, NBC and NBW_1 media decreased after 48 h for the two isolates assessed in this study. However, an increase in the enzyme activity was observed when the microorganisms were cultivated in the NBW_2 and NBW_3 culture media. The increase in the activity in these two culture media after 48 h may be associated to the catabolic repression in the first 24 h of culture, because the culture media contained a higher concentration of cheese whey powder, which is a material rich in carbohydrates (75% w/w lactose) and proteins (13% w/w). Excess protein may stimulate protein synthesis regulators of the GlnR (global nitrogen regulatory protein) type that repress the metabolic activity of *B. subtilis* (Fisher 1999). Regarding lactose, data in the literature states that *B. subtilis* cannot use it as a single carbon source, due to the deficiency in the transport and degradation systems. However, it has a lacA gene that can codify β-galactosidase in function of stress conditions (Stülke and Hillen 2000). This nutritional stress may justify the low enzymatic activity observed in the NBW_3 medium, containing 1% cheese whey powder (Fig. 1a,b). The proteolytic activity of the standard strain was less than that of the wild strain, 97.2 U/mg and 105.6 U/mg, respectively. The growth pattern of the *Bacillus sp.* UFLA 817CF strain in the NBW_1, NBW_2 and NBW_3 culture media showed that the adaptation phase to the conditions of the culture medium also interfered in the population. Kumar et al. (1999) observed that a concentration of 1% of cheese whey powder supplemented with organic and inorganic carbon sources and organic nitrogen sources presented a better response regarding alkaline protease activity. The presence of sodium caseinate and the cheese whey proteins used in this study did not stimulate an increase in the proteolytic activity. This result was also reported by Patel et al. (2006), when they used 0.5% (w/v) casitone (partially hydrolysed casein) and by Joo and Chang (2005) who added 1% casein as supplement in a chemically defined culture medium in *Bacillus* sp. culture. In a previous study, these authors reported that the addition of 1% casein to supplement TSB medium, favored alkaline protease activity of a new *Bacillus* species by 30%, called *B. horikoshii* (Joo et al. 2002). For the standard *B. subtilis* ATCC 6633 strain, the NBW_1 culture medium (Fig. 1b) induced greater enzymatic activity than the NBC culture medium (used as standard inducer) in 24 h, respectively, 555.1 U/mg and 417.7 U/mg. There were no significant differences for the wild strain (Fig. 1a), in the same time, in the proteolytic activity between the NBW_1 and NBC culture media.

Enzyme assay in ammonium sulfate fractions

The supernatants obtained in the five media tested, from both the cultured microorganisms, were precipitated with ammonium sulfate at 40%, 60% and 80% saturation. To culture *Bacillus sp.* UFLA 817CF, the 60% saturation presented the best results from protease specific activity in all the growth culture media tested. Kim and Kim (2005) and Zvidzai and Zvauya (2001) also reported a greater enzymatic activity in the 60% ammonium sulfate fraction when they purified *B. subtilis* protease. The maximum enzymatic activity for *Bacillus sp.* UFLA 817CF was observed in the precipitation obtained from the culture in NB culture medium (926.4 U/mg), while the lowest activity (437.4 U/mg) was reported in the NBW_3 medium (Table 1). This result, nevertheless, exceeded the best results obtained for *B. subtilis* ATCC 6633 culture at the three saturations tested (Table 1). It was also observed for *Bacillus sp.* UFLA 817CF, that the NB culture medium also presented greater proteolytic activity at the 40% and 80% fractions compared to the other culture media tested, 692.7 and 557.7 U/mg, respectively. The fraction obtained with 60% saturation also presented best activity for the *Bacillus subtilis* ATCC6633 strain in most of the culture

Table 1 Partial purification of alkaline protease from *Bacillus subtilis* ATCC 6633 and from *Bacillus sp*. UFLA 817CF

