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Production and purification of novel thermostable alkaline protease from *Anoxybacillus* sp. KP1

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Abstract

In this study, an extracellular novel alkaline protease (EC 3.4.21-24, 99) from a thermophilic and aerobic strain of *Anoxybacillus* sp. KP1 has been studied. Maximum protease activity was obtained at 50 °C at pH 9.0 after 24 hours of incubation. Among the carbon and nitrogen sources used; the optimum protease production was with soluble starch, maltose, urea and casamino acid. The enzyme was purified by ammonium sulphate precipitation and Sephadex G-75 gel chromatography. Molecular weight of purified enzyme was determined as 106 kDa by SDS-PAGE. Purified protease was stable at 50–60 °C and at pH 9.0 for 1 h. The enzyme activity was increased in the presence of Ca^{2+}, Cu^{2+}, Tween 80 and Triton X-100, however the enzyme activity was inhibited in the presence of Hg^{2+}, ethylene diamine tetra acetic acid (EDTA) and H_2O_2. Proteolytic activity was completely inhibited by phenyl methyl sulfonyl fluoride (PMSF). The enzyme seems to be a serine alkaline protease. In the presence of detergents, the protease was clearly stable and residual activity was between 73–82%.

Key words: Thermophiles, *Anoxybacillus*, protease, production, purification.

Introduction

Chemicals, materials and energy producing biological systems are significant for industrial biotechnology that basically uses biocatalysis and fermentation technology, along with breakthroughs in molecular genetics, enzyme engineering and metabolic engineering (1). Because of being eco-friendly and used in various industrial applications, including household products, enzymes used in biotechnology are regarded as "green chemicals" (2).

Thermostable enzymes can produce exceptionally high end-product yields and are advantageous in various applications such as utilization of higher processing temperatures, faster reaction rates, an increase in the solubility of nongaseous reactants and products, and reduced incidence of microbial contamination from mesophilic organisms. Therefore, they offer commercial opportunities (3-6).

Proteases, also known as proteolytic enzymes, are enzymes that catalyse the breakdown of proteins by hydrolysis of peptide bonds (7). The main functions of the extracellular proteases are similar to many other polymers degrading extracellular enzymes in nutrition (8). They are omnipresent and exist in all life forms including plants, animals and microbes (9). Proteases have wide industrial applications, mainly in food, detergent, medical, leather, textile, dairy industries and biotechnology (8, 10-12), peptide synthesis, silver recovery from using X-ray films (13, 14). In recent years, many thermophilic and alkaline proteases have been produced, purified, characterized and commercialized (8, 15-17).

Anoxybacillus is a newly identified genus which consists of 29 species. The enzymes of these species are esterase (18), carboxylesterase (19), amylase (5), helicase (20), glucoisomerase (21), but there is no study on the proteases of these species. This study is the first report on production, purification and characterization of detergent stable thermoalkaline protease by using a species of *Anoxybacillus.* Keeping in view the above essentials, this study aimed to purify and biochemically characterize an thermoalkaline protease from *Anoxybacillus* sp. KP1.

Materials and methods

Microorganism growth medium and preparation of crude enzyme

Anoxybacillus sp. KP1 (NCBI GenBank database accession number is KC525949) used in this study was isolated from a Köprü hot spring in Ağrı/Turkey and identified by morphological, physiological, biochemical and 16S rRNA by Matpan Bekler and Güven (22) previously. To investigate the protease production, the strain was inoculated on skim milk agar plates consisting of peptone (0.1%, w/v), NaCl (0.5%, w/v), skim milk (10%, w/v) and agar (2%, w/v) (pH 8.0) and incubated at 50 °C for 48 h. Colonies with a surrounding clear zone in skim milk agar were selected as protease positive. Cultures were grown in 25 mL of basal medium (BM) containing yeast extract (1%, w/v), peptone (1%, w/v), NaCl (0.5%, w/v), $MgSO_4.7H_2O$ (0.01%, w/v), $K_2HPO_4.3H_2O$ (0.7%, w/v), KH_2PO_4 (0.2%, w/v), (NH_4) SO_4 (0.1%, w/v) and Na-citrate (0.05%, w/v). The flasks were inoculated with 250 µL of a cell suspension ($1.3x10^8$ cells/mL) and incubated at 50 °C for 48 hours in a shaking incubator. The cells were precipited at 10,000 g at 4 °C for 10 min, and the supernatant was used as the enzyme source.

