Characterization of crude proteases of *Bacillus subtilis*EFRL 01

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Abstract

The extracellular proteases produced by *Bacillus subtilis* EFRL01 were characterized for possible use in industry. The bacterium was grown for 8 h without pH control at 45 °C in an inexpensive medium that consisted of molasses, peptone and sodium chloride. The supernatant of the fermentation broth was used as the crude protease preparation. The crude preparation was broadly active against a variety of proteins. Optimal pH and temperature for activity were 8.5 and 50 °C, respectively. The enzyme preparation was quite stable at up to 60 °C. At optimal temperature, the enzyme was stable in the pH range of 7.5 to 9.5. The enzyme was activated by Zn2+, Ca2+ and Mn2+, but inhibited by Ag2+, Co2+, Ni2+ and Cu2+. Metal ion chelating agents suppressed enzyme activity relative to control, but the enzyme was not particularly sensitive to the presence of the serine protease inhibitor phenylmethanesulfonyl fluoride. In conclusion, B. subtilis EFRL01 can be used to produce inexpensive pH- and heat-stable proteases for possible commercial use in detergents and leather processing.

Key words: Proteases, *Bacillus subtilis*, thermostableenzymes

Introduction

Microbial proteases are widely used in commercial applications such as food processing, leathermaking and papermaking [1],[2],[3],[4],[5]. In addition, proteases are commonly added to laundry detergents [6],[5]. Most of the industrial proteases are produced by recombinant microorganisms that yield enzymes with enhanced properties relative to the wildtype enzymes [7]. Bacteria of the genus Bacillus are commonly used for protease production [8],[9],[10],[7]. Commercially available recombinant proteases are made using proprietary
microorganisms and processes and are barely affordable in some of the poor regions of the world. Therefore, interest remains in effective wildtype proteases that could be produced inexpensively for use in household detergents and other applications. Our previous studies are also carried out for protease production from locally isolated microorganisms using cost effective energy sources. [11] reported protease production from Staphylococcus epidermis EFRL 12 using molasses as sole carbon source. In optimized fermentation conditions microorganism produced ~600 U/mL of protease titer. Protease production was also investigated from *Bacillus subtilis* EFRL01 using molasses mineral medium in submerged fermentation condition [12]. In another study, [13] reports open nonsterilized solid state fermentation conditions for protease and amylase coproduction from Bacillus sp BBXS-7. Nonsterilized fermentation simplified the fermentation process and save sterilization cost and energy.

This work reports on extracellular proteases of *Bacillus subtilis* EFRL01 isolated from soil in Sindh, Pakistan. For possible commercial use, the enzyme preparation was characterized in terms of its optimal pH and temperature foractivity; substrate specificity; stability at different pH values and temperatures; andpossible activation/inhibition and stabilization effects of additives such as metal ions. These factors are widely considered to be important in determining the commercial utility of a bulk enzyme [3](Sandhya et al. 2004).

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Materials and methods

Microorganism

Bacillus subtilis EFRL01 isolated from a soil sample from Jamshoro, Sindh, Pakistan, was used. Its identity was confirmed by biochemical and morphological tests carried out by two independent laboratories at the Institute of Biotechnology and Genetic Engineering, University of Sindh and Department of Microbiology, ShahAbdulLatifKhairpurUniversity, Sindh, Pakistan. B. subtilis EFRL 01 was used for protease production [12] and lipase production (manuscript in preparation). The culture was maintained on a nutrient agar medium at 4 °C and subcultured every 7-day. The agar medium contained (per liter of distilled water) the following: meat extracts 3 g, peptone 3 g, sodium chloride 3 g, and agar 15 g.

Enzyme production

Fermentation medium contained the following components (per liter of distilled water): molasses 10 g, peptone 7.5 g and sodium chloride 20 g. The initial pH was adjusted to 8.5 prior to sterilization of 500 mL of medium at 121 °C for 20 min. Shake flasks (1 L each) containing 500 mL of the cooled medium per flask were inoculated with 5 mL of the starter culture and incubated at 45 °C on an orbital shaker (120 rpm). After 8 h of incubation, the bacterial broth was centrifuged (6000-g, 10-min) and the supernatant was recovered for characterizing the proteases.