$(NH4)_2SO_4$ saturation	40%			60%			80%		
Media	Total enzyme activity (U/ml)	Total proteins (mg/ml)	Specific activity (U/mg)	Total enzyme activity (U/ml)	Total proteins (mg/ml)	Specific activity (U/mg)	Total enzyme activity (U/ml)	Total proteins (mg/ml)	Specific activity (U/mg)
Bacillus subtilis ATCC 6633									
NB	59.03	0.258	228.7	111.15	0.414	268.7	78.25	0.552	141.7
NBC	55.21	0.281	196.5	93.12	0.440	211.7	76.76	0.629	122.0
NBW_1	73.32	0.315	232.5	93.96	0.562	167.1	85.58	0.661	129.6
NBW_2	91.98	0.406	226.8	88.40	0.294	300.5	90.27	0.713	126.7
NBW_3	105.46	0.608	173.6	93.08	0.314	296.0	99.94	0.758	131.9
Bacillus sp. UFLA 817CF (wild type)									
NB	80.71	0.087	692.7	89.18	0.096	926.4	95.53	0.128	557.7
NBC	84.27	0.152	415.3	87.98	0.159	553.4	99.19	0.162	459.4
NBW_1	90.34	0.170	398.6	92.10	0.185	497.2	102.85	0.211	365.4
NBW_2	94.00	0.188	374.6	93.08	0.200	465.2	106.51	0.239	333.6
NBW_3	91.59	0.206	334.2	96.78	0.221	437.4	113.74	0.289	295.6

Data presents average of four replicates

media tested (Table 1), except for the NBW_1 medium, where the highest proteolytic activity value, 232.5 U/mg, was observed in the 40% fraction. In the 60% saturation fractions, the greatest enzymatic activity values were observed in the NBW_2 (300.5 U/mg) and NBW_3 (296.0 U/mg) culture media. All samples from 80% saturation presented the lowest proteolytic activity values for both the microorganisms.

Effect of temperature and pH on enzyme activity and stability

The 60% saturation fractions with ammonium sulfate in the NB, NBC and NBW_1 culture media of both the microorganisms were tested for optimal pH and temperature for enzymatic activity. Relative activity was used to compare the cultures, regarding the best pH and temperature, taking as 100% the activity of *B. sp*. UFLA 817CF in the NB culture medium (Table 1). Figure 2a shows the results of the enzymatic activity as a function of variation in pH, for both the microorganisms, in the three culture media that best represented specific activity. *Bacillus sp* UFLA 817CF presented greater enzymatic activity at pH 9.0 in the three culture media tested. In the fraction obtained from the NB culture medium, with greater activity, this strain maintained about 80% activity at pH 7.0 and over 60% at pH 11.0. This optimum activity at values close to 9.0 is characteristic of alkaline proteases (Rao et al. 1998; Kumar and Takagi 1999). Similar values were reported by Gessesse et al. (2003) and Giongo et al. (2007), when culturing *Bacillus* isolates and by Tremacoldi et al. (2007) when culturing *Aspergillus clavatus*. The highest proteolytic activity at pH 9.0 was observed when the fractions precipitated with ammonium sulfate were incubated at 40°C for both the microorganisms in the three culture media tested (Fig. 2b). Optimum alkaline protease activity at 40°C was also detected by other authors when using *Bacillus sp*. strains (Singh et al. 2001; Joo et al. 2002). Stability was observed in the proteolytic activity in both the bacilli at the different pH values, and the best results were observed in *Bacillus sp.* UFLA 817CF culture. The residual activity of this strain, in the three culture media tested was close to 50% of the maximum at pH 5.0. At the values of pH 7.0 and 11.0, about 70% of residual activity was detected after incubation for 30 min at 37°C (Fig. 3a). Greater variation in the culture of *B. subtilis* ATCC6633 was observed in the proteolytic activity in the culture media assessed. In this case the best performance was observed in the NBW_1 culture medium, where about 75%, 83% and 78% of the activity was maintained at pH 5.0, 7.0 and 11.0, respectively (Fig. 3b). The temperature of 40°C was observed as optimum for both the microorganisms in the three culture media (Fig. 4a and b). The residual proteolytic activity of *Bacillus sp.* UFLA 817CF cultivated in NBC culture medium was maintained at temperatures of 50, 60 and 70°C at values 95%, 88% and 77%, respectively, compared to the maximum activity at 40°C (503.4 U/mg) (Fig. 4a). Similar performance was detected in the enzymatic activity of the *B. subtilis* ATCC 6633 strain in the NB and NBW_1 culture media (Fig. 4b), where about 95%, 80% and 70% were maintained at the respective temperatures of 50, 60 and 70°C of the