Protease assay methods

Protease activity with azocasein (Sigma-Aldrich) was experimented according to method of Leighton et al. (23). According to this method, 250 µL of enzyme

solution mixed with 500 µL of 0.5% azocasein solution was prepared in sodium carbonate (Na_2CO_3) -sodium bicarbonate (Na_2HCO_3) buffer (0.1 M, pH 9.0) and incubated at 50 °C for 30 min. At the end of this duration, in order to stop the reaction, 1 mL of trichloroacetic acid (TCA) (10% w/v) was added and incubated at 4 °C for 15 min. This mixture was centrifuged at 10,000 g at 4 °C for 5 min and 400 µL supernatant was mixed with the 800 µL 1.8 N NaOH in a tube and the absorbance was measured at 420 nm by the spectrophotometer. Enzyme activity was denoted as azo units, where one unit (U) is defined as the amount of enzyme that breaks down the amino acid of 1 micromole of azocasein per minute. The protein content was determined according to the Lowry method (24).

Effect of incubation time on bacterial growth and protease production

In order to determine the effect of different incubation time (4-48 h) on protease production, the culture was grown in 250 mL of liquid BM media (pH 8.0). The samples were taken at the end of every 4th hour over a 48-h period. Bacterial growth was measured at OD_{460} and after that the samples were centrifuged at 10,000 g, and the supernatant was used for the enzyme activity.

Effect of carbon and nitrogen sources on the protease production

In order to determine the effect of various carbon and nitrogen sources on the enzyme production, 1 mL of *Anoxybacillus* sp. KP1 was inoculated into 100 mL of BM containing several carbon sources (2%, w/v), such as fructose, glucose, galactose, lactose, maltose, sucrose, and soluble starch and several nitrogen sources (2%, w/v), such as ammonium sulphate, bacto liver, casamino acid, glycine, tryptone, and urea. After incubating time under the optimum conditions, culture was centrifuged and cell-free supernatant was assayed by the azocasein method.

Among the nitrogen and carbon sources tested, which increased enzyme production, were also examined at various concentrations (0.5%, 1%, 1.5%, 2%, and 3%).

Protein purification

Protease was purified by ammonium sulphate precipitation and gel filtration (Sephadex G-75) chromatography. The crude extract was precipitated using ammonium sulphate to 70% (w/v) saturation. Ammonium sulphate fractions were centrifuged at 10,000 g at 4 °C for 15 min and was dissolved in 1 mM NaOH/Glycine buffer (pH 9.0), and dialyzed overnight against the same buffer. Dialyzed sample was applied to a stirred ultrafiltration cell (PBGC membrane, Millipore). The dialysed enzyme samples were loaded on a Sephadex G-75 (1.5X30 cm) column equilibrated with 0.1 M NaOH/Glycine buffer (pH 9.0) and eluted with the same buffer at a flow rate of 3 mL/min. The enzyme containing fractions were collected, concentrated by ultrafiltration. All purification steps were carried out at 4 °C. In subsequent experiments, the purified enzyme was used for the characterization.

Electrophoretic analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) was carried out for the determination of purity and molecular weight of the protease as described by Laemmli (25). For zymogram of protease activity, the sample was not heated before electrophoresis. After electrophoresis, the gel was submerged in NaOH/Glycine buffer (pH 9.0) containing 2.5% TritonX-100 and 5mM $CaCl_2$ for 45 min, for removing SDS. Then Triton X-100 was removed by washing the gel with 0.1 M NaOH/Glycine buffer (pH 9.0). The gel was then incubated with 0.2% (w/v) gelatine in 0.1 M NaOH/Glycine buffer (pH 9.0) at 45 °C for 45 min. Finally, the gel was stained with Coomassie Brilliant Blue R-250. The development of clear zones on the blue background of the gel indicated the presence of protease activity.

Characterization of purified protease

The optimum pH of the purified protease activity was studied in the range of 4.0–11.0, using the appropriate buffers at a concentration of 0.1 M (sodium citrate buffer pH 4.0-6.0; Tris-HCl buffer pH 7.0–9.0; and glycine-NaOH buffer pH 9.0–11.0). For the measurement of pH stability, the purified protease was incubated at 50 °C for 1 h in different buffers (sodium citrate buffer pH 4.0-6.0; Tris-HCl buffer pH 7.0–9.0; and glycine-NaOH buffer pH 9.0–11.0).

