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Characterization of an Oxidant and Detergent Stable Alkaline Protease Produced from a Novel Isolate *Bacillus* sp. Strain

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Abstract

Isolation, partial purification and characterization of a protease from an alkaliphile *Bacillus* sp. strain PX6 isolated from soil sample. For this purpose, the growing and the enzyme producing abilities were researched either in different temperatures or in different pH ranges, and the optimal enzyme production was obtained at pH 8.0 and 40°C. The optimum pH and temperature of the enzyme was determined at pH 8.0 and 50°C. After a pre-incubation of 60 min at 30-50°C, original activity of the enzyme was conserved 69%, and at 60-110°C original activity of the enzyme was conserved 13%. After pre-incubation of 24 h, the original activity was increased 55% at pH 6.0-9.0, retained 88% at pH 10.0 and decreased 13% at pH 11.0-12.0. While the PX6 protease was pre-treated with 2.5 mM $MgCl_2$, $BaCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, NaCl, KCl at ambient temperature for 60 min, the original activity was retained 34%, 23%, 60%, 31%, 43%, 63%, 71%, respectively, it was induced 17% with $FeCl_2$. With a pre-treatment of 60 min, enzyme activity was retained 40%, 29%, 57% in the presence of 2.5 mM EDTA, 4 M Urea, 0.5% SDS respectively, while it increased 260%, 329%, 186%, 280%, 134% in the presence of (0.5%) Triton X-100, Tween 80, Tween 20, H_2O_2, β-Mercaptoethanol, respectively. As a serine protease inhibitor, PMSF (0.5%) almost totally inhibited the activity. After pre-incubation of 60 min with commercial detergents at 30°C, activity of the enzyme PX6 was conserved 100% with 3.5 mg/ml solid laundry detergent, 31% with 0.5% (v/v) 4.6% sodium hypochlorite and 23% with 0.5% (v/v) dishwashing liquid.

According to the results, the PX6 protease is alkaline and thermo-tolerant. To be stable and inductive against various detergents, surfactant and oxidizing agents, shows that this enzyme can be used in detergent, paper and textile industries and also in detergent formulations by having the effectiveness to enhance the effect of commercial detergents.

Key words: *Bacillus* sp., Alkaline protease, Detergent, Thermo-tolerant, Oxidant

INTRODUCTION

Enzymes have attracted attention from researchers all over the world because of the wide range of physiological, analytical and industrial applications, especially, from microorganisms, because of their broad biochemical diversity, feasibility of mass culture and ease of genetic manipulation [1]. Proteases, also known as peptidyl-peptide hydrolases (EC 3.4.21-24 and 99), are industrially useful enzymes, which catalyze the hydrolysis of a peptide bond in a protein molecule [2]. Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially [1]. Alkaline proteases are one of the most widely studied groups of enzymes because of their use in many industrial applications such as in food, pharmaceutical, leather and detergent industries [3]. Their major application is in detergent industry, because the pH of laundry detergents is generally in the range of 9.0–12.0, accounting for about 35% of the total microbial enzymes sales. Bacteria, moulds and yeasts are some of the microorganisms that produce proteases. Most of the commercial extracellular alkaline proteases are isolated from Bacteria of the genus *Bacillus* [3, 4].

In this work we isolated an alkaline protease producing bacteria from a soil sample and identified it as *Bacillus* sp. PX6. This article describes the optimization and characterization of the protease from this strain.

MATERIALS AND METHODS

Organism

Isolation of Bacillus sp.

Bacillus sp. PX6 was isolated from soil samples collected in Adana, Turkey. To ensure the growth of spore forming bacteria by inhibiting the vegetative forms, soil samples were pre-incubated at 80ºC for 10 min [5]. The bacterial strain was grown on N1 medium at 40ºC. The isolated bacteria were identified as *Bacillus* sp. by morphological and some biochemical tests [6].

Screening of proteolytic bacteria

The organism was grown on skim milk agar for determine protease producing ability. Medium reported by Singh et al. (2001) was used to obtain these proteolytic bacteria [8]. The modified composition of the medium was (g/L): peptone 10, meat extract 3, NaCl 5, skim milk 11, agar 15. The initial pH was adjusted with 1 N NaOH to pH 10.0. Colonies with the clearing zone around, showed the protease activity.