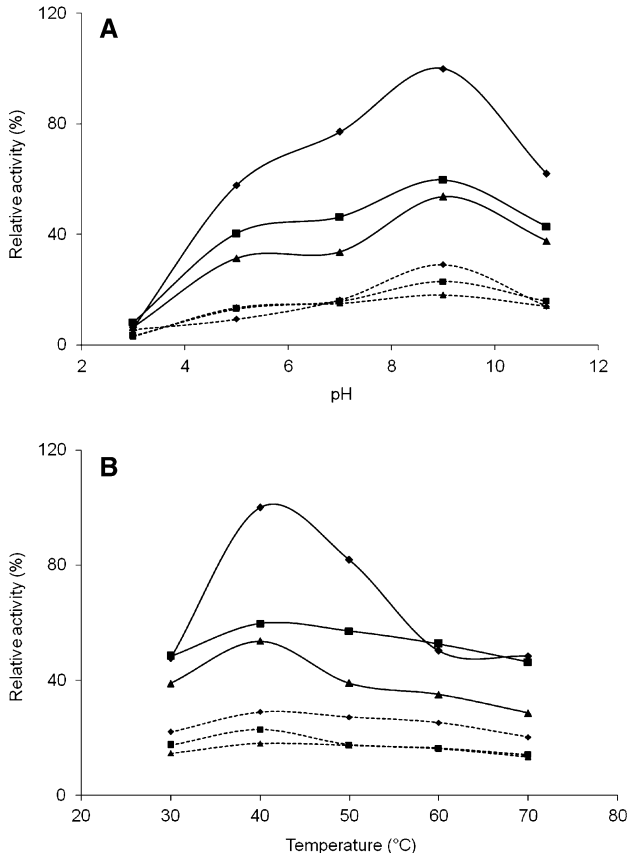

Fig. 2 Effect of pH (**a**) and temperature (**b**) on relative proteolytic activity in ammonium sulphate precipitate (60%) obtained from *Bacillus sp* UFLA 817CF (continuous line) and *B. subtilis* ATCC 6633 (discontinuous line) growing on nutrient broth (NB ♦), nutrient broth plus 0.01% (w/v) sodium caseinate (NBC ■) and nutrient broth plus 0.01% (w/v) cheese whey powder (NBW_1 ▲).

