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Purification and characterization of an extracellular thermotolerant alkaliphilic serine protease secreted from newly isolated *Bacillus* sp. DEM07 from a hot spring in Dehloran, Iran

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ABSTRACT

The current study examines a thermotolerant alkaliphilic serine protease from newly isolated *Bacillus* sp. DEM07, which was purified with a 20% total protein yield and 14.28 fold purification. The molecular weight of the enzyme was estimated to be 27.5 kDa. The K_m and V_{max} values of the purified enzyme were found to be 0.06 mg/ml and 1.25 μmol/min, respectively. The enzyme maintained activity and stability over the 30–55 °C temperature range exhibiting its optimum activity at 50 °C. Furthermore, high activity and stability were found over a wide range of pH from 4 to 11 with a supreme at pH 10. CTAB, TritonX-100, SDS, and H_2O_2 significantly enhanced the protease activity by 300, 266, 136, and 158%, respectively. The protease was inhibited by PMSF, suggesting that it can be a serine protease. Acetone and DMSO appeared as the most potent, increasing protease activity up to 216 and 105%, respectively. The enzyme retained more than 70% of its initial activity in the other organic solvents. DEM07 protease was capable of proteolyzing various substrates, suggesting broad substrate specificity. Considering these properties, the enzyme could be applied as a novel potent protease in industrial and biotechnological processes.

1. Introduction

Nowadays, microbial enzymes are utilized for several applications in various industries (Gurung et al., 2013). Proteases are one of the most commercially available extracellular microbial enzymes that have extensive uses in the detergent, food, pharmaceutical, chemical, leather, and textile industries. Among all industrial enzymes, proteases account for 65% of total enzyme sales worldwide, and alkaline proteases account for 89% of the total sales of proteases (Fujinami and Fujisawa, 2010; Jellouli et al., 2011; Vijayaraghavan et al., 2012).

The use of detergents containing proteases is preferred to conventional synthetic types because of better cleansing properties, higher efficacy, biodegradability, and low toxicity. Carlsberg subtilisin (alkalase) was one of the first proteases used in detergents (Phadtare et al., 1996). Among proteases, only serine proteases are usable in detergents. Bacterial subtilisins are the most appropriate enzymes used in the detergent industry. Currently, most of the detergent subtilisins are derived from *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus alcalophilus*, and *Bacillus amyloliquefaciens*. The industrial demand for highly active proteases with high specificity and stability in alkalic pH scale, hot temperature, organic solvents, bleaching agents, and surfactants drives the effort to discover new catalyst sources. In this course, thermotolerant alkaline proteases are proposed as a crucial additive in the detergent industry (Jellouli et al., 2011). Apart from thermo-alkaline stability, proteases exhibit other invaluable properties including maintaining maximum enzyme activity around chelating agents (for example, EDTA). This feature offers a good advantage when a catalyst is utilized in detergents as an additive because such agents are usually included in the formulation of the majority of detergents for stain removal and water softening purposes (Gupta et al., 2002; Maurer, 2004).

Hot springs as one of the habitats of thermophilic microorganisms are sources for direct isolation of thermotolerant enzymes. Living microorganisms in hot springs are resistant not only to high temperatures but also to high pH and high concentration of certain chemical compounds (Zilda et al., 2013). Hence, exploring, purification, and characterization of hot spring microbial proteases is important for the development of putative industrial enzymes.

In this study, we examine the isolation, purification, and characterization of a novel extracellular thermotolerant serine alkaline protease from newly isolated *Bacillus* sp. DEM07 from a hot spring situated in Dehloran, Iran. The enzyme is a thermo-tolerant and alkaliphilic protease as demonstrated by fine activity in the high temperature

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and pH ranges. In addition, the enzyme showed appropriate activity in the presence of oxidizing agents and some detergents and organic solvents. These features along with the isolation location of the microorganism indicate that this protein could be considered as a novel proteolytic enzyme and could be applied as a novel potent protease in industrial and biotechnological processes.

2. Material and methods

2.1. Microorganisms

Sediment and water samples were collected from the Dehloran hot spring, located in Ilam, in the west of Iran (latitude 32° 42′ 33.8″ N, 47° 18′ 22.6″ E). The Dehloran hot spring is located 3 km from Dehloran city, on the slopes of Siahkouh mountain and close to the Bat Cave. For initial screening, 1 ml water sample was inoculated in preculture medium containing Nutrient broth (8.0 g/l), yeast extract (10.0 g/l), NaCl (5.0 g/l), and sucrose (10.0 g/l). Cultivation flasks were incubated for 48 h at 45 °C under shaker conditions (100 rpm). Samples of repeated cultures were plated onto Skim-milk agar plates (SMA), which contained skimmed milk powder (100 g/l); agar-agar (20 g/l); tryptone (8 g/l); yeast extract (10 g/l), pH 9.0. Plates were incubated at 45 °C for 24–48 h and the colonies that exhibited the highest clear zone around them were isolated as high-yield alkaline protease producers. By repeated streaking on SMA plates, we purified isolate DEM07, and it was used for subsequent experiments. In addition, it was examined for physiological, morphological, and biochemical features according to Bergey's Manual of Systematic Bacteriology (Boone et al., 2001).

2.2. Bacterial identification through 16S rRNA sequencing

To identify the isolated strain using 16S rRNA gene sequence analysis, the genomic DNA was extracted and purified according to Sambrook et al., (1989) and its purity was determined by the A_{260}/A_{280}. Universal forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer (5′-AAGGAGGTGGATCCAGCCGCA-3′) were used to reproduce the maximum possible length of the 16S rRNA genes. Amplification cycles were performed by Techne FT Gene 2D thermocycler under the following conditions: denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 45 s, 58 °C for 30 s for annealing of primers, 72 °C for 45 s for DNA synthesis, and the final cycle was performed at 72 °C for 5 min to full extension of the products. After performing electrophoresis of the PCR product on the agarose gel (1/5%) and ensuring its purity, the purified 16S rRNA was sequenced directly by Bioneer (South Korea). Subsequently, the obtained sequence was aligned with the other sequences of 16S rRNA genes of known Bacillus species present in NCBI (National Center for Biotechnology Information) as well as data banks arb-silva and ribosomal database project; thereby, the phylogenetic tree was designed in MEGA7.