Protease activity

Protease activity in the culture supernatant was assayed according to the following method adapted from [14]. All solutions were equilibrated at 35 °C. A 0.5 mL sample of the culture supernatant was mixed with 0.5 mL of a solution of casein (10 g/L, preadjusted to pH 7.6) and 1.5 mL of sodium phosphate buffer (pH 7.6). This mixture was incubated at 35 °C for 30 min in a shaking water bath. Samples (2 mL) were removed immediately after mixing and at the end of the incubation period and quenched at once by mixing with 2 mL of tricholoroacetic acid (15 g/L). The mixture was centrifuged (4000-g, 10-min) to remove protein precipitate. A 1 mL aliquot of the supernatant was mixed with 4 mL of 0.5 M NaOH, 1 mL of Folin phenol reagent and 4 mL of double distilled water. Spectrophotometric absorbance of the resulting solution was measured at 625 nm against a distilled water blank. The difference in absorbance between the initial reaction mixture and the sample taken at 30 minutes, indicated the proteolytic activity [14] in terms of tyrosine released. One unit of protease activity was defined as the amount of crude culture supernatant that liberated 1 μg of tyrosine under the assay conditions.

Characterization of proteases

Substrate specificity. The substrate specificity of the crude protease preparation was assessed by replacing casein in the enzyme activity assay with 10 g/L solutions of the following proteins: casein hydrolysate, azocasein, tryptone, peptone and albumin.

Substrate and enzyme concentration effects. The effects of substrate and enzyme concentrations on proteolytic activity were characterized by using tryptone (5–30 g/L) as the substrate and 0.1–0.5 mL of crude enzyme solution in the protease activity assay.

pH, temperature and buffer type effects. The effects of pH (pH 3–11, universal buffer) and incubation temperature (25–90 °C) on protease activity was assessed by measuring the activity at different specified pH values and temperatures. Effects of buffer types on activity at a given pH of 7.6 was assessed by using universal buffer [15], sodium phosphate buffer and sodium citrate buffer. The universal buffer of a given pH value was made by mixing a Solution A, and a Solution B (0.2 M NaOH) in various proportions [15]. Solution A contained (g per liter of distilled water) the following components: citric acid 6.008, potassium dihydrogen phosphate 3.893, boric acid 1.769 and diethylbarbituric acid 5.266.

pH stability and thermostability of proteases. The pH stability of the crude enzyme was characterized by determining the fraction of the initial activity that remained 10 minutes after incubation (35 °C) of 0.5 mL of the enzyme solution with 1.5 mL solutions of the above specified universal buffer at pH values ranging between 3 and 11. Thermal stability of the enzyme was determined by measuring the activity at 35 °C by the above specified assay after the enzyme solution had been incubated at specified temperatures (25–90 °C) for 10 minutes.

In separate experiments, the

thermostability was characterized by activity measurements (35 °C) after the enzyme solution had been incubated at 50 and 70 °C for various lengths of time (10–60 minutes) with and without an activator (7.5 mM ZnCl2).

Effects of metal ions and other compounds. Effects of metal ions and other compounds on proteolytic activity were characterized by incubating the enzyme solution (0.5 mL) at 35 °C with 5 mM of the specified compound dissolved in 1.5 mL of the universal buffer at pH 8.5 for 10 minutes prior to measuring the activity at 50 °C against 0.5 mL of a tryptone solution (15 g/L). Effects of concentrations of potential activator salts (ZnCl2, MnCl2, CaCl2) on protease activity were also characterized.