To analyse the effect of temperature, the purified protease activity was experimented at different temperatures between 30 to 90 °C for 15 min at pH 9.0. In order to determine enzyme thermostability, the purified enzyme was incubated at different temperatures (50, 55, and 60 °C) for different time intervals (15-120 min). The comparisons were made using the unheated crude enzyme activity as 100%. The remaining proteolytic activity was measured under standard assay conditions.

Effects of metal ions and inhibitors on the proteolytic activity

The effects of metals and enzyme inhibitors on the purified protease activity were studied by preincubating the enzyme in the presence of substances such as Ca^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+}, ethylene diamine tetra acetic acid (EDTA) and phenyl methyl sulfonyl fluoride (PMSF) with a final concentration of 1.5 mM at 37 °C for 30 min, and then performing the assay at the optimum temperature. All of the metals used were in the chloride form. The comparisons were made using the crude enzyme activity as 100% in the absence of any additives. The residual activity was measured using the standard assay conditions.

Effects of surfactants, oxidizing agent and some commercially detergents on the proteolytic activity

The effects of some polysorbate surfactants (=surface acting agents) such as Tween 40 1% (v/v), Tween 80 1% (v/v) and typical nonionic surfactant Triton X-100 1% (v/v), oxidizing agents (H_2O_2) 1% (v/v) and various commercially laundry detergents such as Omo Matic (Unilever), Ariel (Procter and Gamble) and Persil (Henkel CEE GmbH) (each as 5% w/v) and diluted in tap water) on enzyme stability were studied. The enzyme was pre-incubated at 50 °C for 30 min, after which the

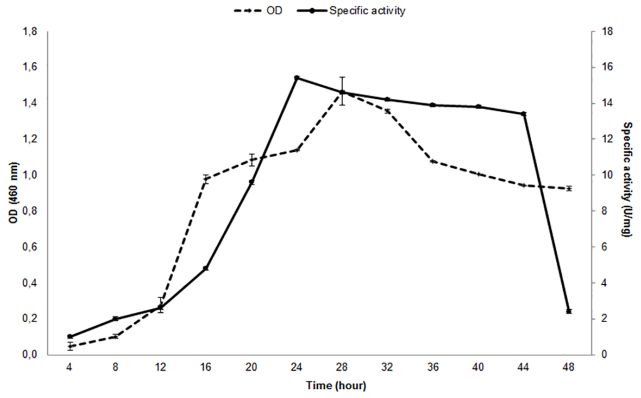

Figure 1. Effect of time-course on bacterial growth and protease production. The cells were incubated at 160 rpm, pH 8.0, at 50 °C for 48 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

assay was performed at the optimum temperature. The comparisons were made using the crude enzyme activity as 100% in the absence of any additives. The residual activities were measured at pH 9.0 and 50 °C.

Statistical analysis

The results are represented as the mean ± SD of at least 3 experiments. The Kruskal Wallis test was done with the SPSS Statistical software for Windows.

Results and Discussion

The *Anoxybacillus* sp. KP1 strain used in this study was isolated from Köprü hot water spring of Diyadin township of Ağri Province in northeastern Turkey. The 16S rRNA gene sequence of the thermophilic *Anoxybacillus* sp. KP1 (accession number: KC525949) was very similar (99.27%) to that of *Anoxybacillus kamchatkensis* JW/VK-KG4 (accession number: AF510985). Since no studies have been made on Köprü hot water spring, our study is significant for the isolation of new thermophilic microorganisms which are of biotechnological importance. In addition, this study presents novelty due to the isolation and characterization of alkaline protease obtained from a thermophilic *Anoxybacillus* species, which has not been reported previously.

Effect of incubation time on bacterial growth and protease production

As shown in Figure 1, enzyme production by *Anoxybacillus* sp. KP1 increases to maximum level up to 24 h incubation (15.4 U/mg) and after 44 h, it decreases sharply to minimum levels (2.4 U/mg). Abusham et al. (26) and Akhavan Sepahy and Jabalameli (27) have also reported that maximum protease production occurred at 24 hour for *Bacillus subtilis* and *Bacillus* sp., respectively. As can be seen from Figure 1, maximum bacterial growth is obtained at 28 hour. Obtaining the maximum bacterial growth and enzyme production in a short time period is of importance in possible industrial use.