Using the Neighbor-joining method, the evolutionary history of Bacillus sp. DEM07 was obtained (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.29776874 is shown. The bootstrap consensus tree taken from 1000 replicates (Felsenstein, 1985) represents the evolutionary history of the taxa analyzed in this study (Felsenstein, 1985). It should be noted that the branches associated with partitions reproduced in less than 50% of the bootstrap replicates are collapsed. “The proportion of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches” (Felsenstein, 1985). The Kimura 2-parameter method (Kimura, 1980) was used to calculate the evolutionary distances based on the units of base substitutions per site. Twelve nucleotide sequences were involved in the analysis, in which the positions having gaps and missing data were removed, which left 1421 positions in the refined dataset. Evolutionary analyses were carried out using MEGA7 (Kumar et al., 2016).

2.3. Enzyme purification

2.3.1. Protease production

A loop of DEM07 isolate culture from agar plates was inoculated. The liquid preculture medium comprised nutrient broth (8 g/l), yeast extract (10 g/l), NaCl (5 g/l), and sucrose (10 g/l). It was placed in an incubator at 45 °C with an agitation rate of 120 rpm for 24 h. Subsequently, 5% preculture medium was transferred to the production medium containing yeast extract (10 g/l), citric acid (5 g/l), sucrose (5 g/l), and $CaCl_2.2H_2O$ (0.1 g/l). The initial pH of the medium was adjusted to 7.0 using 10% Na_2CO_3 solution that was autoclaved separately. The selected bacterial strain was studied at interval time to determine the optimum time for bacterial growth and enzyme production. Correspondingly, the effect of different temperatures (37–50 °C) and pH (3.0–11) values on enzyme production was investigated to determine the optimal conditions.

2.3.2. Protease enzyme purification

The organism was grown for 48 h in the production medium. The culture comprising extracellular enzyme was collected following centrifugation at 15000 rpm for 20 min. The supernatant was then harvested by precipitation with 85% saturated ammonium sulfate. The acquired deposit was dissolved in Tris-HCl buffer (20 mM, pH 8) and was subsequently dialyzed overnight against the same buffer. Further steps were carried out at 4 °C. The dialyzed solution was loaded at a flow rate of 1 ml/min onto a DEAE-Sepharose column, which equilibrated with a pH 8 Tris buffer. By increasing NaCl concentrations (0.0–1.0 M), the proteins were eluted and each fraction (2.0 ml) was collected. All the resulting fractions were subsequently measured regarding their protein content by taking absorbance at 280 nm using a spectrophotometer device, and the active fractions were then pooled for succeeding experiments. All the steps were carried out at 4 °C. Molecular weight and purity of purified enzyme were performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli method (Laemmli, 1970). Electrophoresis was carried out using 12.5% separating and 5% stacking gels. Coomassie blue R-250 was used to observe protein bands on the gels. Albumin serum (66 kDa), Carbonic anhydrase (28 kDa), β-lactoglobulin (18 kDa), and Lysozyme (14 kDa) were used as the standard molecular markers.

2.3.3. Zymography analysis

Protease activity was revealed by zymogram using casein as a substrate. After electrophoresis, the gel was washed using the following solutions at 4 °C: SDS was removed by 2.5% Triton X-100 solution for 60 min. T gel was then immersed three times in 100 mM Tris-HCl buffer (pH 7.6) to eliminate Triton X-100. Then, to activate the enzyme, the gel was incubated with 2% (w/v) casein substrate in 100 mM Tris-HCl and 4 mM calcium chloride at 37 °C for 24 h. Finally, the gel was stained with Coomassie brilliant blue R250 and was then destained. The advent of clear halo on the dark background of the gel confirmed the existence of proteolytic activity.

2.4. Analytical methods

2.4.1. Alkaline protease assay

Proteolytic activity was investigated using the modified procedure based on the Venugopal et al. method (Venugopal and Saramma, 2007). 0.5 ml casein (0.75% w/v) prepared in glycine-NaOH buffer (50 mM, pH 10.0) as a substrate was added to 0.2 ml crude enzyme and was incubated for 10 min at 37 °C. The enzyme reaction was halted by the addition of 0.5 ml of 10% trichloroacetic acid (TCA) solution, and the mixture was kept at 50 °C for 30 min, followed by centrifugation at 10000 rpm for 5 min to remove suspended particles. Then, 0.2 ml fractional of the supernatant was mixed with 0.5 ml Na_2CO_3 and 0.1 ml of Lowry reagent (Folin-Ciocalteu's) and was permitted to stand at 37 °C

for 30 min. The absorbance of the resulting solution was observed at 660 nm. A single unit of alkaline protease activity was considered as the amount of enzyme able to hydrolyze casein as substrate and produced a color comparable to released 1 μg of pH 8.0 tyrosine per minute at 37 °C. The amount of tyrosine was calculated according to the tyrosine standard curve. All the experiments were carried out in triplicates. Results are mean of ± SD of triplicate experiments.

2.5. Protein measurement

The protein amount in all samples was measured according to the Bradford method using bovine serum albumin as a standard solution (Bradford, 1976).

2.6. Characterization of purified enzyme

2.6.1. Effect of pH on the activity and stability of protease

To examine the influence of pH on protease activity, the purified enzyme was assayed in the presence of different pH ranging from 4 to 11 in one of the following buffers (50 mM): sodium acetate (for pH 4-5.5), potassium phosphate (for pH 6-7.5), Tris-HCl (for pH 8–9), and glycine-NaOH (for pH 9.5–11).

2.6.2. The effect of temperature on the activity and stability of protease

To investigate the influence of temperature on protease activity, the enzyme was assayed at different temperatures ranging from 30 to 60 °C using casein as the substrate for 30 min in 50 mM glycine-NaOH buffer. Thermostability of the protease was studied by incubating the enzyme at different temperatures and relative activity was assayed for 120 min at 30-min intervals. Subsequent analysis of the remaining activity was performed at standard conditions.

2.6.3. Effect of metal ions on protease

Effect of some metal ions (5 mM and 10 mM) such as Na^+, K^+, Zn^{2+}, Ca^{2+}, Co^{2+} and Fe^{3+} (all ions in chloride form) on enzyme activity was studied. The mixtures were preincubated for 30 min at 50 °C prior to adding the substrate. Under standard assay conditions, the remaining protease activity was measured in which enzyme activity in the absence of these compounds was considered as control.