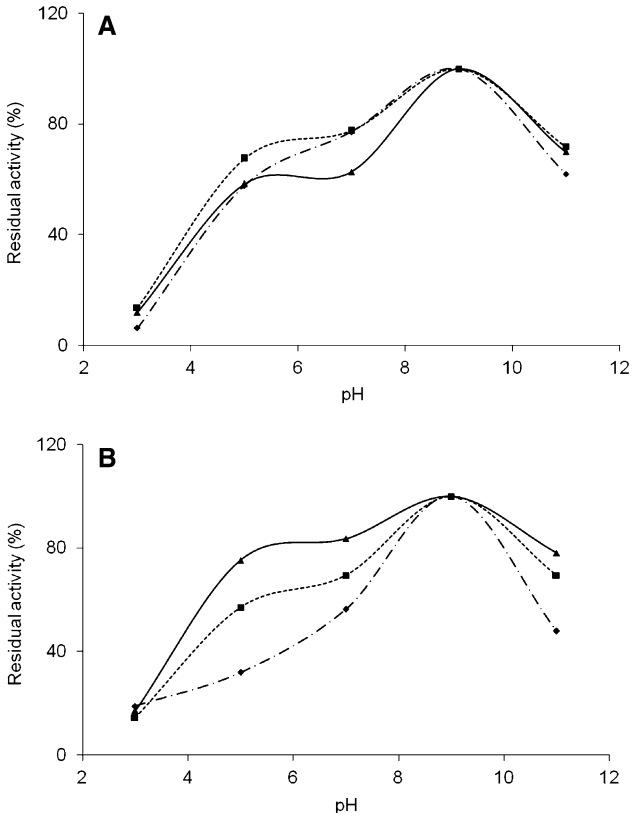

Fig. 3 Effect of pH on residual proteolytic activity in ammonium sulphate precipitates (60%) obtained from *Bacillus sp* UFLA 817CF (**a**) and *B. subtilis* ATCC 6633 (**b**) growing on nutrient broth (NB ♦), nutrient broth plus 0.01% (w/v) sodium caseinate (NBC ■) and nutrient broth plus 0.01% (w/v) cheese whey powder (NBW_1 ▲)

maximum activity at 40°C (268.7 U/mg for NB and 167.1 for NBW_1).

Stability at different temperatures and pH was verified in the NB medium for both the organisms, at the 60% saturation fraction with ammonium sulfate. The samples were incubated for 24 h at the pH tested before proteolytic activity was determined at pH 9.0. There was stability at pH 7.0 for both the microorganisms and more than 90% of the activity was maintained (Fig. 5) at the optimum pH (9.0).

The temperature stability of the enzyme was verified by incubating the samples at the tested temperatures for up to 120 min before assaying the proteolytic activity at 37°C (Fig. 6). At the incubation temperature of 40°C, the proteolytic activity of the enzyme obtained from the *Bacillus sp.* UFLA 817CF culture presented 62% activity after a 120-min incubation period in the same conditions that the *B. subtilis* ATCC 6633 enzyme presented 53% residual activity. At 50°C the enzymes from the *Bacillus sp.* UFLA 817CF culture were more stable than those from the *B. subtilis* ATCC 6633 culture. At 60°C it was observed that the *B. subtilis* ATCC 6633 culture presented greater enzymatic activity than the wild strain, but with a sharp loss in activity in the first 30 min of incubation for both the microorganisms (Fig. 6).

The precipitates obtained from saturation with 60% ammonium sulfate in the NB media for both the bacilli were characterized in polyacrylamide gel. Bands were detected between the 30 kDa and 45 kDa standards in both the cultures, approximately 36 kDa for the protease of the wild strain and 40 kDa for the standard *B. subtilis* ATCC 6633 strain. The bands observed were close to the band of protease molecular mass that in general, ranged between 15 and 45 kDa (Kumar and Takagi 1999; Gupta et al. 2002b). Zvidzai and Zvauya (2001) detected a similar band in a precipitated sample derived from saturation with 60% ammonium sulfate when they cultured a new species of *Bacillus*.

Based on the results in this study, the wild *Bacillus sp.* UFLA 817CF strain isolated in coffee beans was a potential producer of alkaline protease when cultivated either in nutrient broth or in other culture media tested. The NBW_1 culture presented good alkaline protease production, and was superior or equivalent to the culture medium with