Results and discussion

Although *Bacillus subtilis* produces both extracellular and intracellular proteases [16], this work focused exclusively on extracellular proteases because they are easier to recover from a fermentation broth than are intracellular enzymes [17] and therefore are less expensive to produce. In our previous study [12], fermentation conditions were optimized for protease production from *Bacillus subtilis* EFRL 01.In present study crude protease of same strain is characterized for its applicability in local detergent formulations.

Under identical conditions, the crude proteases were broadly effective against various protein substrates as shown in (Figure 1). Activity was highest on tryptone. A broad activity spectrum is useful in applications such as detergency.

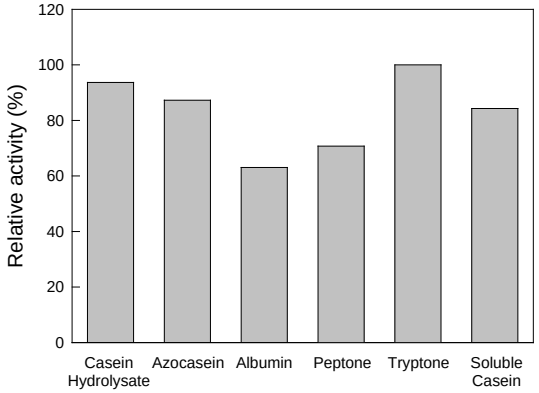

Fig. 1. Proteolytic activity on various substrates relative to activity on tryptone. Measured at 35 °C, pH 7.6 (sodium phosphate buffer) and a substrate concentration of 10 g/L.

The effect of tryptone concentration on protease activity is shown in Figure 2. For a fixed concentration of the enzyme, activity generally increased with increasing concentration of the substrate. Occurrence of a peak activity (Figure 2) suggests that the enzyme was subject to substrate inhibition. For a fixed concentration of the enzyme

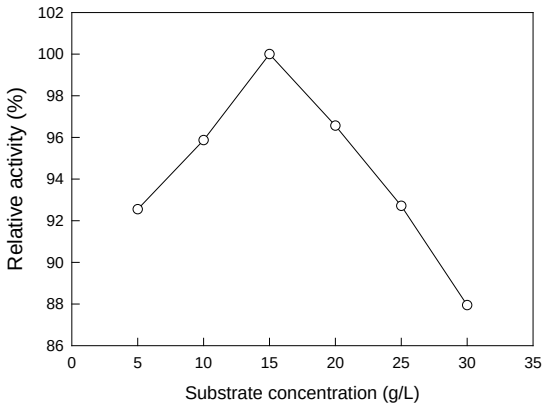

Fig. 2. Effect of tryptone concentration on relative proteolytic activity (35 °C, pH 7.6, sodium phosphate buffer). Activity is shown relative to the maximum value.

as in Figure 2, an increasing concentration of the substrate decreased the molar ratio of the enzyme to substrate [18]. Consequently, the enzyme became saturated with the substrate and the activity approached a maximum value. A further increase in substrate concentration inhibited the enzyme and hence the observed decline in relative activity (Figure 2). Similar results have been reported for alkaline proteases of Bacillus pantotheneticus [7]. For the latter, a peak activity was observed at a substrate concentration of ~15 g/L, or quite consistent with the present study for a totally different Bacillus species.

Effect of the volume of the crude enzyme solution used, on activity against tryptone is shown in Figure 3. A 5-fold increase in the concentration of the enzyme increased the activity by only 20% (Figure 3), suggesting that a small quantity of enzyme is sufficient for use in most applications.

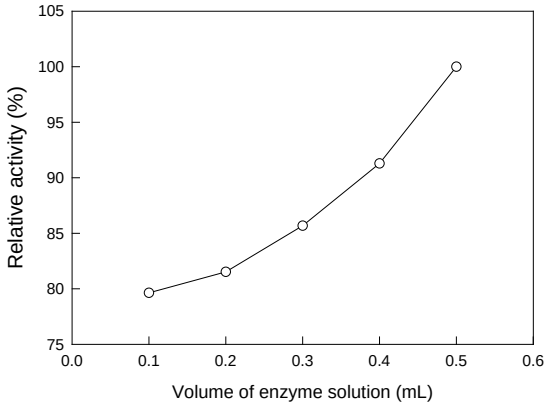

Fig. 3. Effect of enzyme solution volume on activity against tryptone (10 g/L, 35 °C, pH 7.6, sodium phosphate buffer). Activity is shown relative to the maximum value.