2.6.4. The effect of protease inhibitors on enzyme activity

To determine the nature of protease, the influence of various inhibitors, including phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and β-mercaptoethanol (β-ME) on protease activity was investigated. The purified protease was pre-incubated at concentrations of 5 and 10 mM in each inhibitor for 1 h at 50 °C. Protease activity in the absence of an inhibitor was used as the control.

2.6.5. Protease activity in surfactants and oxidizing agents

To examine the influence of different surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, cetyl trimethyl ammonium bromide (CTAB), and an oxidizing agent (hydrogen peroxide), the enzyme was studied at final concentrations of 5% of the mentioned surfactants and pre-incubated for 1 h at 50 °C. The enzyme activity without additives was considered as the control.

2.6.6. Protease activity in organic solvents

The activity of the purified enzyme was investigated in different organic solvents including ethanol, methanol, isopropanol, chloroform, diethyl ether, toluene, acetone, hexane, and dimethyl sulfoxide (DMSO). The purified enzyme was mixed with each organic solvent with the final concentration of 50% and was pre-incubated at 50 °C for 30 min. Relative activity was compared with the control (without any organic solvent).

2.6.7. Substrate specificity of the enzyme

Protease activity against various protein substrates such as BSA, Gelatin, Skim Milk Agar and Casein was estimated by measuring residual enzyme activity after 30 min of incubation at 50 °C.

2.7. Kinetic parameters of the enzyme

To assess the kinetic parameters, K_m and V_{max}, the activity of the purified enzyme was assayed in the presence of different substrate concentrations, ranging from 0.01 to 1 mg/ml. K_m and V_{max} values were calculated using the reciprocal Lineweaver-Burke plot.

3. Results and discussion

3.1. Isolation and identification of the microorganism

Protease producing bacteria were isolated using growing colonies on skim milk agar plates. Some of the colonies that generate high clear zones of hydrolysis around them on skim milk agar plates were chosen as extracellular alkaline protease producers. The colonies were purified via repetitive streaking on fresh agar plates. Lastly, one of the colonies was established as the best strain with protease activity (Fig. 1). The newly isolated strain was a spore-forming, Gram-positive, short rod-shaped and motile bacterium, catalase-positive as well as thermotolerant with a growth temperature ranging from 37 to 60 °C. The relevant strain is sensitive to penicillin and sodium azide and grows on the agar plate at pH values ranging from 9 to 11.

The 16S rRNA sequence was analyzed by the DECIPHER web-based tool to detect any possible chimeras and was then aligned using the NCBI 16S ribosomal RNA sequences database to identify the isolated strain. The isolate showed the most homology (95% identity) with *Bacillus licheniformis* strain DSM 13 (Accession Number NR 118996.1), and this result was confirmed by means of aligning with the Silva database. *Bacillus licheniformis* strain BCRC 11702 (NR 116023.1), *Bacillus sonorensis* strain NBRC 101234 (NR 113993.1), *Bacillus licheniformis* strain ATCC 14580 (NR 074923.1), *Bacillus licheniformis* strain NBRC 12200 (NR 113588.1), and *Bacillus aerius* strain 24 K (NR 042338.1) were the next closest strains; all by 95% identity, but decreasing Max Score, as mentioned, respectively. A phylogenetic tree was constructed using MEGA 7 software and the neighbor-joining method (Fig. 2). Regarding BLAST-based results, the strain belongs to the genus Bacillus but requires additional study based on the polyphasic approach to identify at the species level because of the sequence identity less than 97%. In the end, based on the morphological and biochemical characteristics of the strain, phylogenetic data, and the 16S rRNA sequence, the present isolate was recognized as *Bacillus* sp. DEM07.

Fig. 1. The clear zone of hydrolysis of Bacillus sp. DEM07 on skim-milk agar.

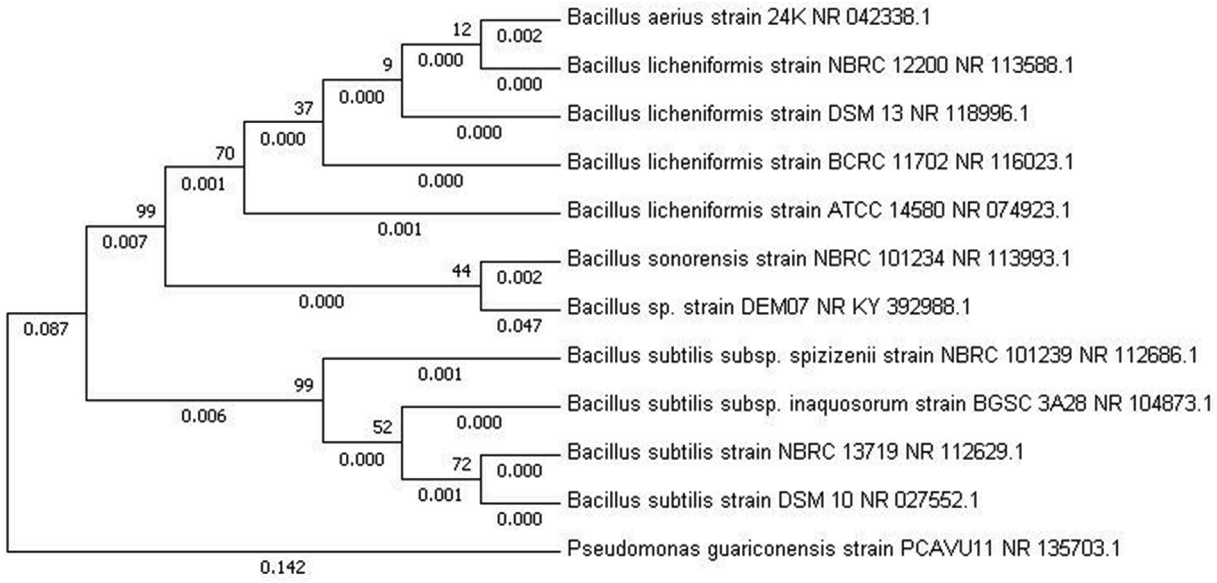

Fig. 2. Evolutionary relationships of taxa. The Neighbor-joining method was used to obtain the evolutionary history of the taxa. The optimal tree with the sum of branch length = 0.29776874 is shown. The bootstrap consensus tree inferred from 1000 replicates is selected to characterize the evolutionary history of the analyzed taxa. Branches resultant from partitions reproduced in less than 50% of the bootstrap replicates are collapsed. The percentage of replicate trees in which the related taxa clustered together in the bootstrap test (1000 replicates) are displayed next to the branches. The evolutionary distances were calculated using the Kimura 2-parameter method and are presented in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions with gaps and missing data were excluded. A total of 1421 positions were identified in the final dataset. Evolutionary analysis was carried out using MEGA7 software.