Effect of carbon and nitrogen sources on the protease production

In the present study, 2% (w/v) soluble starch was found to be the best substrate for protease production (24 U/mg; Fig. 2). Johnvesly and Naik (28) and Chi and

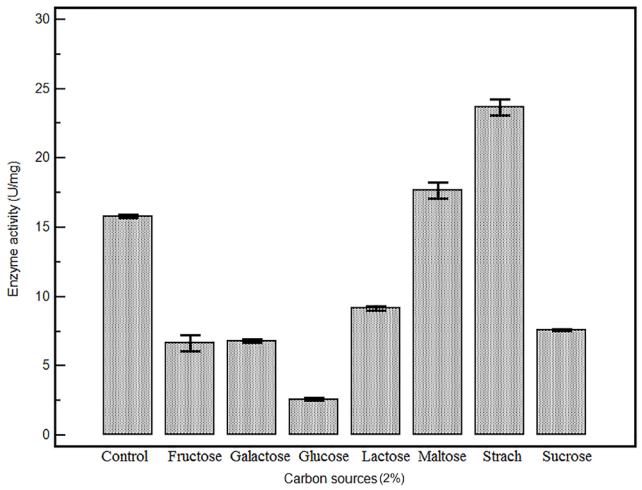

Figure 2. Effect of carbon sources on protease production. For this experiment, different carbon sources were added into the medium at 2.0% (w/v) and incubated at 160 rpm, pH 8.0, at 50 °C for 28 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

Zhao (29) also found that extracellular amylase hydrolyzes starch as sole carbon source for protease production in the medium and claimed that starch is the best carbon source for the fermentation industry due to its low cost and being an easily obtained material. Protease production was also increased in the presence of 2% (w/v) maltose (17 U/mg), while glucose, galactose, fructose, lactose and sucrose inhibited enzyme production. Phadatare et al. (30) and Bajbaj and Sharma (31) determined maltose being the best carbon source for the ability to induce protease production. Chu (32) has reported that glucose decreases protease production. This could be due to catabolite repression by high glucose available in the medium. We have also tested the effects of various concentrations of soluble starch and maltose that do not increase the enzyme production at concentrations higher than 2% (w/v).

The effect of different nitrogen sources, including ammonium sulphate, bacto liver, casamino acid, gly-

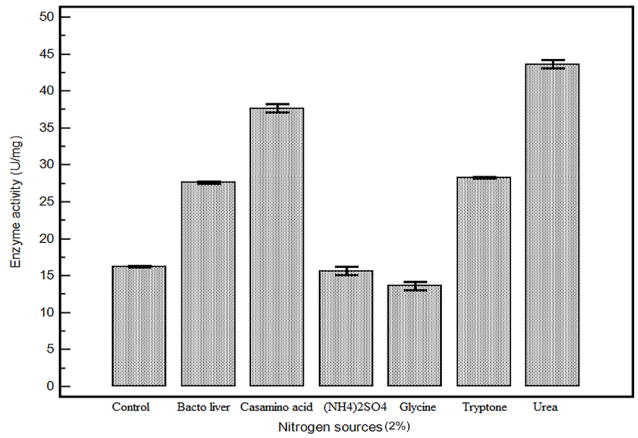

Figure 3. Effect of nitrogen sources on protease production. For this experiment, different nitrogen sources were added into the medium at 2.0% (w/v) and incubated at 160 rpm, pH 8.0, at 50 °C for 28 hours. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols.

Table 1. Purification steps of protease.

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	13.31	218.14	16.39	1	100
Ammonium sulphate precipitation and dialysis	0.385	45.81	188.78	7.2	21
Sephadex G-75	0.024	6.10	254.16	15.5	2.8

cine, peptone, tryptone, urea was also studied. In Figure 3, 2% (w/v) of urea and casamino acid seems to be best nitrogen sources for enzyme production (44 U/mg and 38 U/mg, respectively). Moreover, bacto liver and tryptone increase the protease production to some levels (27.6 U/mg and 28.2 U/mg, respectively). The addition of glycine and ammonium sulphate showed no effect on the production of protease for *Anoxybacillus* sp. KP1. We have also tested the effects of various concentrations of urea and casamino acid that do not increase enzyme production at concentrations higher than 2% (w/v). Suganthi et al. (33) investigated the effect of various nitrogen sources for protease production and they determined that high yield of protease production was observed in the presence of urea. Chauhan and Gupta (34) and Patel et al. (35) have reported that casamino acids were found as effective ingredients for the protease production. Most microorganisms are capable of metabolizing organic and inorganic nitrogen sources to biosynthesize amino acids, nucleotides, proteins and other cell components (35). This study shows that there must be carbon and nitrogen sources in the medium for the stimulation of alkaline protease production for use in industrial applications, which can easily and cheaply be obtained.

