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Alkaline protease production of *Bacillus cohnii* APT5

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Abstract: An obligate alkaliphilic *Bacillus* strain was isolated from a soil sample and identified as *Bacillus cohnii* APT5 based upon phylogenetic and phenotypic analyses. The optimum growth pH of *B. cohnii* APT5 was 10.0. *B. cohnii* APT5 was also found capable of producing an extracellular alkaline protease that showed optimum activity (693,318 U/min) at 50 °C and pH 11.0 when grown in a medium containing casein. The enzyme was partially purified 3.22-fold with a yield of 78.74% after acetone precipitation and cation exchange column chromatography, respectively. The partially purified enzyme maintained its activity when incubated at 50 °C for 2 h. It was stable at pH 11.0 at room temperature for 72 h. Furthermore, the alkaline protease activity was not completely inactivated by the specific inhibitors of serine alkaline proteases and metalloproteases such as PMSF and EDTA. While 1 mM PMSF had no effect on the enzyme activity, a 10 mM concentration of PMSF slightly decreased the activity, producing a relative activity of 76%. In addition, the enzyme was found to be very stable toward surface active agents such as SDS, Tween 20, Tween 80, and Triton X-100. None of the metal ions used (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Mn^{2+}) showed an inhibitory effect on enzyme activity. On the contrary, APT5 alkaline protease activity increased 53% when treated with Mn^{+2}. It was concluded that the active center of APT5 alkaline protease required Mn^{+2} as a cofactor. Based on the thermostability, pH stability, and resistance of *B. cohnii* APT5 alkaline protease against inhibitors, this enzyme may be very useful in commercial applications, particularly in the detergent industry.

Key words: Alkaliphilic *Bacillus*, alkaline protease, enzyme purification, 16S rRNA gene sequence, phylogenetic analysis

Introduction

Proteolytic enzymes play a specific catalytic role in the hydrolysis of proteins. They are widespread in all living organisms as they are essential for cell growth and differentiation (1). Proteases are categorized as serine proteases, aspartic proteases, cysteine proteases, and metalloproteases depending on their catalytic mechanism. They are also classified into different families and clans depending on their amino acid sequences and evolutionary relationships. Based on their optimal pH activity, they are referred to as acidic, neutral, or alkaline proteases (2). Moreover, bacterial proteases are now among the largest classes of industrial enzymes, accounting for 60% of total worldwide enzyme sales (2).

Among proteases, alkaline proteases (EC 3.4.21-24) are defined as enzymes that are active from the neutral to the alkaline pH range (1). These enzymes are generally active between pH 9.0 and 11.0, with the exception of a few higher pH values of about 12.0

and 13.0 (3). Alkaline proteases are a physiologically and commercially important group of enzymes used in various branches of industry, such as the detergent, food, leather tanning, pharmaceutical, and textile industries (3,4).

Alkaliphilic *Bacillus* strains constitute an important source of extracellular enzymes that are useful for numerous industrial processes (5,6). This group includes facultative and obligate alkaliphilic species that grow well at pH values higher than 9.0. Alkaliphilic *Bacillus* strains secrete large amounts of alkaline proteases with a high stability at elevated pH and temperature values. In the 1960s, the first detergent containing a bacterial protease appeared on the market. This enzyme, subtilisin Carlsberg, is one of the validly characterized subtilisins from *Bacillus licheniformis* (7). Because of the extreme properties of bacterial - alkaline proteases produced by *Bacillus* strains in particular are considered as compatible additives for laundry detergents (2,8). Some examples of industrially important alkaliphilic *Bacillus* strains were also studied, including a protease-producing strain, *Bacillus* sp. D-6 (JCM 9154) (9); a cyclomaltodextrinase producer strain, *Bacillus* sp. 199 (10); a serine protease-producing strain, *Bacillus* sp. Y (11); an oxidatively stable alkaline protease-producing strain, *Bacillus* sp. SD 521 (12); an alkaline protease-producing strain, *Bacillus pumilus* (13); an alkaline protease-producing strain, *Bacillus cereus* (14); an alkaline serine protease-producing strain, *Bacillus licheniformis* BA17 (15); and an alkaline serine protease-producing strain, *Bacillus clausii* GMBE 22 (16). Among the above-mentioned enzymes, the alkaline protease from *Bacillus* sp. SD 521 was also patented (US 7.371.839.B2).

Here we report the physiologic and phylogenetic characters of an obligate alkaliphilic, *Bacillus cohnii* APT5, as well as the partial purification and characterization results from its novel, extremely stable alkaline protease. Data including thermal and pH activity and stability and the effect of some inhibitors, metal ions, surface active agents, and oxidizing agents on APT5 alkaline protease are also reported. Results show that enzymatic properties distinct from other characterized alkaliphilic bacilli alkaline proteases were produced.