3.2. Production of extracellular protease

Maximum protease production was observed after 48 h of cultivation, during the stationary phase of microbial growth (Fig. 3). However, maximum protease production was proportional to the maximum growth of the bacterium. Our results are in agreement with previous investigations. In many bacterial species, substantial increases in protease production occurred during the stationary phase of growth (Bhaskar et al., 2007; Ferrero et al., 1996; Jain et al., 2012; Shivanand and Jayaraman, 2009).

3.3. Purification of protease

The supernatant (1 L) of the bacterial culture was collected and concentrated with 85% ammonium sulfate. After centrifugation, the obtained pellet was dissolved in a minimum volume of Tris-HCl buffer (20 mM, pH 8) and was dialyzed against the same buffer overnight at 4 °C. The dialyzed solution was loaded onto a DEAE-Sepharose column, which equilibrated with a pH 8 Tris buffer. The proteins were eluted by increasing concentrations of NaCl (0-1.0 M). Then, all fractions were checked for their protein content and protease activity. Fractions showing high protease activity were pooled and applied again for DEAE-sepharose column chromatography (Fig. 4a and b). The purity of the active fraction was checked by SDS-PAGE (Fig. 5). Table 1 summarizes the results of the purification stages. The fractionation of Ammonium Sulfate resulted in 3.19-fold purification with the specific activity of 4.92 Umg^{-1}. In the final stage, a specific activity of 22 U/mg was attained with a yield of 14.28 fold purification and 20% total protein. The purity of the protease was analyzed using SDS-PAGE. As shown in Fig. 5a, purified enzyme appeared as a single band of molecular weight of about 27.5 kDa. As observed in the zymogram analysis, the zone of clearance at the same position confirmed the purity and molecular weight of the enzyme (Fig. 5). Generally, in previous investigations, the molecular weights of bacterial alkaline proteases lay within 15–40 kDa (Doddapaneni et al., 2009; Joo et al., 2001; Kumar and Takagi, 1999; Shah et al., 2010). In this sense, a protease from *Bacillus circulans* M34 possesses the same molecular weight as the DEM07 protease (Sari et al., 2015) and the molecular weight of the alkaline protease produced by DEM07 was higher than those of the *Bacillus mojavensis* A21 (20 kDa) (Haddar et al., 2009) and lower than *Geobacillus toebii* LBT 77 (30 kDa) (Thebti et al., 2016).

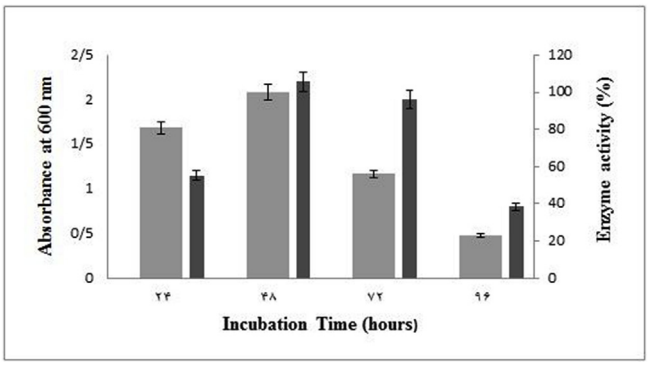

Fig. 3. Protease production by *Bacillus* sp. DEM07 in comparison with bacterial growth.

3.3.1. The effect of temperature on enzyme activity and stability

To study the effect of temperature on protease activity, as described in the materials and methods section, the temperature range of 25–60 °C was selected for evaluation of protease activity. As shown in Fig. 6a, maximum protease activity was achieved at 50 °C. However, protease activity dropped significantly at temperatures above 50 °C. Though almost 40% of the initial enzyme activity was maintained at 55 °C (Fig. 6a), DEM07 can grow up to 55 °C showing that this strain is a thermotolerant organism. This finding is in accordance with the previous report for *B. licheniformis* S-40 (Sen and Satyanarayana, 1993) and *B. circulans* M34 (Sari et al., 2015). Declined residual activity arises from conformational changes and thermal unfolding of proteins by a strong increase in entropy changes (Adinarayana et al., 2003; Niyonzima and More, 2014).

Study on the thermal stability of the purified protease revealed that even after 120 min of incubation, the enzyme was extremely stable

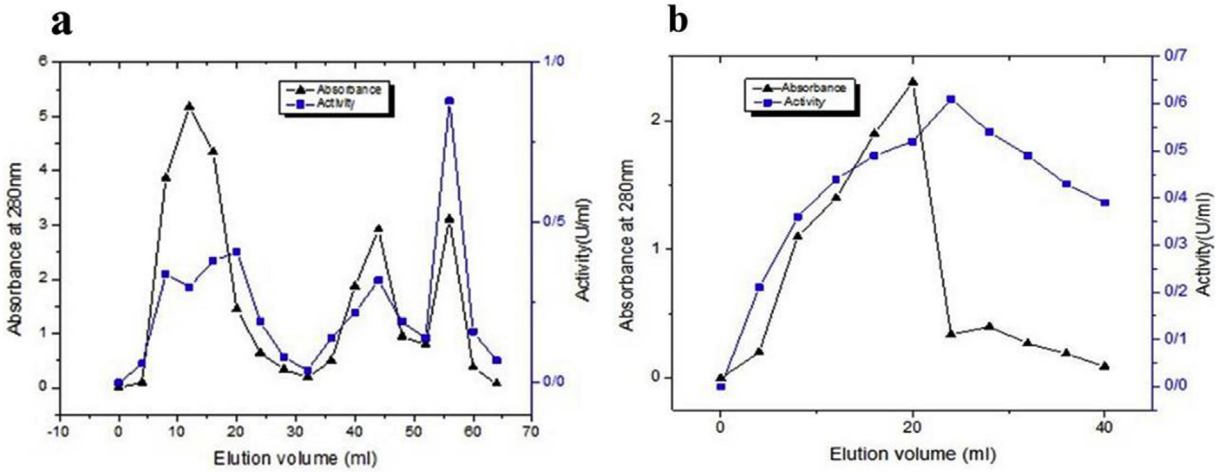

Fig. 4. Elution profile of protease from Bacillus sp. DEM07 on DEAE-Sepharose anion exchange chromatography.