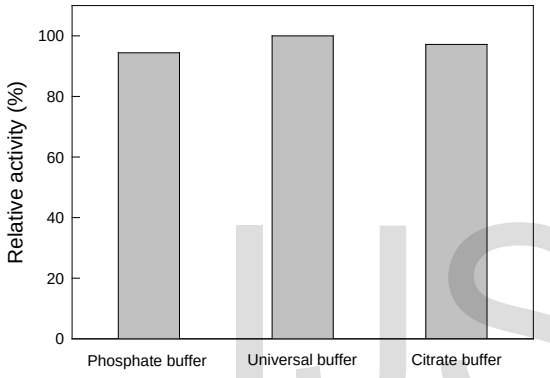

Fig. 4. Effect of buffer type on protease activity on tryptone (10 g/L) at pH 7.6 and 35 °C. Activity is shown relative to universal buffer.

At a given pH, different buffer formulations are known to affect the activity of an enzyme. Therefore, the effect of three different buffers at a pH of 7.6 on the crude protease activity was assessed, as shown in Figure 4. The maximum activity was observed in the universal buffer. Activity in citrate and phosphate buffers was approximately 97% and 94% of the activity in universal buffer. This suggests that all these buffers may be used in an enzyme formulation without affecting the proteolytic activity significantly.

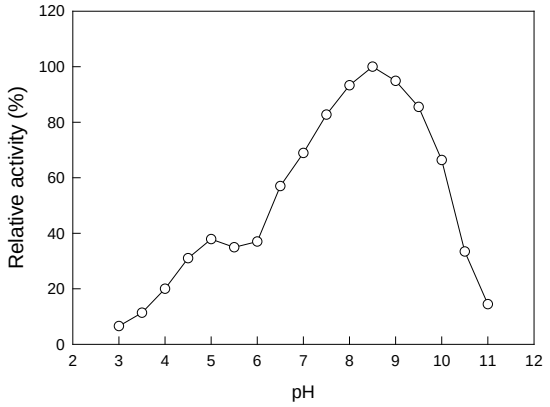

Fig. 5. Effect of pH (universal buffer) on protease activity on tryptone (10 g/L) at 35 °C. Activity is shown relative to the maximum value.

The effect of pH (universal buffer) on proteolytic activity is shown in Figure 5. The activity profile had two peaks, but the optimal pH was clearly around 8.5. Proteases that are active at alkaline pH's are common among bacilli. A pH optimum of 8.4 has been reported for proteases of B. pantotheneticus [7]. [19]noted an optimum pH of 9.0 for proteases of Bacillus licheniformis. [9]reported an optimum pH of 8.0 for Bacillus cereus KCTC 3674 proteases. For proteases of a thermophilic Bacillus species, [20] reported an optimal pH of 8.5. Alkaline proteases have been reported in other bacterial species [21],[22].

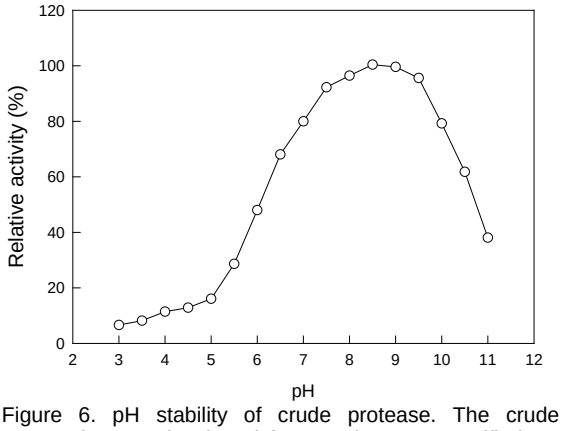

Figure 6. pH stability of crude protease. The crude preparation was incubated for 10 minute at specified pH values in universal buffer at 35 °C. Activity is shown relative to the maximum observed. Activity was measured in the universal buffer at a pH of 7.6 and 35 °C.