Materials and methods

Bacterial strains: In this study, a total of 57 bacterial isolates were screened for alkaline protease activity. Of these isolates, 46 thermophilic and 2 halophilic isolates were obtained from our microbiology research laboratory culture stocks. In addition, 9 isolates from APT1 to APT9, including APT5, were newly isolated from a soil sample taken at a car wash in the Beşevler area of Ankara, Turkey. The protease-producing standard strain, *Bacillus licheniformis* DSM 13^T, was obtained from the DSMZ (the German Collection of Microorganisms and Cell Cultures).

Isolation and the screening for protease production: The soil samples (0.2-0.4 g) were inoculated in nutrient broth for all isolation and enrichment procedures. The pH of the medium was adjusted to 10.0 by adding 1 N NaOH solution aseptically after sterilization. Inoculated samples were cultivated at 37 °C by shaking at 200 rpm for 48 h. The turbid cultures were diluted with saline solution and transferred onto skim-milk agar (pH 10.0) plates (17). Skim-milk agar medium (pH 10.0) was also used for purification and screening isolates for protease production. The inoculated plates were incubated for 72 h at 37 °C for mesophilic isolates and at 55 °C for thermophilic isolates. Protease-producing strains, which gave a clear zone around colonies due to the hydrolysis of skim milk, were selected. The zone diameters were also compared with standard strain *B. licheniformis* DSM 13^T.

Culture conditions for alkaline protease production and determination of alkaline protease activity: Medium containing 0.5% casein was used for enzyme production as described by Gessesse and Gashe (18). The pH of the medium was adjusted to pH 7.0 and 9.0 by adding sterile 10% Na_2CO_3 solution after sterilization. Actively growing cells were suspended in 0.85% sterile NaCl to an absorbance value of 0.2-0.4 at 660 nm, and then 500 µL of the resulting suspension was inoculated into 5 mL of enzyme production medium and cultivated at 37 °C for mesophilic isolates and at 55 °C for thermophilic isolates by shaking at 200 rpm for 72 h. The cells were removed from the culture medium by centrifugation at 5000 rpm for 20 min at 4 °C, and the wet weights of the cells were determined. The resulting supernatants were used for the extracellular enzyme source. In

addition, alkaline protease activity was measured as described by Takami et al. (19), by using casein (Sigma C7078) as a substrate. One unit of alkaline protease activity was defined as the amount of the enzyme capable of producing 1 µg of tyrosine in 1 min under standard assay conditions. All enzyme activity assays were carried out in triplicate.

Morphologic and physiologic characterization analysis: Cells actively growing on nutrient agar plates (pH 7.0 and 9.0) were used for cell and colony morphology. The formation of spores was tested by using nutrient broth cultures of 18-24 h supplemented with 5 mg/L of $MnSO_4.4H_2O$ and observed under a phase-contrast microscope. Temperature (15-60 °C), pH (6.5-12.5), and salinity (2%-10% NaCl) ranges for growth were tested in nutrient broth, and after 24 h of incubation at 37 °C the optic density of the cells at 600 nm was measured. Physiological characterization tests including Gram staining; anaerobic growth; catalase and amylase activities; casein, citrate, starch, tyrosine, gelatin, and urea utilization; reduction of nitrate to nitrite; N_2 gas production from nitrate; acid production from sugars; the methyl red test; the Voges-Proskauer test; indole and H_2S production; and susceptibility to lysozyme were carried out according to the methods of Claus and Berkeley (20), as previously described. A disk diffusion test was performed using nutrient agar plates for antimicrobial susceptibility testing. The disks (Oxoid) and their contents were as follows: vancomycin (30 µg), bacitracin (10 µg), chloramphenicol (30 µg), rifampicin (30 µg), tetracycline (30 µg), penicillin G (10 U), neomycin (30 µg), novobiocin (30 µg), kanamycin (30 µg), and azithromycin (15 µg). Plasmid DNA isolation was carried out according to the method of Anderson and McKay (21). All assays were performed with pure cultures in triplicate.