Protease assay

Protease activity was performed according to the method reported by Shah et al. (2010) with minor modification. Casein used as a substrate. The enzyme assay was carried out using 500 µL of Na-phosphate buffer (100 mM, pH 8.0), 500µL of 2% casein solution and 1000 µL of enzyme solution and incubating at 40°C for 60 min. The reaction was terminated by adding 2000 µL 10% trichloroacetic acid (TCA) solution and samples were kept on ice for 15 min. The non-hydrolyzed casein then removed by centrifugation at 10,000 rpm for 10 min and the absorbance was measured at 280 nm. Proteolytic activity in U/mL is defined as increase in absorbance caused by 1 mL enzyme/h under the given assay conditions.

Identification of the bacteria

The strain that showed the highest protease activity was determined through VITEK-2 (bioMerieux) bacteria identification system. For this purpose, bacteria from stock culture was inoculated to trypticase soy broth and incubated for 18 h at 37°C under reciprocal agitation at 150 rev/min for activation. Then inoculation from this culture to trypticase soy broth was held and incubated for 18-24 h at 37°C. Fresh bacteria culture was homogenized with 0.45% sterile NaCl and loaded to the system as required. The results were obtained in 12-16 h after loading.

Optimization of media for growth and protease production

Bacillus sp. strain PX6 was cultivated at different temperatures (20-60ºC) and pH ranges (6.0-13.0) to find out the optimal pH and temperature condition for growth on skim milk agar plates. After an incubation of 40 h, the maximum activity zone of protease secreted from PX6 detected at 40ºC and pH 8.0 [10].

Characterization of protease

Optimum pH and temperature

The effect of pH on protease activity was investigated at pH 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 with an incubation of 60 min at 40ºC. For this purpose three different pH buffer solutions were utilized; 0.1 M Na-phosphate buffer (pH 6.0-8.0), Glycine-NaOH buffer (pH 8.5-10.5) and Borax-NaOH buffer (pH 11.0-13.0) [11]. The pH value that showed the highest activity was selected as the optimum pH for the enzyme.

Effect of temperature on protease activity was determined by performing an activity assay for protease at 30-100°C at 10 degree intervals. Temperature exhibited the highest protease activity was defined as the optimum temperature for this enzyme.

Effect of pH

The effect of pH on protease activity was examined using different pH buffer systems (0.1 M) Na-phosphate buffer (pH 6.0-8.0), Glycine-NaOH buffer (pH 8.5-10.5) and Borax-NaOH buffer (pH 11.0-13.0). For determining stability of the protease at different pH values, the enzyme was pre-incubated for 24 h at different pH values (6.0-12.0) using the appropriate buffer, and the residual activity was assayed.

Effect of temperature

Thermo-stability of the protease was examined by pre-incubation of the enzyme preparation at different temperatures ranging from 30 to 110°C at 10 degree intervals for 60 min, and then by determining the residual activity.

Effect of metal ions, inhibitors and surfactants

To investigate the effect of metal ions on protease activity, the following chemicals were tested at the concentration of 2.5 mM; $MgCl_2$, $BaCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, NaCl, KCl and $FeCl_2$. Inhibitors including 2.5 mM EDTA, 0.5% PMSF and β-Mercaptoethanol, 4 M Urea, and surfactants such as SDS, Triton X-100, Tween 80, Tween 20 and H_2O_2 in a final concentration of 0.5% were investigated. The protease samples were incubated for 60 min at ambient temperature with the presence of various chemical agents described above. The activity of the enzyme preparation in the absence of chemicals was defined as the 100% level and the activity of the enzyme pre-incubated with the chemicals were estimated according to this level.

Effect of commercial detergents

The compatibility of PX6 protease with some commercial detergents was studied using solid laundry detergent, 4.6% sodium hypochlorite and dishwashing liquid. The detergents were diluted in tap water to give a final concentration of 3.5 mg/ml for solid laundry detergent, 0.5% (v/v) for sodium hypochlorite, 0.5% (v/v) for dishwashing liquid. The enzyme preparation was incubated with diluted detergents for 60 min at 30°C and the residual activity was determined at pH 8.0 and 50°C. The enzyme activity of a control (without detergent) incubated under the similar conditions, was taken as 100% [12].