As shown in Figure 6, the crude protease was quite stable at its optimal pH of 8.5 (universal buffer). The stability maximum for the enzyme was fairly broad (pH 7.5 to 9.5). A 10 min exposure to a pH value of less than 6 or greater than 10 significantly denatured the enzyme. This

was consistent with earlier observation of proteases from Bacillus species [8],[20].

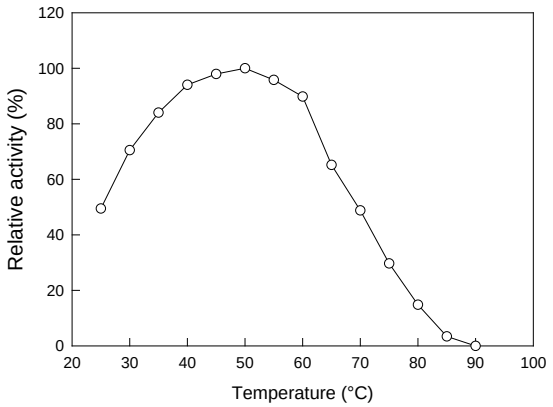

Fig. 7. Effect of temperature on protease activity. Activity was measured on tryptone (15 g/L) in phosphate buffer, pH 7.6, at the specified temperature. Activity is shown relative to the maximum observed.

Effect of reaction temperature on the catalytic activity of the crude enzyme is shown in Figure 7. The enzyme activity increased with increasing temperature until the optimum temperature of ~50 °C. Further increase in temperature caused a gradual and progressive loss in activity. For example, incubation at 70 °C for only 10 min reduced activity to 50% of the initial value (Figure 7). These results are generally consistent with the findings reported for proteases of a thermophilic Bacillus species [20]. For practical purposes in applications such as detergents, the enzyme was active at up to 60 °C (Figure 7). The behavior in Figure 7 is fairly common for enzymes.

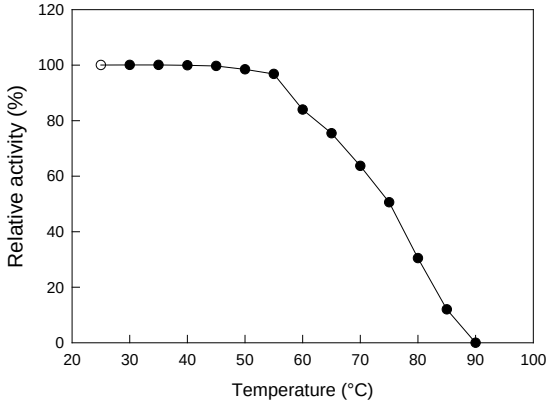

Fig. 8. Temperature stability of protease activity. The enzyme was incubated at specified temperatures for 10 minutes prior to activity measurement (15 g/L tryptone as substrate, pH 8.5, universal buffer) at 35 °C. Activity is shown relative to the observed maximum.

The thermal stability of the crude enzyme is shown in Figure 8. The enzyme was quite stable for 10 minutes at up to 60 °C. At 70 °C, 40% of the initial activity was lost within 10 minutes; however, 50% of the initial activity remained after 10 minute incubation at 75 °C. The enzyme is therefore satisfactory for use in detergents where a high activity is needed only for relatively short periods at temperatures of ≤70 °C.