16S rRNA gene amplification and sequencing: Genomic DNA was extracted from the cultures growing in nutrient broth for 18 h at 37 °C using a genomic DNA purification kit (Fermentas). The gene encoding 16S rRNA was amplified by polymerase chain reaction (PCR) with the 16S bacteria-specific primers of 27F forward and the 1492R reverse primer (22). The reaction mixture contained 1X Taq buffer with $(NH4)_2SO4$, 0.2 mM dNTP mix, 0.25 µM forward and 0.25 µM reverse primer, 1.25 U Taq polymerase (Fermentas), 200 ng genomic DNA, and 1 mM $MgCl_2$ for a total volume of 50 µL. The reaction conditions were as follows: after initial denaturation at 94 °C for 2 min, 30 cycles were carried out with a denaturation step at 94 °C for 45 s, an annealing step at 55 °C for 45 s, and an elongation step at 72 °C for 1.5 min. Amplification was completed with a final extension step at 72 °C for 7 min. The amplification products were purified with a PCR purification kit (Promega, Wizard SV Gel and PCR Clean-Up System). The sequence of the PCR-amplified 16S rRNA gene (1420 bp) was determined with the Beckman Coulter CEQ Genetic Analyzer.

Phylogenetic analysis: Evolutionary history was designated using the neighbor-joining method (23). The bootstrap consensus tree inferred from 1000 replicates was taken into the evolutionary history of the taxa analyzed (24). Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates were collapsed. Evolutionary distances were computed using the maximum composite likelihood method (25) and are given in the units of the number of base substitutions per site. The phylogenetic tree was constructed with the MEGA package, version 4 (26).

Partial purification of the enzyme: Alkaline protease production was carried out using medium (pH 10.0) containing 0.5% casein as described by Gessesse and Gashe (18). Erlenmeyer flasks (500 mL) containing 100 mL of medium were inoculated by a culture of 18 h old with a 1% v/v ratio and cultivated at 37 °C by shaking at 200 rpm for 72 h. After cultivation, the culture was centrifuged (5000 rpm, 20 min, 4 °C), and the resulting supernatant was used as an enzyme source for further purification procedures, which were carried out at 4 °C. The supernatant was precipitated by the addition of cold acetone (−20 °C) with a saturation of 85% and stored at −20 °C overnight. The protein aggregates were collected by centrifugation, washed with 100% acetone, dried at room temperature, and then dissolved in 4.7 mL of 50 mM glycine-NaOH buffer (pH 10.0). This sample was applied to a Bio-Rad UNO S column (Bio-Rad Duo Flow System) for cation exchange chromatography. The loading buffer of 50 mM glycine-NaOH buffer (pH 9.0) was used with a flow rate of 1.0 m/min. The column was eluted with a linear gradient of the

same buffer containing 1 M NaCl with a flow rate of 1.0 mL/min. The fractions (0.4 mL) were collected using a fraction collector (Bio-Rad BioFrac Fraction Collector). The protein content and the proteolytic activity of the fractions were measured, and those showing proteolytic activity were pooled and used for enzyme characterization.

Electrophoretic analysis: Nondenaturing polyacrylamide gel electrophoresis (native PAGE) was carried out according to the modified method of Kazan et al. (27). A 10% (w/v) concentration of resolving gel containing 1% skim milk was used in electrophoresis, which was run in duplicate. One side of the gel was stained using silver staining according to the manufacturer's instructions (Bio-Rad Silver Stain Plus Kit, 161-0449), while the other was developed for activity staining. The gel used for activity staining was stained with 0.2% Coomassie blue G250 solution as described by Kazan et al. (27), and a clear zone on the gel indicated the presence of alkaline protease activity.

Effect of temperature and pH on enzyme activity: Experiments on the effect of temperature and pH on purified enzyme activity were performed as described for the standard protease assay by using casein as a substrate. The temperature profile of the enzyme activity was measured from 30 to 85 °C using 50 mM glycine-NaOH buffer at pH 10.0. The effect of pH on the activity was studied over a range of pH levels, from 7.0 to 13.0, at 50 °C. When determining the pH profile, casein was dissolved in 50 mM glycine-NaOH buffer at pH values from 8.0 to 12.0, whereas 200 mM glycine-NaOH buffer was used for testing the activity at pH 12.5 and 13.0 (27).

Temperature and pH stability of enzyme: Thermal stability was determined by incubating the purified enzyme at its optimum temperature for 20 min and 4 h at 30 °C for 3 days. The pH stability of the enzyme was tested by treating the enzyme with 50 mM glycine-NaOH buffer at its optimum pH for 3 days at room temperature. The residual activities of the enzyme were then measured. The activity of the nonheated and non-pH-treated enzyme was expressed as 100% (27).

Effects of metal ions on enzyme activity: To determine the effect of metal ions on enzyme activity, $CaCl_2$, $MgSO_4$, $ZnSO_4$, $FeSO_4$, and $MnSO_4$ were used by adding these ions to the assay mixture at a concentration of 5 mM. Relative enzyme activities were measured at optimum temperature. The activity under these conditions in the absence of metal ions was expressed as 100%.