Electrophoresis and zymogram analysis

SDS-PAGE was performed by the modified method of Laemmli (1970) using 5% stacking gel and 10% separating gel. 3% casein solution was added into the separating gel for the later zymogram analysis. The samples loaded were prepared by mixing enzyme with the sample buffer in a ratio of 5:1 (v/v) and boiled for 5 min. Following the electrophoresis the gel was cut into two and marker column stained with Coomassie Brilliant Blue R-250 (1 g/L) in methanol-acetic acid-water (45:10:45) for 2 h and destained with the solution containing; methanol, acetic acid and water (10:10:80) [14].

After SDS-PAGE analysis the gel submerged in 2.5% Triton X-100 for 60 min with constant agitation to remove SDS. The gel was incubated with 50 mM Tris-HCl buffer (pH 8.0) at 50°C for 30 min. This gel was then stained with Coomassie Brilliant Blue R-250 and destained with the same destaining solution. Finally, the clear zones appeared on the blue background of the gel indicated the presence of protease activity. Molecular weight of the enzyme was estimated using bovine serum albumin (66,000 Da) as the marker.

RESULTS AND DISCUSSION

Organism

Isolation and Screening proteolytic bacteria of Bacillus sp.

Protease producer strains of *Bacillus* were determined after 24 h at 40°C by visualizing the clear zone formed surrounding of the colonies on the skim milk agar. Depending upon the zone of clearance, PX6 strain was selected for further experimental studies. The isolated proteolytic strain was a spore-forming gram-positive rod, identified as 88% of *Bacillus cereus/ thuringiensis/ mycoides* by VITEK2 bacteria identification system, and it was termed as *Bacillus* sp. PX6.

Optimization of production medium for growth and protease secretion

Effect of pH and temperature

PX6 was grown well within the pH range 6.0-12.0 at between 20-60ºC temperatures on skim milk agar medium for 40h. The optimum growth and protease activity on agar plates were observed at 40ºC and pH 8.0 (Fig. 1, 2). *Bacillus* sp. JB-99 was able to grow over a wide range of temperature (30–65°C) and pH (6.0-12.0). The enzyme secretion was maximum at 55°C and pH 8.0-10.0 [15]. *B. subtilis* PE-11, maximum growth and maximum enzyme production was observed at 48 hours on milk agar at 37°C [10].

Characterization of protease

Optimum pH

The effects of pH on the enzyme activity was examined over a pH range of 6.0-12.0 at 40°C. The maximum activity obtained at pH 8.0 was considered 100%. PX6 protease showed more than 90% activity over a broad pH range 6.0-12.0 (Fig. 3). The optimum pH of *Bacillus cereus* AK1871 purified protease was 8.0 with 60–90% activity over a broad range of pH 6.0–9.0 [9]. The optimum activity of *Bacillus polymyxa* B-17 enzyme was observed at pH 7.5 [16]. Besides, BM1 protease displayed activity over a broad pH range 6.0–12.0 with an optimum from pH 8.0 to 10.0, BM2 protease exhibited optimum activity at pH 10.0 and more than 50% of the activity was detected at pH 7.0 and 11.0 [12]. The optimum pH and high activity over a broad pH range for proteases of *Bacillus* sp. were reported to might be candidate for detergent industry which is in agreement with the present study.

Optimum temperature

The optimum temperature of PX6 protease activity was determined by assaying enzyme activity at various temperatures for 60 min at pH 8.0. The optimum activity of enzyme was observed at 50°C. PX6 retained averagely 43% and 41% of its activity between 30°C to 40°C and 60°C, respectively. However relative activity of the enzyme decreased to 19% at 70-100°C (Fig. 4). Maximum activity of B-17 protease was also determined at 50°C [16]. The optimum temperature for BM1 and BM2 purified proteases were 60°C [11]. *Bacillus* sp. JB-99 showed maximum activity at 70 °C [15]. For applications in detergents and for tanning processes, alkaline proteases with an optimal temperature of 50–70°C and an optimal pH of 9.0–12.0 are desirable [12]. According these properties, *Bacillus* sp. PX6 protease termed as thermo-tolerant enzyme.