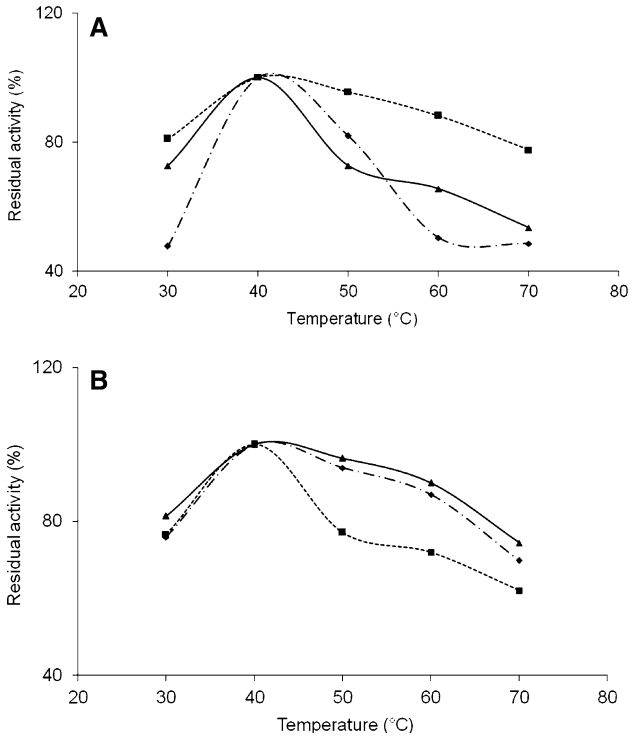

Fig. 4 Effect of temperature on residual proteolytic activity in ammonium sulphate precipitates (60%) obtained from *Bacillus sp* UFLA 817CF (**a**) and *B. subtilis* ATCC 6633 (**b**) growing on nutrient broth (NB ♦), nutrient broth plus 0.01% (w/v) sodium caseinate (NBC ■) and nutrient broth plus 0.01% (w/v) cheese whey powder (NBW_1 ▲)

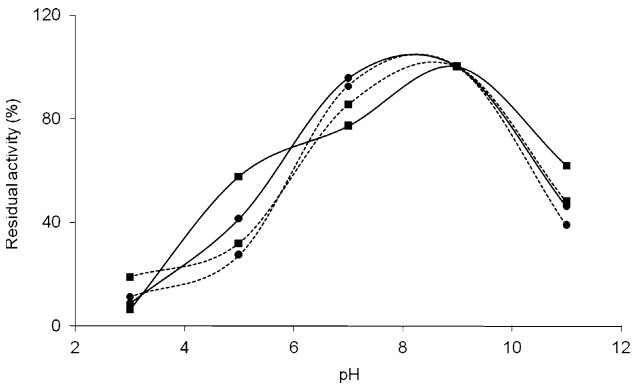

Fig. 5 Effect of pH on residual proteolytic activity (■) and stability (●) in ammonium sulphate precipitate (60%) obtained from *Bacillus sp* UFLA 817CF (continuous line) and *B. subtilis* ATCC 6633 (discontinuous line) growing on nutrient broth

addition of sodium caseinate. This opens perspectives for use of cheese whey powder, an effluent of the dairy industry and therefore an inexpensive source, as protease synthesis inducer, and for new research to optimize growth culture media based on cheese whey powder. The protease obtained from culture of wild strain *Bacillus sp.* UFLA 817CF presented stability at pH 7.0 and considerable heat

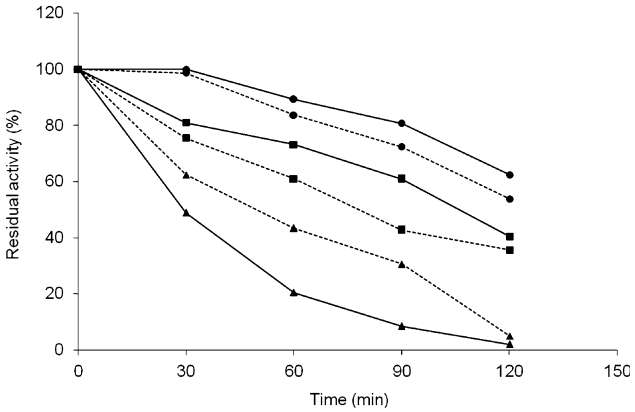

Fig. 6 Effect of temperature on residual proteolytic activity in ammonium sulphate precipitate (60%) obtained from *Bacillus sp* UFLA 817CF (continuous line) and *B. subtilis* ATCC 6633 (discontinuous line), growing on nutrient broth, at 40°C (●), at 50°C (■) and at 60°C (▲)

stability at 40°C and 50°C, and could be an alternative for the various industrial sectors to produce proteolytic enzymes using cheese whey.

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