Effect of inhibitors, surfactants, and hydrogen peroxide on enzyme activity: Inhibitors such as phenylmethylsulfonyl fluoride (PMSF, 1 and 10 mM), ethylenediamine tetra acetic acid (EDTA, 2.5 mM), and urea (1 M); surfactants such as sodium dodecyl sulfate (SDS, 0.5% and 1%), Tween 20 (1%), Tween 80 (1%), and Triton X-100 (1%); and oxidizing agent H_2O_2 (1% and 5%) were used for sensitivity experiments. The proteases of *Bacillus* sp. (Sigma P5985) and *B. licheniformis* (Sigma P4860) were also treated with a 10 mM concentration of PMSF. When determining sensitivity to inhibitors, the purified protease was preincubated with the stock solutions of each reagent at a ratio of 1:1 at 30 °C for 2 h in 50 mM glycine-NaOH buffer (pH 10.5), and the protease activity was assayed as described previously in the standard assay. The residual activities were then determined, and activity under these conditions in the absence of these reagents was expressed as 100%.

Results and discussion

Alkaline protease producer isolates: Of the 57 thermophilic, halophilic, and alkaliphilic bacterial isolates, 14 isolates were found to grow and produce a proteolytic zone on skim-milk agar plates at pH 9.0 and 10.0, as in the case of reference strain *B. licheniformis* DSM 13. These isolates were selected for further spectrophotometric protease assays. When determining the alkaline protease production capacities of these 14 isolates, enzyme activity values per pellet wet weight (U/mg) were determined, and it was revealed that an obligate alkaliphilic bacterium, APT5, produced higher amounts of alkaline protease (1555 U/mg) than reference strain *B. licheniformis* DSM 13 (1197 U/mg) at 37 °C and at pH 10.0 when their 48-h-old cultures were used. As a result, further purification and enzyme characterization experiments were carried out using strain APT5.

Phenotypic characteristics of strain APT5: Cells of isolate APT5 were gram-positive, motile, and rod-shaped with terminal, ellipsoidal endospores

occurring in a swollen sporangia. The APT5 strain was aerobic and obligate alkaliphilic. Growth was observed at 25-55 °C (optimum: 37 °C) and pH 9.0-12.0 (optimum pH 10.0), but the strain could not be grown below pH 9.0. Growth occurred under 2%-10% concentrations of NaCl. Isolate APT5 utilized a wide range of carbon sources including maltose, D-fructose, D-glucose, saccharose, and D-mannitol, but was not able to ferment lactose, D-xylose, raffinose, D-(+)-galactose, D-sorbitol, or L-arabinose. Casein, gelatin, starch, citrate utilizations and amylase and catalase activities were all positive, but urea and tyrosine could not be utilized. It was able to reduce nitrate to nitrite, but gas production was not observed from nitrate. The methyl red and Voges-Proskauer tests were negative. Indole and H_2S were not produced. Isolate APT5 was found to be sensitive to lysozyme and all of the antibiotics tested.

Phylogenetic analysis: The 16S rRNA gene sequence of strain APT5 was analyzed to determine its phylogenetic position. The 16S rRNA gene sequence data of strain APT5 were deposited in the GenBank (accession number: JF689927). A 1406-bp 16S rRNA gene sequence was compared with those of other related bacteria. The phylogenetic tree is shown in Figure 1. The 16S rRNA gene sequence similarity demonstrated that strain APT5 was a member of the genus *Bacillus*. A 100% 16S rRNA gene sequence similarity value with *B. cohnii* DSM 6307^T and nonvalidly published strains of *Bacillus* sp. D6 (JCM 9154), *Bacillus* sp. 199 (JCM 9163), *Bacillus* sp. Y, *Bacillus* sp. SD521, and *Bacillus* sp. 12-1 was observed. In the 16S rRNA gene sequence analysis, strain APT5 was clustered within these industrially important alkaliphilic strains, and thus strain APT5 was identified as a species of *B. cohnii*.

Partial purification of alkaline protease: After cation exchange chromatography, alkaline protease activity was measured in the first 2 major protein peaks that came with the NaCl gradient in the elution activity profile. Fractions showing alkaline protease activity from fraction numbers 16 to 30 and 31 to 40 of peak 1 and peak 2, respectively, were pooled for anionic *Bacillus cohnii* APT5 enzyme. The elution profile of *B. cohnii* APT5 alkaline protease after cation exchange chromatography is shown in Figure 2.