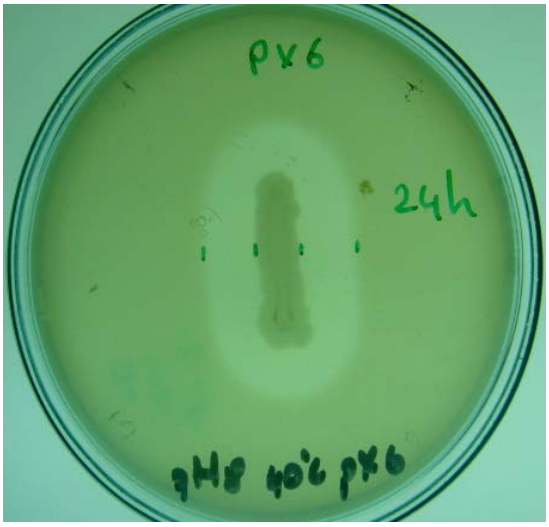

Fig 1. The highest activity zone was detected at pH 8.0, 40°C on skim milk agar after a 24 h of incubation.

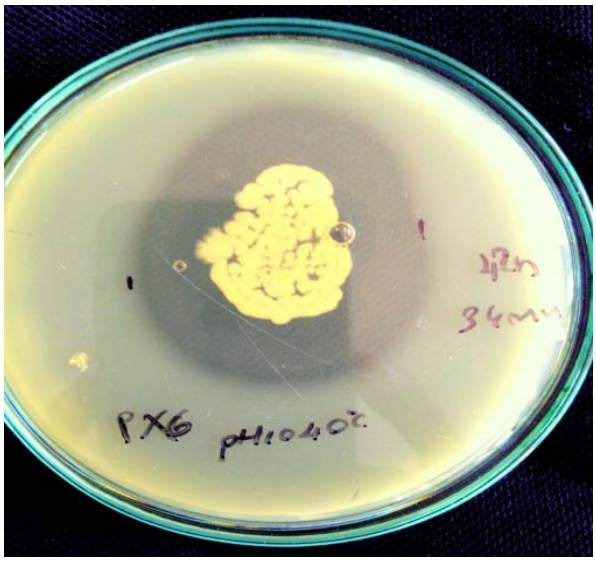

Fig 2. The activity zone after an incubation of partially purified enzyme (500 µl) dropped onto the skim milk agar at pH 10.0, 40°C for 10 h.