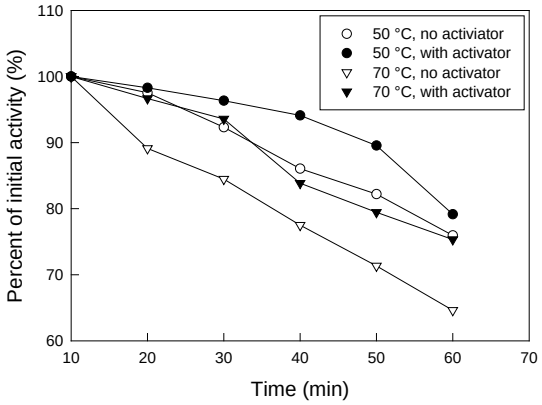

Fig. 9. Stability of protease at: a) 50 °C with and without ZnCl2 (7.5 mM) activator; b) 70 °C with and without ZnCl2 (7.5 mM) activator. The enzyme solutions were preincubated with or without the activator at 35 °C for 10 min at pH 8.5 (universal buffer) prior to activity measurements on tryptone (15 g/L) at the specified temperatures.

Thermal stability at 50 and 70 °C could be improved measurably relative to controls, by adding 7.5 mM ZnCl2 to the crude enzyme (Figure 9). For example, at 70 °C <65% of the initial activity remained after 60 min in the absence of ZnCl2 whereas in its presence >75% of the initial activity was retained. ZnCl2 has previously been used to substantially enhance the thermostability of a fungal protease [23]. Many enzymes are known to be stabilized by divalent metal ions and other additives [24].

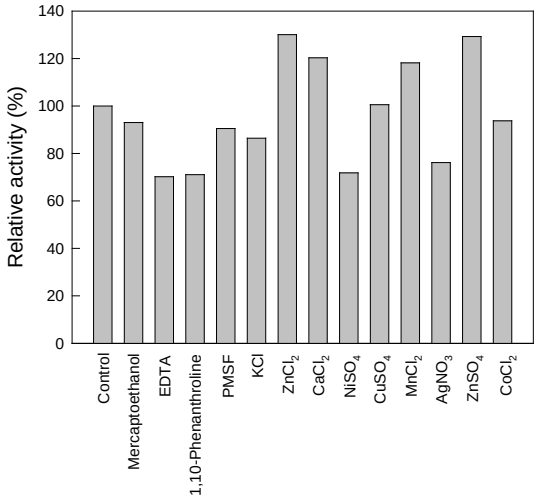

Fig. 10. Effect of preincubation (10 min, 35 °C, pH of 8.5, universal buffer) of the crude enzyme with various salts and organic compounds (5 mM concentration) on activity. Activity was measured at 50 °C using tryptone (15 g/L) in universal buffer at pH 8.5. Activity is shown relative to control that was preincubated as above, but without any additive. (EDTA = ethylenediaminetetraacetic acid, a chelator of metal ions; PMSF = phenylmethanesulfonyl fluoride, an inhibitor of serine proteases.)

Figure 10 shows the effect of the presence of various compounds on protease activity relative to control (no additives). Relative to control, the presence of Zn2+, Ca2+ and Mn2+ at 5 mM concentration enhanced activity, but the presence of K+, Ni2+, Ag2+ and Co2+ did not (Figure 10). Cu2+ at 5 mM level had no effect on activity.

Divalent metal ions are known to enhance activity of many microbial proteases [23],[10],[25],[7],[22],[26]. This is because many proteases are metalloproteins which require a divalent metal ion for proteolytic activity [24]. Chelating agents such as EDTA and 1,10-phenanthroline inactivate metalloproteases by binding the metal ions [24]. This explains the activity reducing effect of EDTA and 1,10-phenanthroline (Figure 10). Both compounds reduced activity by roughly 30%, suggesting that both are equally effective in binding the specific metal ions that are essential for activity. The results unequivocally suggest that at least part of the observed proteolytic activity is associated with the presence of metalloproteases in the crude enzyme preparation.

Some alkaline proteases of Bacillus sp. contain serine at the active site and are not affected by EDTA, but are inhibited by the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) [27],[24]. In the present study, PMSF did reduce activity measurably relative to control suggesting that a relatively small portion of the proteolytic activity in the crude enzyme preparation is contributed by serine proteases.