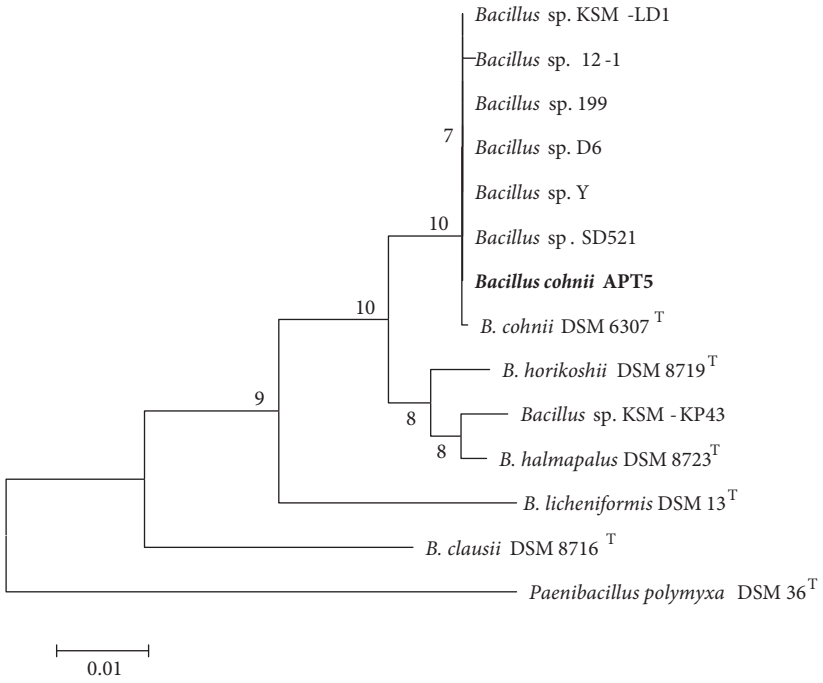

Figure 1. Phylogenetic tree of *Bacillus cohnii* APT5 associated with the other members of the genus *Bacillus*. Bootstrap values (%) are based on 1000 replicates and shown for branches with more than 50% bootstrap support. Bar indicates 0.01 substitutions per nucleotide position. The 16S rRNA gene sequence of *Paenibacillus polymyxa* DSM 36^T was chosen arbitrarily as an outgroup.

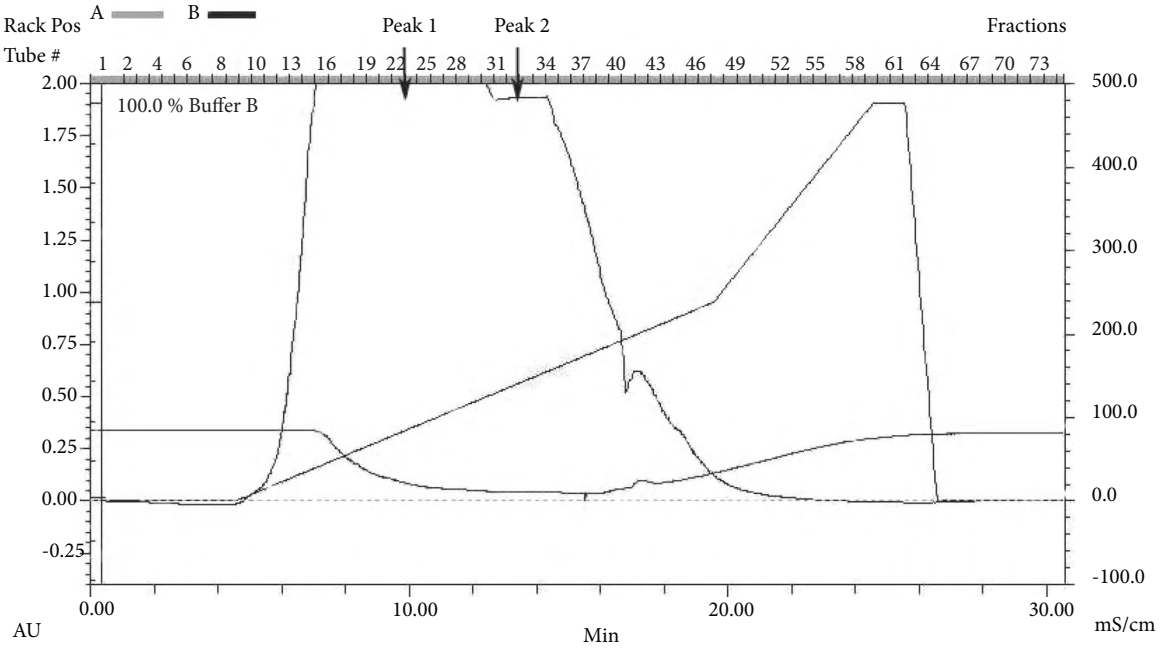

Figure 2. The elution profile of *Bacillus cohnii* APT5 alkaline protease after cation exchange chromatography. The first 2 major protein peaks show enzyme activity (peak 1: fractions showing alkaline protease activity from fraction numbers 16 to 30; peak 2: fractions showing alkaline protease activity from fraction numbers 31 to 40).