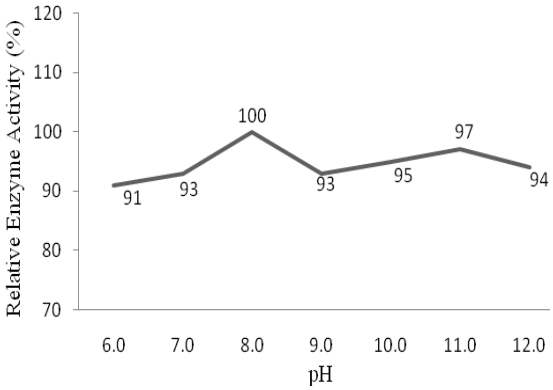

Fig. 3. Effect of pH on the activity of protease

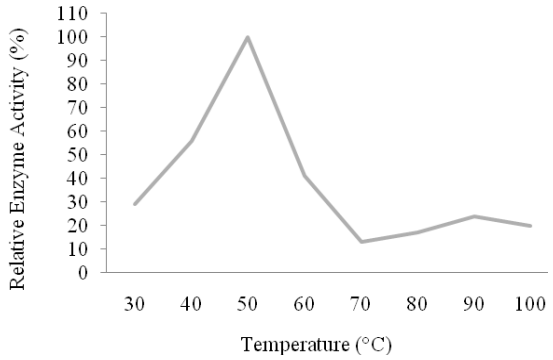

Fig. 4. Effect of temperature on the activity of protease

Effect of pH

The purified protease enzyme was pre-incubated in buffers at various pH values for 24 h at 30°C. PX6 protease was highly stable over a pH range of 6.0-9.0 and it was also stimulated 55% approximately. The enzyme retained 88% of its original activity at pH 10.0. However, the activity decreased rapidly to 13% at pH 11.0-12.0 (Fig. 5). BM1 and BM2 proteases are highly stable over a broad pH range, maintaining 100% of its original activity at pH values between 7.0-10.0 and pH 8.0–9.0 after incubation at 40°C for 60 min, respectively [12]. AprB was stable between pH 5.0 and 12.0 and retained about 90% of its activity after incubation at 30°C for 6 h [17]. *Bacillus licheniformis* NH1 protease enzyme was stable between pH 5.0 and 12.0, and higher stability was observed in the range of pH 10.0–12.0 [3]. NG312 was fairly stable at a wide range of pH values from 7.0–11.0, with maximum stability being exhibited at pH 9.0–10.0 [18]. According to literature datas, PX6 protease enzyme has higher stable properties than other alkaline protease enzymes and also over a broad pH values are significantly induced its activity.

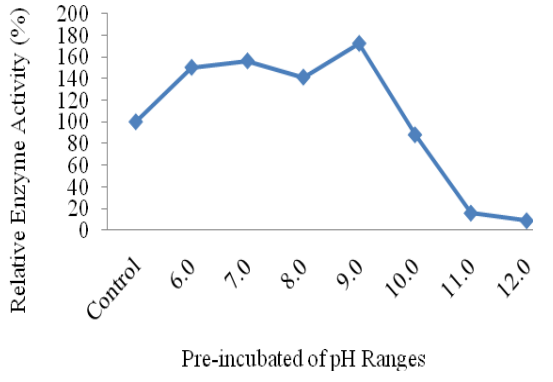

Fig. 5. Effect of pH on the stability of protease from *Bacillus* sp. PX6

Effect of temperature

For effect of temperature, samples were pre-incubated for 60 min in 0.1M glycine–NaOH buffer (pH 8.0) at different temperatures. The thermal stability analysis showed that PX6 enzyme was highly stable at temperatures below 50°C but was inactivated at higher temperatures. It retained 78%, 82% and 42% of its initial activity at 30°C, 40°C and 50°C, respectively and orjinal activity inhibited about 13% at temperatures ranging from 60 to 110°C (Fig. 6). BM1 and BM2 proteases are highly stable at temperatures below 50°C but were inactivated at higher temperatures. Both enzymes retained more than 55% of their initial activity after 1 h incubation at 50°C, while 11.76% and 19% of the maximal activity of BM1 and BM2 proteases remained within 10 min of incubation at 60°C, respectively [12]. *Bacillus polymyxa* B-17 purified enzyme retained more than 95% residual activity after exposure to 35–50°C for 10 min. The enzyme activity however declined at higher temperatures, but it retained 35% activity even after heat treatment at 70°C for 10 min. After 10 min at 85°C no activity was recorded [16]. The *Bacillus* sp. JB-99 protease enzyme was stable from 40 to 70°C and beyond this temperature activity decreased [15]. Up to 60°C *Bacillus licheniformis* MIR 29 alkaline protease activity was stable (100%), but about 70% was lost at 90°C for 10 min [19].

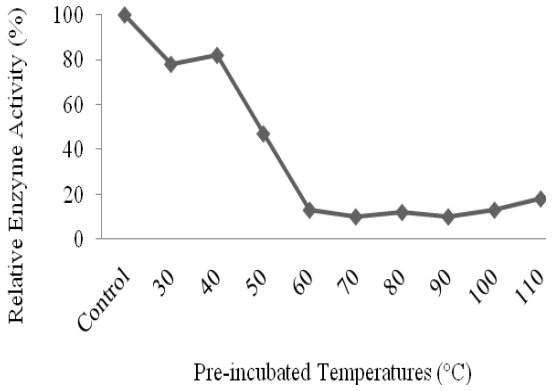

Fig. 6. Effect of temperature on the stability of protease from *Bacillus* sp. PX6