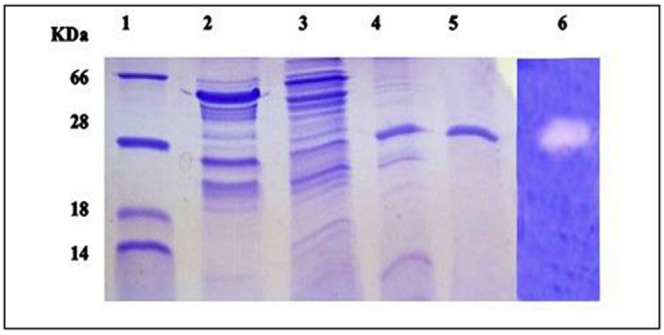

Fig. 5. SDS-PAGE and zymography of purified Bacillus sp. DEM07 protease. Lane 1: Molecular weight markers; Lane 2: dialysis solution after ammonium sulfate precipitation; Lane 3: crude extract, Lane 4: the first step of purification of protease by DEAE-sepharose chromatography; Lane 5: Purified enzyme, and Lane 6: zymogram of purified protease.

between 37 and 50 °C. The enzyme remained more than 97% active after 2-h incubation at 37 °C. Furthermore, protease reserved above 80% of its primary activity at 50 °C following 2-h incubation. It is noteworthy to say that at 60 °C, it still retains approximately 65% of its activity, signifying that the enzyme could be utilized under mild or harsh heating conditions (Fig. 6b).

3.3.2. The effect of pH on protease activity and stability

Fig. 7a shows that *Bacillus* sp. DEM07 protease was active throughout an extensive pH range of 4–12 showing maximum activity at pH 10 (Fig. 7a). A slight reduction in activity was observed at pH higher than 10. The enzyme maintained almost 87% of its activity at pH 11. The result is in agreement with earlier research that described an optimum pH of 10 and 10.5 for proteases *B. licheniformis* NH1 and *B. mojavensis*, respectively (Beg and Gupta, 2003; El Hadj-Ali et al., 2007). Subtilisin NOVO or BPN ad Subtilisin Carlsberg (Horikoshi, 1990) and other strain established their maximum activity at pH 10.5 (Badoei-Dalfard et al., 2015; Prakash et al., 2005). As a matter of fact, all detergent-compatible enzymes are required to stand alkaline conditions (Sana et al., 2006). These characteristics show that this enzyme has the potential ability to be active at high temperatures and alkaline pH conditions. Therefore, it could be used as a detergent additive.

Remarkably, protease was stable over a wide range of pH from 4 to 10. The enzyme retained more than 80% of its primary activity after 2 h of incubation (Fig. 7b). The subtilisin-like serine protease from *T. kodakaraensis* proved to be stable over a broader range of pH from 2 to 12 compared to our enzyme. Meanwhile, other proteases from the Bacillus species are shown to be stable in a narrower pH range.

3.3.3. Determining K_m and V_{max}

The kinetic parameter on DEM07 alkaline protease activity was assayed using casein as the substrate and data are shown in Fig. 8a. According to the results, K_m and V_{max} values of purified enzyme were found by Lineweaver-Burk plot to be 0.06 mg/ml and 1.25 μmol/min, respectively (Fig. 8b). The low K_m value of this protease indicates the high affinity of the enzyme for its substrate and when followed by high amounts of V_{max} results in a higher efficiency of the enzyme in hydrolyzing the substrate (Sari et al., 2015). Our findings were in agreement with previous studies that showed K_m values in the range of 0.08–5.1 mg/ml and V_{max} in the range of 1.6–6.346 μmol/min for different types of pure protease (Dodia et al., 2008; Gupta et al., 2005; Jain et al., 2012; Raval et al., 2014; Shankar et al., 2011). Regarding the DEM07 protease, its high catalytic efficiency demonstrates its suitability for industrial applications.

3.3.4. The effect of metal ions on protease activity

Many investigations have confirmed that divalent cations like Ca^{2+} and Mn^{2+} are required to optimize the activity of proteases. Indeed, these cations play a vital role in maintaining the active conformation of the enzyme at high temperatures via protection of the enzyme structure against thermal denaturation (Bhatiya and Jadeja, 2010; Reddy et al., 2008). For this purpose, two concentrations of metal ions, 5 and 10 mM, were used to investigate the effects of different metal ions (Na^+, Ca^{2+}, K^+, Co^{2+}, Zn^{2+}, Fe^{3+}) on the alkaline protease activity (Fig. 9). Enzyme activity in the absence of metal ions was considered as 100%. The

Table 1
Purification of protease from Bacillus sp. DEM07.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	2990	1942	1.54	100	1
Ammonium sulfate precipitation	1826	371	4.92	61	3.19
DEAE-Sepharose (1)	880	142	6.19	29	4
DEAE-Sepharose (2)	616	28	22	20	14.28