A 3.22-fold purification was achieved from the crude culture broth, with a recovery of 78.74% activity. The specific activity of the partially purified enzyme was 1342.17 U/mg (enzyme activity measured in glycine-NaOH buffer, pH 10.0, at 37 °C).

The partially purified fractions from each purification step were loaded onto native PAGE; activity-stained and silver-stained gel electrophoresis results are shown in Figures 3a and 3b, respectively. The culture supernatant, the acetone-precipitated enzyme sample, and the cation exchange-subjected enzyme fraction of *B. cohnii* APT5 all showed 4 bands with proteolytic activity on the skim milk-containing native gel developed at pH 10. The protease activity bands were visualized as clear colorless bands against a blue background on the destained side of the native PAGE (Figure 3a). The other side of the gel, which was silver-stained, also confirmed the presence of 4 bands, as shown in Figure 3b. In conclusion, the cation exchange chromatography profile was in congruence with the native PAGE results. These bands may belong to different proteases, may be different isomers of the same enzyme, or may be the protease artifacts of the same enzyme.

Rai and Mukherjee reported that a bacterium may produce arrays of extracellular protease isoenzymes for its survival and growth in a particular habitat (28). In relation to these findings, there are only a limited number of studies dealing with the isoenzymes of alkaline proteases produced by genus *Bacillus*. Among these studies, Mala and Srividya reported isolating 2 different isoenzymes with molecular weights of 66 kDa and 18 kDa from *Bacillus* sp. Y, which had a close homology with *Bacillus cohnii* YN-2000 protease (29).

Effect of temperature on enzyme activity and stability: Alkaline protease from *B. cohnii* APT5 was found to be active at a wide range of temperatures, between 30 and 75 °C. *B. cohnii* APT5 alkaline protease showed optimal activity at 50 °C and pH 10.0 (Figure 4). The enzyme was found to be stable from 40 to 75 °C and had a half-life of 4 h at 75 °C. It also showed 100% stability for up to 4 h at 50 °C as its optimal temperature and retained all of its activity and stability after 72 h at 30 °C.

In general, most of the commercially available alkaline proteases show optimum activity at temperatures ranging from 50 to 70 °C (1,3). The

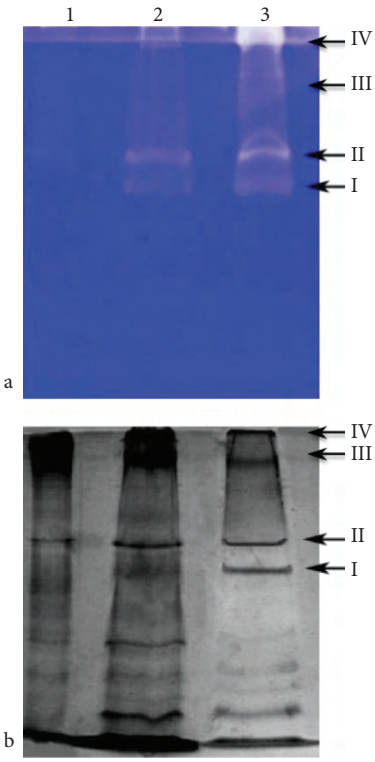

Figure 3. Native PAGE of partially purified *Bacillus cohnii* APT5 alkaline protease. Arrows I, II, III, and IV show 4 different proteolytic activity bands. a) Samples stained with Coomassie brilliant blue R-250, b) samples stained with silver native gel. Lane 1, crude extract; lane 2, acetone precipitation of crude extract; lane 3, partially purified enzyme from cation exchange chromatography.

alkaline protease of *Bacillus* sp. B18 is the enzyme with the highest optimal temperature value (85 °C) among alkaline protease-producing species from the genus *Bacillus* (3). Ferrero et al. reported an alkaline protease from *B. licheniformis* with optimum activity at 60 °C and stability for 10 min between 30 and 60 °C (30). A serine alkaline protease from *B. pumilus* was found to be most active at 55 °C; nevertheless, it retained only 50% of its activity after 30 min of incubation at 50 °C (31). Other studies of serine alkaline proteases from 2 different *B. clausii* strains revealed that both of their enzymes had optimum activity at 60 °C, but that they had different thermostability profiles. While *B. clausii* I-52 was found to be stable for 1 h at 55 °C, *B. clausii* GMBAE 42 showed 55% activity after 30 min of incubation at 60 °C and lost all its activity after 2 h of incubation (27,32). Therefore, it can be concluded