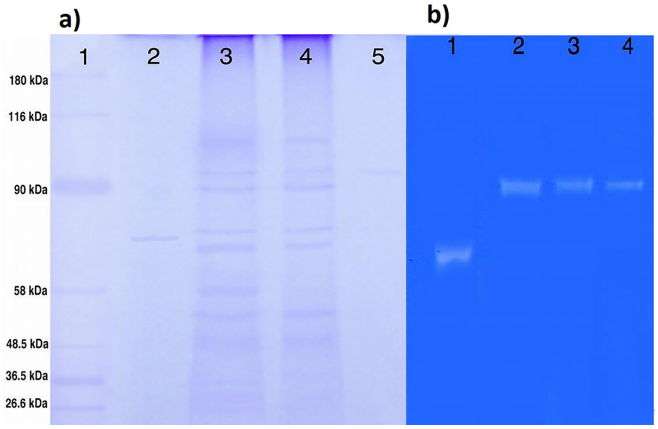

Figure 4. (a): SDS-PAGE Lane 1, molecular mass markers [Sigma SDS7B2: α_2-macroglobulin (180 kDa), β-galactosidase (116 kDa), lactoferrin (90 kDa), pyruvate kinase (58 kDa), fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triosephospate isomerase (26.6 kDa)]; lane 2, protease from *Bacillus polymyxa* (75 kDa); lane 3, crude extract; lane 4, ammonium sulphate precipitation/dialysis and lane 5, Sephadex G-75 **(b):** Zymogram with the nondenatured polyacrylamide gel electrophoresis containing 1% Gelatine (lanes 1, protease from *Bacillus polymyxa* (75 kDa); lane purified enzyme; lane 2, crude extract; lane 3, ammonium sulphate precipitation/dialysis and lane 4, Sephadex G-75.).

Biochemical properties of the purified protease

Protease was purified by ammonium sulphate and gel filtration (Sephadex G-75) chromatography methods (Table 1). The results showed that enzyme was purified to 15.5 fold with an activity yield of 2.8%. The characterization of enzyme in the following experiments was carried out on the purified enzyme. The molecular weight of purified enzyme was calculated as 106 kDa by SDS-PAGE (Fig. 4). Hernández-Martínez et al. (36), Cho et al. (37), Sookkheo et al. (6), Zhu et al. (38), Waghmare et al. (39) and Jain et al. (40) found the molecular weight of some proteases as 88kDa, 102 kDa, 36, 53-71 kDa, 59.2 kDa, 98 kDa and 71 kDa from *Aspergillus fumigatus, Aeromonas hydrophila, Bacillus stearothermophilus* TLS33, *Geobacillus* sp. YMTC 1049, *Stenotrophomonas maltophilia* and *Bacillus* sp. respectively.

Effect of temperature and pH on the activity and stability of the purified protease

As can be seen from Figure 5, the enzyme activity was examined at pH values between 4.0 and 11.0. The enzyme activity increased to optimum value of pH 9.0, after that the activity decreased sharply. The protease activity remained about 70% when the enzyme was incubated between pH 6.0 and 10.0 at 50 °C for 1 h (Fig. 5). The enzyme produced by *Anoxybacillus* sp. KP1 seems to be an alkaline protease. There are several stu-