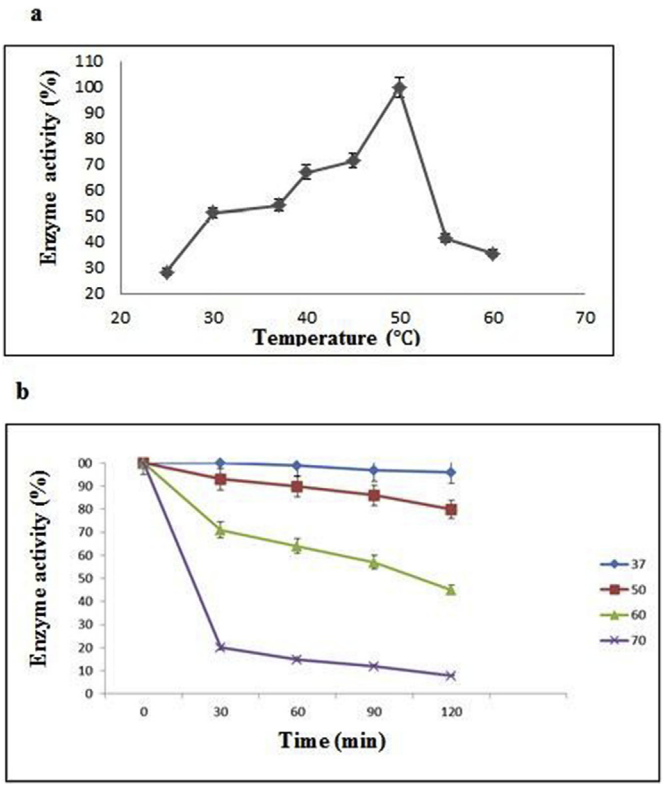

Fig. 6. The effect of temperature on the activity (a) and thermal inactivation (b) of the purified protease. The temperature profile was defined via the assay of protease activity at temperatures ranging from 25 to 60 °C. Enzyme activity at 50 °C was taken as 100%. Temperature stability was also defined via incubating the purified protease at temperatures ranging from 37 to 70 °C for 120 min. Residual enzyme activity was measured under standard assay conditions for each of the enzymes for 30 min. The initial activity of the enzyme at the start of the experiment was taken as 100% for each temperature. 37 (♦), 50 (■), 60 (▲), 70 (×). Each data point represents the mean of three independent assays (the standard errors were less than 5% of means).

Bacillus sp. DEM07 alkaline protease activity increased in the presence of 5 mM of some metal ions including Na^+ and Ca^{2+} at about 130% and 105%, respectively. Enzyme activity reduced to 50% by increasing the concentration of these ions to 10 mM. Similar effects of $CaCl_2$ on protease activity were also observed by Usharani et al. (Usharani and Muthuraj, 2010). Other metal ions decreased enzyme activity. As shown in Fig. 9, K^+, Co^{2+}, and Fe^{3+} ions in both concentrations reduced the activity of the enzyme up to 60%. Zn^{2+} ion increased enzyme activity by increasing its concentration from 5 to 10 mM. It may be inferred that this metal ion plays an important role in maintaining the active site conformation of the enzyme (Shivanand and Jayaraman, 2011).

3.3.5. The effect of inhibitors on protease activity

Inhibition studies usually offer insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center (Sigman and Mooser, 1975). The effect of different inhibitors on the activity of the purified alkaline protease DEM07 is presented in Fig. 10. Among the inhibitors tested (at 5 and 10 mM concentrations), PMSF was able to inhibit the enzyme completely. In the case of other inhibitors, such as ??-mercaptoethanol, not only did they not inhibit protease activity, but they also improved protease activity by 150% of its original activity. Activity was increased by ??-mercaptoethanol suggesting that it is a thiol-depending serine protease and thiol-disulfide conversion regulates the activity of the protease (Beg and Gupta, 2003; Oberoi et al., 2001; Paliwal et al., 1994; Sana et al., 2006; Shivanand and Jayaraman, 2011; Sigman and Mooser, 1975; Usharani and Muthuraj, 2010). The activity

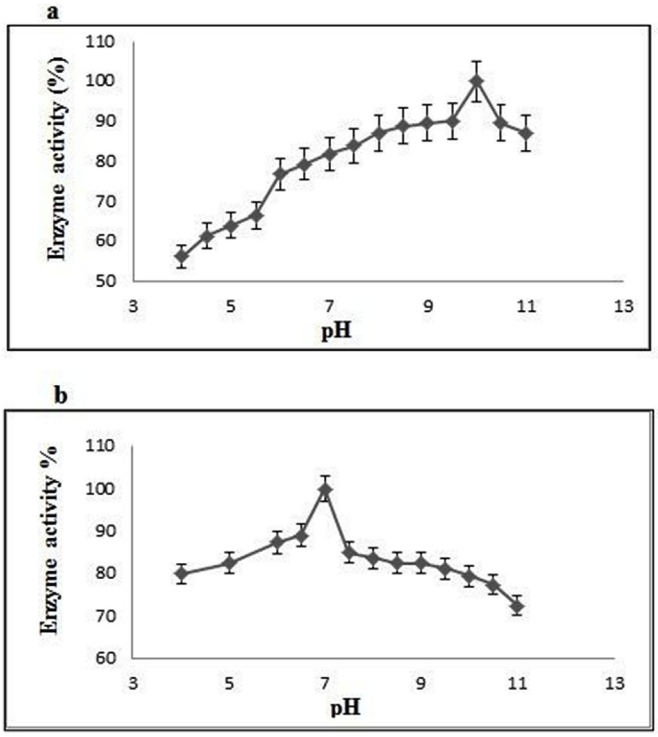

Fig. 7. The effect of pH on the activity (a) and stability (b) of the purified protease. Protease activity was evaluated in the pH range 4.0–11 using different buffers. The highest activity attained at pH 10 was reflected as 100% activity. By incubating the enzyme in various buffers for 2 h, the pH stability of the enzyme was determined. Subsequently, retained activity was measured at pH 9.5. Activity before incubation of the enzyme was taken as 100%. Section 2 presents the buffer solutions used for pH activity and stability. Each data point denotes the mean of three different assays (SEM < 5%).