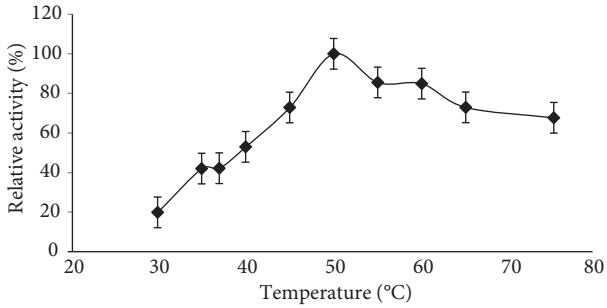

Figure 4. Effect of temperature on *Bacillus cohnii* APT5 alkaline protease.

that the alkaline protease of *B. cohnii* APT5 differs from the above-mentioned proteases of the *Bacillus* species due to its high thermostability at its optimal temperature.

Effect of pH on enzyme activity and stability: The effect of pH on enzyme activity is shown in Figure 5. The enzyme was active over a broad pH range, from 8.0 to 13.0, with an optimum of pH 11.0. Enzyme activity decreased below pH 8.0, and relative activity was measured as 9% at pH 7.0. On the other hand, relative activity began to increase at pH 8 (Figure 5). The highest enzyme activity was between pH 10.0 and 12.0, and at these pH values, the relative activity was 92% and 87%, respectively. The enzyme retained 100% of its activity after incubation at its optimum pH of 11.0 at 30 °C for up to 72 h.

The pH range for alkaline proteases were usually between pH 8.0 and 12.0, and the optimum pH values of various *Bacillus* species differed within the pH ranges from pH 10.0 to 11.0 (1). Nevertheless Nilegaonkar et al. reported that the dehairing

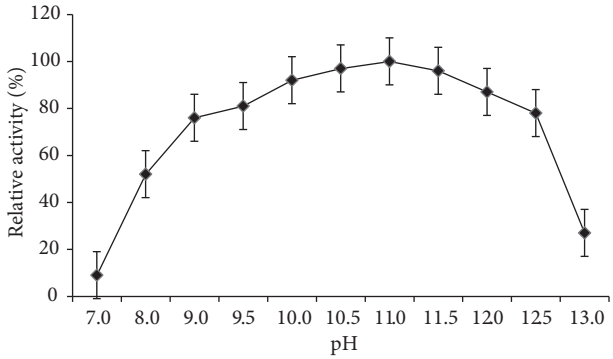

Figure 5. Effect of pH on the activity of *Bacillus cohnii* APT5 alkaline protease.

protease of *B. cereus* MCM B-326 had an optimum pH of 9.0 (33). Apart from this exception, optimal pH values for alkaline proteases from *Bacillus* sp. and *B. licheniformis* were in the range of pH 12.0-13.0 (30,34). On the other hand, the pH stabilities of *Bacillus* alkaline proteases were measured at temperatures varying from 25 to 40 °C for 60 min (34-36). Joo et al. reported a serine alkaline protease from *B. clausii* I-52, which was stable from pH 5.0 to 12.0 after incubation at 30 °C for 3 days (32). An alkaline protease from *B. mojavensis* was found to be stable at pH values ranging from 7.0 to 11.5 at 25 °C for 2 days (37). In addition, the pH stability of a serine alkaline protease from *B. clausii* GMBAE 42 was determined within the pH range of 9.0-11.5 after incubation at room temperature (27). The optimum pH of alkaline protease from *B. cohnii* APT5 was in the range of other bacilli proteases and was also found to be very stable at its optimal pH, which offers great potential, especially in the detergent industry.

Effect of metal ions on enzyme activity: The effect of metal ions on *B. cohnii* APT5 alkaline protease activity is presented in Table 1. The ion Ca^{2+} is known to play a major role in enzyme stability by increasing the activity and thermal stability of alkaline proteases (38,39). In contrast to this finding, there was no effect of the metal ions Ca^{2+} and Zn^{2+} on *B. cohnii* APT5 alkaline protease. In addition, the ions Mg^{2+} and Fe^{2+} showed only a slight inhibitory effect on enzyme activity (4% and 3%, respectively). On the other hand, Mn^{2+} increased enzyme activity by up to 53%. As in previous reports on alkaline proteases from *Bacillus* sp. RGR-14 and *B. clausii* GMBAE 42, *B. cohnii* APT5 alkaline protease activity was stimulated when treated with Mn^{2+} ion, and therefore it was revealed that the enzyme had a requirement for Mn^{2+} as a cofactor (27,40).

Table 1. The effect of metal ions on *Bacillus cohnii* APT5 alkaline protease activity.