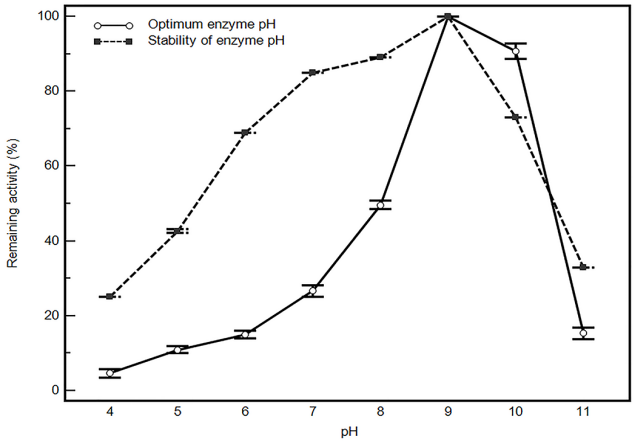

Figure 5. Effect of pH on activity of *Anoxybacillus* sp. KP1 protease. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols. The values are shown as percentages of the maximum activity of enzyme observed at pH 9.0 and 50 °C, which is taken as 100%. For the measurement of pH stability, the purified protease was incubated at 50 °C for 1 h in different buffers. The remaining proteolytic activity was measured under standard assay conditions.

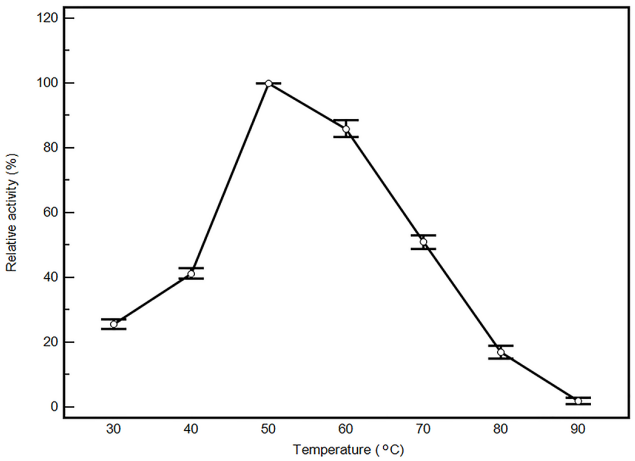

Figure 6. Effect of temperature on activity of *Anoxybacillus* sp. KP1 protease. The results represent the means of three experiments, and bars indicate ± standard deviation. Absence of bars indicates that errors were smaller than symbols. The values are shown as percentages of the maximum activity of enzyme observed at 50 °C, which is taken as 100%.

dies on different *Bacillus* species, where optimum pH for the protease activity was 9.0 (14, 27, 41-44).

The effect of temperature on the activity of the purified *Anoxybacillus* sp. KP1 protease was tested at different temperatures at pH 9.0. The enzyme was active between 50 and 70 °C temperature range, with an optimum at 50 °C (Fig. 6). The relative activities at various temperatures (50, 55 and 60 °C) for studying enzyme thermostability, using azocasein as a substrate, are depicted in Fig. 7. The enzyme is highly stable at 50 °C for 120 min. Almas et al. (45) determined that protease was able to maintain its stability at 50 °C for 1 h for *Bacillus* strain SAL1. Thermostability is a critical feature required of proteases for industrial applications such as detergent and leather processing (11, 46, 47). Hence, stability of *Anoxybacillus* sp. KP1 protease may have an advantage of using it in laundry detergent formulations.

Effects of metal ions and inhibitors on the purified protease

The effects of inhibitors and metal ions on protease activity were investigated (Table 2). The protease activity was increased in the presence of Ca^{2+} (121%) and Cu^{2+} (113%). In previous studies, Sookkheo et al. (6), Ghorbel et al. (48), Nascimento and Martins (49), Arulmani et al. (50) and Ahmetoğlu et al. (51) have reported that protease activity increased in the presence of Ca^{2+}. An increase in the activity in the presence of Ca^{2+} may be due to stabilization of enzymes in its active conformation rather than it being involved in the catalytic reaction. It probably acts as a salt or an ion bridge via a cluster of carboxylic groups (12). In addition, Johnvesly and Naik (28) reported that protease activity was increased in the presence of Cu^{2+}. Table 2 also shows that enzyme activity was reduced in presence of Hg^{2+} (a thiol group inhibitor), Zn^{2+} and EDTA (residual activities of 57%, 57% and 19%, respectively). Tsuchya et al. (52) and Shah et al. (44) stated that a stronger inhibitory effect was observed in the presence of Hg^{2+}. Lamed et al. (53) also explained that the enzyme inhibition by Hg^{2+} is not only through binding to thiol groups, but may be the result of interaction with tryptophan residue or the