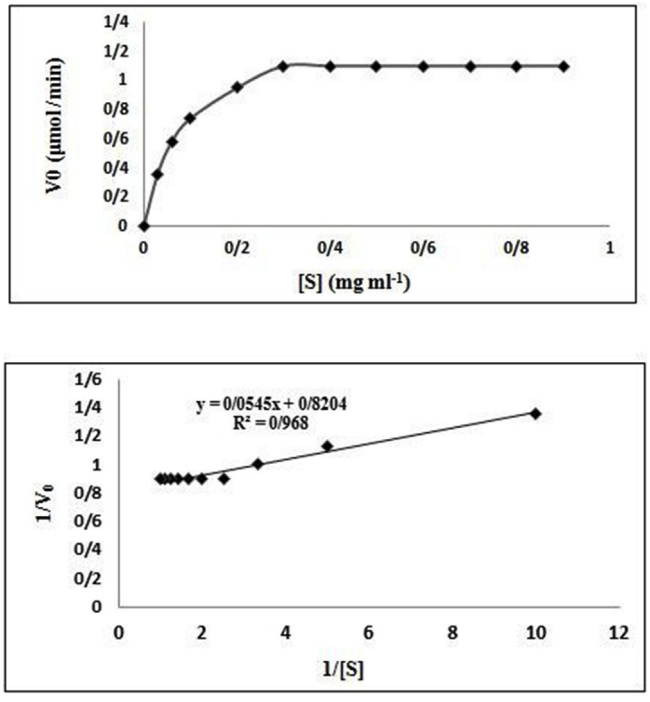

Fig. 8. Michaelis–Menten (a) and Lineweaver- Burke plot and kinetic parameters of Bacillus sp. DEM02 protease. The Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) were examined within various substrate concentrations [S] ranging from 0.01 to 1 mg/ml. K_m and V_{max} values were calculated using the reciprocal Lineweaver- Burke plot (b).

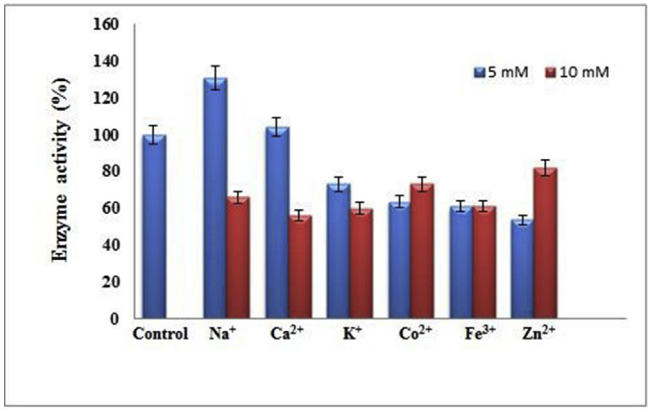

Fig. 9. Effect of different concentrations of metal ions on enzyme activity. Two concentrations of metal ions, 5 and 10 mM, were used to investigate the effects of different metal ions (Na^+, Ca^{2+}, K^+, Co^{2+}, Zn^{2+}, Fe^{3+}) on the alkaline protease activity. Prior to adding the substrate, the mixtures were pre-incubated at 50 °C for 30 min. Subsequently, protease activity was measured according to standard assay conditions. To validate reproducibility, the displayed results are the mean of a minimum of three tests in a typical run.

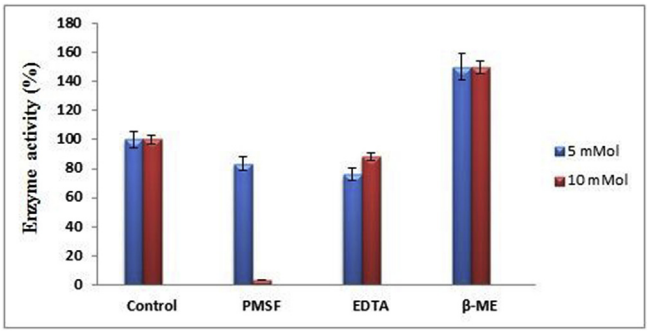

Fig. 10. Effect of protease inhibitors on enzyme activity. The influence of various inhibitors including phenylmethanesulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and β-mercaptoethanol (β-ME) on the protease activity was investigated. The purified protease was pre-incubated with the additives at concentrations of 5 and 10 Mm, each inhibitor for 1 h at 50 °C. The activity of the protease without any inhibitor was used as the control.

was slightly decreased to 83% and 76% in the presence of 5 and 10 mM EDTA, respectively. Slight change in the retained activity of the protease in presence of EDTA is a suitable feature for application as a detergent additive as chelating agents offer extensive applications in detergents for stain removal and water softening. Similar results were previously reported for *Bacillus licheniformis* (Jellouli et al., 2011; Joshi and Satyanarayana, 2013). Given the evidence, the protease of the present study belongs to the family of the serine protease, and it was highly active in the presence of the chelating agent.

3.3.6. The effect of surfactants/detergents and oxidizing agents on protease activity

An efficient protease enzyme requires resistance to oxidizing agents and surfactants used in the detergent formulation. To consider the influence of surfactants/detergents and oxidizing agents on Bacillus sp. DEM07 alkaline protease activity, the enzyme was pre-incubated with cationic, nonionic and strong anionic detergents like CTAB, Triton X-100, SDS and Oxidizing Agent hydrogen peroxide (H_2O_2) at final concentrations of 5% for 1 h at 50 °C. The remaining activity of the enzyme in the presence of surfactants/detergents and the oxidizing agent is shown in Fig. 11. The outcome revealed the high stability of the enzyme in the presence of selected compounds. These compounds not only failed to reduce the activity of the enzyme, but also improved it. All tested detergents show significant effects on the protease activity.

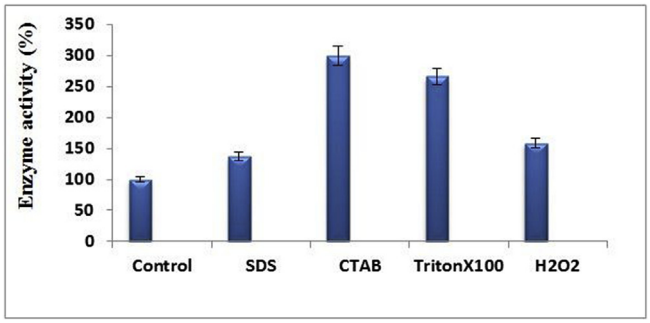

Fig. 11. Effect of detergents/surfactants and oxidizing agents on protease activity. To examine the influence of different surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, cetyl trimethyl ammonium bromide (CTAB), and an oxidizing agent (hydrogen peroxide) on the enzyme, they were studied at final concentrations of 5% and pre-incubated for 1 h at 50 °C. The enzyme activity determined without additives was considered as the control.