Metal ions	Relative activity (%)
Ca^{2+}	101
Mg^{2+}	96
Zn^{2+}	100
Fe^{2+}	97
Mn^{2+}	153

Effect of inhibitors, surfactants, and hydrogen peroxide on enzyme activity: Inhibitors known to be specific to the different classes of proteases were used to determine the nature of protease produced by *B. cohnii* APT5. The effects of inhibitors, surfactants, and hydrogen peroxide on enzyme activity are shown in Table 2. Serine alkaline proteases are known to be completely inhibited by PMSF (41). Most of the earliest studies reported that 1-5 mM concentrations of PMSF generally completely inactivated serine alkaline proteases (31,34,36,42).

Table 2. The effect of some inhibitors, surfactants, and hydrogen peroxide on *Bacillus cohnii* APT5 alkaline protease activity.

Additives	Relative activity (%)
Oxidizing agent	
1% H_2O_2	113
5% H_2O_2	102
Surface active agents	
0.5% (w/v) SDS	105
1% (w/v) SDS	107
1% (w/v) Tween 20	100
1% (w/v) Tween 80	108
1% (w/v) Triton X-100	116
Inhibitors	
2.5 M EDTA	105
1 M Urea	108
1 mM PMSF	100
10 mM PMSF	76
Bacillus sp. (Sigma P5985) + 10 mM PMSF	23
Bacillus licheniformis (Sigma P4860) + 10 mM PMSF	62

However, when the alkaline protease of *B. cohnii* APT5 was treated with 1 and 10 mM concentrations of PMSF, the 1 mM PMSF concentration had no effect on enzyme activity, whereas 10 mM PMSF slightly inhibited the activity with a relative enzyme activity value of 76%. Protease from *Bacillus* sp. (Sigma P5985) and protease from *B. licheniformis* (Sigma P4860) were also subjected to a 10 mM concentration of PMSF, and residual activities were measured at 23% and 62%, respectively (Table 2). Based on previous reports and findings, including the effect of PMSF on alkaline protease from *B. cohnii* APT5, we suggest that APT5 alkaline protease does not belong to the family of serine proteases because of its resistance to high concentrations of PMSF.

Some of the alkaline proteases were found to be metal ion-dependent in view of their sensitivity to chelating agents such as EDTA (43,44). No inhibition was detected when the metalloprotease inhibitor EDTA was added to the alkaline protease of *B. cohnii* APT5. The enzyme was also not inactivated with a 1 M concentration of urea. On the contrary, EDTA and urea activated the alkaline protease, producing relative activity values of 5% and 8%, respectively (Table 2). According to these results, the alkaline protease of *B. cohnii* APT5 does not seem to belong to the metalloproteases.

In addition to temperature and pH stability, a good detergent protease should be stable against various surface active agents and oxidizing agents used as detergent components (27). Investigations of *Bacillus* sp. alkaline protease revealed that a 0.5% concentration of SDS completely inhibited its activity (34). As in the case of *Bacillus* sp. alkaline protease (34), *Bacillus* sp. RKY3 protease was found to be inactivated by 23% with 1% SDS (42). On the other hand, Joo et al. reported a serine alkaline protease from *Bacillus clausii* I-52 that was stable under 1% SDS and could protect 78% of its activity when treated with 5% SDS (32). The enzyme of *B. cohnii* APT5 was treated with up to 1% concentrations of SDS and retained 93% of its activity (Table 2). It is obvious that the alkaline protease of *B. cohnii* APT5 is more resistant to SDS than most of the other bacilli proteases.

When the alkaline protease was subjected to Triton X-100 and the nonionic surfactants Tween 20 and Tween 80, no loss of enzyme activity was detected. Triton X-100 and Tween 80 activated the enzyme, producing relative activity of 16% and 8%, respectively (Table 2). This increase in enzyme activity may be due to the effect of surfactants on the unfolding of the substrate moiety as reported previously (45,46).

The oxidizing agent H_2O_2 in 1% and 5% concentrations showed no inhibitory effect on enzyme activity. By contrast, the enzyme activated 2% and 13% with these H_2O_2 concentrations (Table 2). A previous investigation of alkaline protease from *Bacillus* sp. RGR-14 by Oberoi et al. (40), which was incongruous with our findings, showed a 40% activity loss with 1% H_2O_2 after 1 h of incubation.

In this study, we partially purified and characterized the alkaline protease from a newly isolated *B. cohnii* APT5 strain that was found to be resistant to inhibitors, surfactants, and H_2O_2. It also possesses high pH stability, thermostability, and laundry detergent compatibility. All of these results make *B. cohnii* APT5 alkaline protease suitable for use in the detergent industry as a potential additive.

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