Presence of mercaptoethanol at 5 mM level reduced activity, but only slightly relative to control. Mercaptoethanol is a reducing agent that is commonly used to break disulfide bonds between protein chains. Although at 5 mM the concentration of mercaptoethanol was only about 5% of the concentration that is commonly used in reducing disulfide links [24], the results suggest that the preservation of any disulfide links is not necessary for preserving most of the proteolytic activity.

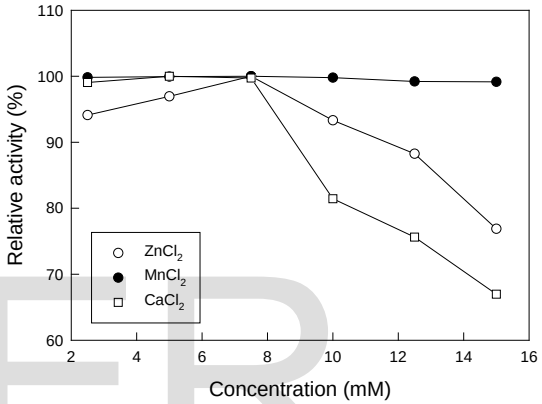

Fig. 11. Effects of ZnCl2, MnCl2 and CaCl2 concentrations on protease activity. Activity is shown relative to the maximum value observed for a given salt. Activity was measured at 50 °C, pH 8.5, universal buffer, using tryptone (15 g/L) as substrate.

In view of the strong activity enhancing effect of ZnCl2, CaCl2 and MnCl2 observed at a fixed concentration of 5 mM (Figure 10), the concentration of these salts was varied (2.5–15.0 mM) in separate experiments to characterize any concentration dependence of this effect. The results are shown in Figure 11. At 50 °C, the activity enhancing effect of Mn2+ was independent of the concentration over the range tested. In contrast, Ca2+ and Zn2+ had an activatory effect only in the 2.5 to 7.0 mM concentration range. Higher concentrations of Ca2+ and Zn2+ actually reduced proteolytic activity. Similar results have been reported for other bacterial proteases. For example, MgCl2 and CaCl2 added in 1 to 5 mM range have enhanced the activity of proteases of the halophilic bacterium Salinivibrio sp. in an essentially concentration independent manner, but CaCl2 which was an activator at ≤7.5 mM proved to be a strong inhibitor at higher

concentrations [22](Karbalaei-Heidari et al. 2007). These properties of protease make it suitable candidate for local detergent formulations.

Conclusions

Bacillus subtilis EFRL01 was a good producer of potentially useful proteases. A protease activity of >5,000 U/mL on tyrosine was attained in the crude cell-free culture broth within an 8-h fermentation using an inexpensive medium. The crude enzyme was broadly effective on a variety of protein substrates. The enzyme was apparently inhibited by the substrate but only at a high substrate concentration of ~15 g/L. The pH for optimal activity was found to be 8.5, but the enzyme was stable over the pH range from 7.5 to 9.5. The enzyme was almost equally active in phosphate, citrate and universal buffers at a pH of 7.6. The optimal temperature for enzyme activity was ~50 °C, but the enzyme was quite stable up to a temperature of 60 °C. The enzyme activity was enhanced by certain divalent metal ions (Zn2+, Ca2+, Mn2+), but not by others (Ag2+, Co2+, Ni2+, Cu2+). The serine protease inhibitor phenylmethanesulfonyl fluoride reduced activity relatively to control, but only marginally. Metal ion chelators such as EDTA and 1, 10-phenanthroline at a concentration of 5 mM reduced activity by about 30% relative to controls. Thermal stability of the enzyme at 50 and 70 °C was significantly improved in the presence of 7.5 mM Zn2+ relative to controls. In view of these results, the crude extracellular proteases of B. subtilis EFRL01 contained mostly relatively thermostable alkaline metalloproteases that are potentially useful for leather processing and as additives in detergents.

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