CTAB, TritonX-100, and SDS significantly enhanced the protease activity by 300, 266 and 136%, respectively. Incubation of the enzyme with H_2O_2 improved the enzyme activity by 158%. These results are relatively similar to earlier reports on serine protease produced by *Bacillus* sp. and *Bacillus clausii* (Joo et al., 2003; Patel et al., 2006) and *Geobacillus toebii* LBT 77 (Thebti et al., 2016). Enhanced activity in the presence of these surfactants and bleaching agents makes the protease highly compatible with detergent ingredients.

3.3.7. Substrate specificity

As shown in Fig. 12, the DEM07 protease was capable of proteolysis of all of the selected substrates with different degrees of specificity, suggesting broad substrate specificity. In examining substrate specificity of the enzyme, it was found that in the presence of 1% concentration of the four protease substrate casein, gelatin, BSA and SMA, the enzyme had the highest activity in presence of the casein substrate, which was considered 100%, and the other results are compared to that shown in the diagram. The activity of the purified protease enzyme in the presence of BSA substrate was 67%. When gelatin was used as a substrate, the activity of the enzyme protease reached 61% in comparison with the control. The enzyme had the least activity in presence of the SMA substrate where its activity dropped by about 60% and settled at 40%. Previous reports on various substrates have indicated that alkaline proteases have the highest activity toward casein relative to other substrates like BSA and gelatin (Adinarayana et al., 2003; Rahman et al., 1994; Yossan et al., 2006). Hydrolysis of various substrates has been reported as one of the important criteria for selecting

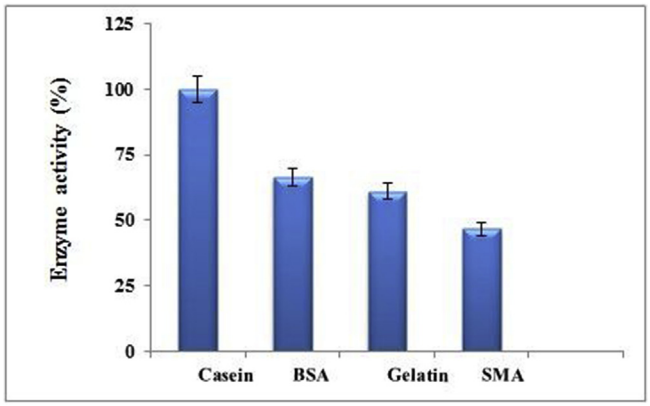

Fig. 12. Substrate specificity of the enzyme. Protease activity against various protein substrates such as BSA, Gelatin, Skim Milk Agar, except Casein was estimated by measuring the residual enzyme activity after 30 min of incubation at 50 °C.

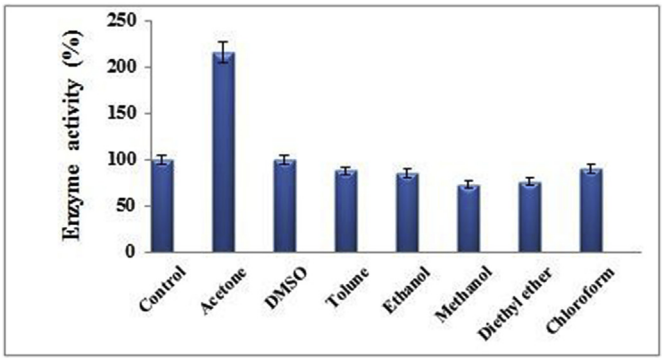

Fig. 13. Protease activity in the organic solvent. The activity of the purified enzyme was investigated in some organic solvents including ethanol, methanol, isopropanol, chloroform, diethyl ether, toluene, acetone, hexane, and dimethyl sulfoxide (DMSO). The purified enzyme was mixed with each organic solvent with the final concentration of 50% and was pre-incubated at 50 °C for 30 min. Relative activity was compared with the control (without any organic solvent).

proteases for application in laundry detergents (Gouda, 2006).

3.3.8. The effect of organic solvents on protease activity

The application of protease in organic media has gained momentum in the last decade considering their possible use in ester and peptide synthesis in non-aqueous conditions (Jellouli et al., 2011). The tested solvents showed various effects. Acetone and DMSO appeared as the most potent, increasing protease activity up to 216 and 100%, respectively (at 50% concentration v/v). The enzyme retained 88, 85, 76, 73 and 90% of its initial activity in the other organic solvents such as toluene, ethanol, methanol, diethyl ether, and chloroform, respectively (Fig. 13). These results validate the previous findings in which maximum activity was shown in the presence of acetone, which may be due to positive interactions between serine residues on the catalytic site and the carbonyl group in acetone (Ahmed et al., 2016). The reason behind enzyme stability alongside such organic solvents could well be the availability of a large number of negatively charged acidic amino acids in comparison to scarce basic amino acids on the surface. These negative charges are believed to maintain the solubility of the protein either by establishing a hydrated ion network with cations or by inhibiting protein aggregation through electrostatic repulsive charges at the protein surface (Jain et al., 2012).

4. Conclusion

Industrial demand for proteases is highly dependent on high enzyme stability and activity in different temperatures and pH. In addition, robust properties against surfactants, organic solvents, and bleaching agents is an important concern. In the present study, a thermotolerant alkaline serine-protease produced by *Bacillus* sp. DEM07 was purified and characterized. The purification steps gave an overall purification of 14.28 -fold. The overall activity yield of the purified protease was 20%, with specific protease activity of 22 U/mg. The molecular weight of DEM07 protease was estimated to be 27,500 Da by SDS–PAGE. The purified enzyme maintained activity and stability over the 30–55 °C temperature range. The optimum temperature for protease activity was 50 °C. The enzyme was highly stable and active over a wide range of pH from 4 to 11 with a supreme at pH 10. The enzyme was stable towards anionic detergent SDS and oxidizing agent and showed excellent stability and compatibility with CTAB and TritonX-100 detergents. DEM07 protease was inhibited by PMSF, suggesting that it can be a serine protease. The enzyme retained more than 70% of its initial activity in the several organic solvents. Hence, considering the high activity and stability in high temperature and alkaline pH and in the presence of various detergents and organic solvents, DEM07 protease may find potential application in medicine, biotechnology, and the laundry detergent industry.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101053.

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