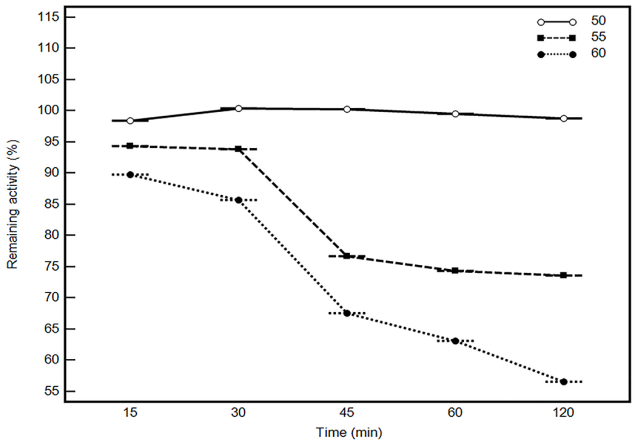

Figure 7. Effect of temperature on stability of *Anoxybacillus* sp. KP1 protease. The unheated crude enzyme was taken as 100%. The remaining proteolytic activity was measured under standard assay conditions.

Table 2. Effects of metal ions, inhibitors, surfactans, oxidizing agent, some commercial detergents on the proteolytic activity.

Metal ions and inhibiors (1,5 mM)	Residual Activity (%)
Ca^{2+}	121±1.25
Cu^{2+}	113±0.84
Hg^{2+}	57±0.91
Mn^{2+}	94±1.03
Zn^{2+}	57±1.01
EDTA	19±0.79
PMSF	0
Surfactans, oxidizing agent, some commercially detergents	**Residual Activity (%)**
Tween 40 (1%)	102.1±0.89
Tween 80 (1%)	105.1±0.73
Triton X-100 (1%)	111.3±1.01
H_2O_2 (1%)	25.1±1.55
Omo matic (5%)	73.5±0.76
Ariel (5%)	82.4±1.14
Persil (5%)	78.2±1.07

*Residual activity was determined as percentage of control with no additions.

carboxy group of amino acid in the enzyme. Moreover, proteolytic activity of *Anoxybacillus* sp. KP1 protease was completely inhibited by PMSF, which is known as a protease inhibitor (54-56). Adinarayana et al. (57) and Matpan Bekler et al. (58) have stated that PMSF blocks the active site of proteases by sulfonating the essential serine residue, resulting in complete inhibition of protease activity. The results obtained show that *Anoxybacillus* sp. KP1 protease is a member of alkaline serine proteases.

Effects of surfactants, oxidizing agent, some commercially detergents on the proteolytic activity

Among nonionic surfactants such as Tween 40, Tween 80 and Triton X-100 (each in 1% w/v) tested, the protease activity was increased in the presence of Tween 40, Tween 80 and Triton X-100 as 102%, 105% and 111%, respectively. In contrast, oxidizing agent H_2O_2 inhibited protease activity to a great extent (residual activities of 25%; Table 2). Tween 40 and Tween 80 are generally known as gentle surfactants, they do not affect protein activity and they are effective in solubilization and may have improved the permeability of

the cell membrane through disruption of lipid bilayer (59). Subba Rao et al. (60) and Divakar et al. (12) also found that the protease activity increased in the presence of Triton X-100. Nonionic surfactant such as Triton X-100 is considered mild surfactant as it breaks protein-lipid, lipid-lipid associations, but not protein-protein interactions, and does not denature proteins. Therefore, proteins are solubilized and isolated in their native and active form, retaining their protein interactions (61).

We have also tested the stability of laundry detergents, and found that purified alkaline protease activity remained 73%, 82% and 78% in the presence of various commercial laundry detergents such as Omo Matic, Ariel and Persil, respectively (Table 2). Several studies have also reported the stability of protease in the presence of laundry detergents for *Bacillus mojavensis* A21 (14), *Bacillus licheniformis* MP1 (46) and *Bacillus circulans* (60). The detergent stability of proteases is an important feature for their industrial applications.

Conclusion

Our study shows that thermophilic *Anoxybacillus* sp. KP1 secretes extracellular protease which seems to be the first report for *Anoxybacillus* species. Our study also shows that this enzyme can be used in industrial applications such as detergent industry as extracellular protease due to its production in a short time and for lower costs, and in addition, for being active in alkaline pH and in high temperature, and particularly for being stable in laundry detergents.

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