Effect of metal ions, inhibitors and surfactants

By using specific inhibitors the class of enzyme active site involved in reaction and its cofactor requirements may be studied [15]. To investigate the effect of metal ions on *Bacillus* sp. PX6 enzyme activity, the following chemicals were tested at the concentration of 2.5 mM: $MgCl_2$, $BaCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, NaCl, KCl and $FeCl_2$ for 60 min at 30°C. The original activity was conserved %34, %23, %60, %31, %43, %63, %71 after pre-incubation at $MgCl_2$, $BaCl_2$, $ZnCl_2$, $CaCl_2$, $MnCl_2$, NaCl, KCl, respectively. $FeCl_2$ was enhanced 17% of protease activity (Table 1). Ferrero et al. (1996) reported that no increase of the activity after pre-incubation of $CaCl_2$. In the presence of 5M sodium chloride *Bacillus* sp. JB-99 protease retained 61% of activity after 12h pre-incubation at 30°C [15]. A serine alkaline protease from *Bacillus* sp. SSR1 was reported to retain about 221.3–237.2% relative activities with 10–20 mM of Fe_2^+ [7]. The activity of PX6 enzyme was decreased to 6% by PMSF which is a serine protease inhibitor (Table 2). PMSF, almost or completely inhibited the protease enzyme activity [9, 4, 18]. This result indicated that the protease PX6 is a kind of serine protease [20]. In the presence of nonionic surfactants as Tween 20, Tween 80 and Triton X-100, PX6 protease activity was increased by 186%, 329% and 280%, respectively (Table 3). Oxidizing and bleaching agent of hydrogen peroxide enhanced protease activity by 280%, showing that alkaline protease is also excellent bleach stable. Deng et al. (2010) reported that upon incubation with 5% of Tween 20, Tween 80, and Triton X-100 at 30°C for 24–72 h, AprB exhibited enhanced residual activities

between 105–112%. In the presence of 3% Triton X-100, *Bacillus* sp. JB-99 enzyme retained 95% of activity and 5% hydrogen peroxide enhanced enzyme activity by 107% [15]. In the detergent industry, several oxidizing agents like sodium perborate used as detergent compositions, which may release hydrogen peroxide and hence bleach stable enzymes are prepared for detergent industry [15]. Due to these properties, *Bacillus* sp. PX6 alkaline protease seems to be suitable for the detergent industry. However, the PX6 enzyme was unstable against one of the strong anionic surfactant of SDS and retained 57% of its activity at concentrations 0.5% after 60min pre-incubation at 30°C. SDS significantly reduced the AK1871 protease activity by 55% [9]. In the presence of 0.5% SDS (w/v) *Bacillus* sp. JB-99 enzyme retained 78% activity at 40 °C after 2 h pre-incubation [15]. EDTA (2.5 mM) and urea (4 M) inhibited PX6 enzyme activity by 60% and 71%, respectively. On the other hand, another inhibitor β-mercaptoetanol stimulated protease activity to 134%. Metalloprotease inhibitor EDTA (5 mM) inhibited the NH1 protease enzyme activity by 70% [4]. Matta and Punj (1998) were reported that *Bacillus polymyxa* B-17 purified protease enzyme retained 92.7% of its activity after pre-incubation at concentration of 1mM β-mercaptoetanol at 50°C for 30 min. In this respect, *Bacillus* sp. PX6 protease is similiar to proteases of *Bacillus* sp. were not or only slightly inhibited by β-mercaptoetanol [10, 17, 12]. Increase of PX6 protease activity by β-mercaptoetanol indicates that active site of enzyme may include significant amount of cystine aminoacids [8].

Table 1. Effect of Metal Ions

Metal Ions (2.5 mM)	Relative activity (%)
Control	100
$MgCl_2$	34
$BaCl_2$	23
$ZnCl_2$	60
$CaCl_2$	31
$MnCl_2$	43
$FeCl_2$	117
$NaCl_2$	63
KCl	71

Table 2. Effect of Inhibitors

Inhibitors	Relative activity (%)
Control	100
EDTA (2.5 mM)	40
PMSF (0.5%)	6
β-mercaptoethanol (0.5%)	234
Urea (0.5%)	29

Table 3. Effect of Surfactants

Surfactants (0.5%)	Relative activity (%)
Control	100
SDS	57
Triton X-100	360
Tween 80	429
Tween 20	286
H_2O_2	380

Effect of commercial detergents

To analysis the compatibility and stability of the *Bacillus* sp. PX6 protease towards commercial detergents, enzyme was pre-incubated in the presence of solid laundry detergent, %4.6 sodium hypochlorite and dishwashing liquid for 1 h at 30°C. The alkaline protease was highly stable in the presence of solid detergent at concentration of 3.5 mg/ml. The enzyme showed 100% stability and compatibility with Ariel. PX6 protease retained 31% and 23% of original activity after pre-incubation at 0.5% (v/v) concentration of a kind of surface disinfectant which includes 4.6% sodium hypochlorite and 0.5% (v/v) dishwashing liquid, respectively (Table 4). BM1 and BM2 proteases are highly stable in the presence of solid detergents at concentration of 7 mg/ml. The alkaline proteases exhibited higher stability in Axion (100%) than Ariel, Dixan and Nadhif. However, both proteases were found to be less stable in the presence of New Det, retaining 79.4% and 87.5% of their initial activity, respectively [12]. NG312 protease enzyme was almost 100% stable in presence of 5 g/L solution of Nirma, Tatachem, and Super Wheel detergents. However, it was relatively less stable in presence of Surf Ultra [18]. *B.* brevis protease retained about 60% activity after 1.5 h in the presence of Surf Excel® at 60°C and was completely inactivated after 3 h in the absence of any stabilizer [21]. *Bacillus licheniformis* NH1 enzyme is extremely stable in the presence of New Dex, Axion, and Dixan, retaining about 96, 95 and 93% of its initial activity. In the presence of Schems and Ariel the enzyme retained about 80% of the original activity. However, the enzyme was found to be least stable in the presence of Nadhif, retaining about 64% of its initial activity [4]. Considering the high compatibility and stability in the presence of various commercial liquid and solid detergents, *Bacillus* sp. PX6 protease may find potential application in laundry detergents.

Table 4. Effect of Commercial detergents

Commercial detergents	Relative activity (%)
Control	100
Solid laundry detergent (3.5 mg/ml)	100
4.6% Sodium hypochlorite (0.5% v/v)	31
Dishwashing liquid (0.5% v/v)	23

Electrophoresis and zymogram analysis

The crude PX6 protease enzyme appeared as two independent activity bands on SDS-polyacrylamide gel containing 3% casein, corresponding to molecular masses of 471 kDa and 206 kDa (Fig. 7). Molecular weight of the enzyme was calculated using bovine serum albumin (66 kDa) as the marker. The purified *Bacillus polymyxa* B-17 protease was found to be a homogeneous preparation of molecular weight of 30 kDa as determined by SDS-PAGE using molecular weight standards [16]. Zhu et al. (2007) reported that the purified enzyme appeared as a single band which was 56.5 kDa on SDS-PAGE. The apparent molecular mass of the purified protease AK1871 as revealed by SDS-PAGE was about 38 kDa [9]. The purified BM1 and BM2 proteases were estimated to be approximately 29 kDa and 15,5 kDa, respectively [12]. Ferrero et al. (1996) were reported that the proteolytic activity found in the supernatant resulted in the isolation of two active protein bands which were estimated 25 kDa and 40kDa in SDS gels, were close

in molecular mass and were possibly isoenzymes. Because both of them showed alkaline protease activity.

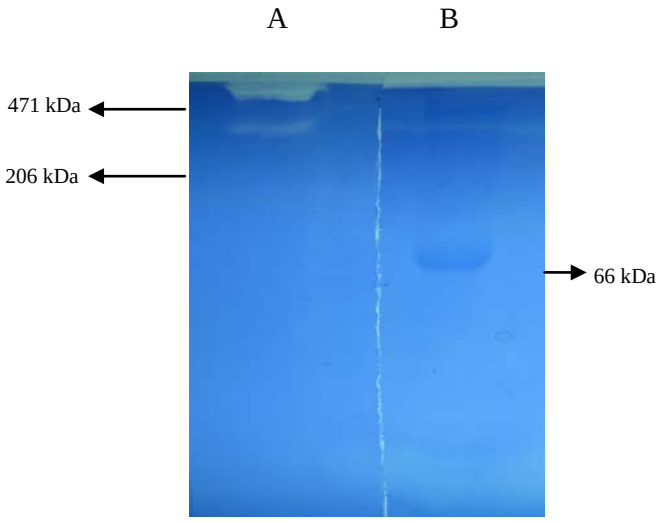

Fig 7. SDS-PAGE and Zymogram analysis.
A: Enzyme B: Marker protein

REFERENCES

[1] Patel R, Dodia M, Singh SP. 2005. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. Process Biochemistry. 40: 3569-3575.

[2] Beg QK, Sahai V, Gupta R. 2003. Statistical media optimization and alkaline protease production from Bacillus mojavensis in a bioreactor. 39: 203-209.

[3] Singh J, Vohra RM, Sahoo DK. 2004. Enhanced production of alkaline proteases by Bacillus sphaericus using fed-batch culture. Process Biochemistry. 39: 1093-1101.

[4] Hadj-Ali N, Agrebi R, Ghorbel-Frikha B, Sellami-Kamoun A, Kanoun S, Nasri M. 2007. Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. Enzyme and Microbial Technology. 40: 515-523.

[5] Hamilton LM, Kelly CT, Fogarty WM. 1999. Purification and properties of the raw starch degrading α-amylase of *Bacillus* sp. IMD434. Biotechnol. Lett. 2:111-115.

[6] Jin F, Cheng X, Shi Y, Zhang C. 1990. Isolation of New Thermophilic Aerobic Bacteria Which Produce Thermostable a-Amylase. J. Gen. Appl. Microbiol. 36:415-424.

[7] Singh J, Batra N, Sobri C. 2001. Serine alkaline proteaz from a newly isolated *Bacillus* sp. SSR1. Process Biochemistry, 36: 781-785.

[8] Shah K, Mody K, Keshri J, Jha B. 2010. Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from Bacillus cereus isolated from the Gulf of Khambhat. Journal of Molecular Catalysis B: Enzymatic. 67: 85-91.

[9] Turus N. 2011. Isolation of Cold-Active Protease *Bacillus* Sp. Strains, Enzyme Production, Characterization and Investigation of Biotechnological Applications. University of Cukurova. Department of Biotechnology. Institute of Natural and Applied Sciences. M.Sc Thesis.

[10] Adinarayana K, Ellaiah P, Prasad SD. 2003. Prufication and Partial Characterization of Thermostable Serine Alkaline Protease from A Newly Isolation *Bacillus suptilis* PE-11. AAPS Pharmscitech, India, 4(4): 56.

[11] Mctigue MA, Kelly CT, Doyle EM, Fogarty WM. 1995. The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370. Enzyme and Microbial Technology. 17:570-573.

[12] Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. 2009. Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: Purification, characterization and potential application as a laundry detergent additive. Bioresource Technology 100: 3366-3373.

[13] Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680-685.

[14] Bollag DM, Rozycki MD, Edelstein SJ. 1996. Protein Methods, 2nd edition, pp 414. Viley-Liss Press, USA.

[15] Johnvesly B and Naik G.R. 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB-99 in a chemically defined medium. Process Biochemistry. 37: 139–144.

[16] Matta H, Punj V. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. International Journal of Food Microbiology. 42: 139–145.

[17] Deng A, Wu J, Zhang Y, Zhang G, Wen T. 2010. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. Bioresource Technology. 101: 7100–7106.

[18] S J, Gill N, Devasahayam G, Sahoo D.K. 1999. Studies on Alkaline Protease Produced by *Bacillus* sp. NG312. Applied Biochemistry and Biotechnology. 76: 57-63.

[19] Ferrero M.A, Castro G.R, Abate C.M, Baigorí B.D, Siñeriz F. 1996. Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: isolation, production and characterization. Appl Microbiol Biotechnol. 45: 327-332.

[20] Zhu W, Cha D, Cheng G, Peng Q, Shen P. 2007. Purification and characterization of a thermostable protease from a newly isolated *Geobacillus* sp. YMTC 1049. Enzyme and Microbial Technology 40 : 1592–1597.

[21] Banerjee U.C, Sani R.K, Azmi W, Soni R. 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. Process Biochemistry. 35: 213–219.