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Research Article

Purification and Characterization of an Extracellular Alkaline Solvent-stable Metalloprotease Secreted from Newly Isolated *Bacillus* sp. DEM05: Optimization of Protease Production

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Background: Proteases play an important role in food, leather, detergent, and medical technologies.

Objectives: In the current study, an alkaliphilic solvent-stable thermotolerant metalloprotease was isolated from *Bacillus* sp. DEM05.

Material and Methods: For culture optimization, carbon, and nitrogen sources as well as incubation temperature, pH, and time were examined.

Results: Herein the highest outcome for bacterial growth and protease production was obtained after 72 h incubation (pH 7) at 37 °C. DEM05 protease was successfully purified and the specific activity of the protease was 1075 $U.mg^{-1}$. The purity of the enzyme was verified by SDS-PAGE electrophoresis as a single band of 30 kDa. The optimal activity of the enzyme was at pH 10 and 50 °C. H_2O_2, SDS, Triton X-100, Zn^{2+}, Co^{2+}, and Cu^{2+} could increase the protease activity. EDTA inhibited the protease activity, revealed that it can be classified as a metalloprotease. The enzyme was compatible with the water-miscible and water-immiscible organic solvents and proteolyzed several substrates, implying the wide substrate specificity.

Conclusions: The results brought convincing evidence that DEM05 protease could be recruited as a novel prevailing protease that can be earmarked on industrial and medical technologies.

Keywords: Alkaline; *Bacillus* sp; Optimization; Protease; Purification

1. Background

Proteases are the class of hydrolyzing enzymes that catalyze the cleavage of proteins (1). Proteases can be isolated from animals, herbs, Fungi, and microorganisms which are used with special capabilities in various fields such as detergents, leather, food and pharmaceutical industries, peptide synthesis, and protein processing (post-translational protein modification) (2). Proteases also play an important role in medical technology and among the medical applications of proteases is their role in producing therapeutic agents against diseases such as AIDS and cancer (3). Organic solvent-resistant proteases achieved great interest due to their application as the main ingredient in pharmaceutical and industrial compounds. Therefore, finding organic solvent-resistant protease-producing bacteria can contribute to the development of industrial and pharmaceutical biotechnology.

Microorganisms are the main sources of intracellular and extracellular proteases. Among them, extracellular proteases are required for hydrolysis of proteins in non-cellular conditions such as industrial and *in vitro* experiments. Given the high distribution, resistance, and survival potential as well as environmental stability, *Bacillus* species are considered as the most

important sources of extracellular proteases required for industry (3). Two-third of commercial enzymes and antibiotics are produced from different species of *Bacillus* (4, 5). Despite the identification of a large number of proteases with unique characteristics, it is more important to optimize the production process of these enzymes to increase their industrial efficiency.

One of the convenience strategies for achieving the maximum cellular density, optimum metabolic products, and enzymatic activity level is the one-factor-at-a-time approach that is used commonly in biotechnology. The disadvantages of this method are time-consuming and ignorance of interactions among different physicochemical parameters. On the other hand, the response surface method is performed to find effective factors, development of models for interaction studies as well as selecting the optimal conditions of variables to get convenient responses (6, 7).

2. Objectives

We have previously isolated and characterized a thermotolerant alkaline protease from Bacillus sp., DEM07, from a hot spring in the west of Iran (8). In the current study, the aim was to isolate and investigate an alkaline protease-resistant to organic solvent from *Bacillus* sp. DEM05 found in the same hot spring as well as optimizing the production process of the protease.

3. Material and Methods

3.1. Isolation and Screening of Protease Producing Bacterial Strain With Tolerability in the Organic Solvent

Samples were collected from the bottom sediment and surface of Dehloran hot spring (Ilam, in the west of Iran; latitude 32° 42′ 33.8″ N, 47° 18′ 22.6″ E). At first, 1 mL of water sample was inoculated in a cultivation flask. The Luria–Bertani (LB) medium enriched by organic solvents (10% (v/v) toluene and 30% (v/v) cyclohexane) was used to screen organic solvent-tolerant strains. To isolate strains with protease-producing capability, Skim-milk agar plates (SMA) at pH 9.0, 45 °C for 24-48 h were used to plate samples. The colonies which produced the clear zone with a high diameter were selected, cultured with exposure upon organic solvent in which protease activity was assayed. The strain with higher protease activity, DEM05, was isolated and used for the next experiments. Further, the morphological, physiological, and biochemical features were investigated using Bergey's Manual of Systematic Bacteriology (9).

3.2. Production of Crude Protease

One colony of DEM05 isolate was transferred from agar plates to the liquid preculture medium containing ($g.L^{-1}$): 5.0 NaCl, 10.0 yeast extract, 10.0 sucrose, and 8.0 Nutrient broth and maintained at 37 °C with constant shaking at 120 rpm for 24 h. Afterward, 5% of preculture medium was inoculated into production medium with these compounds ($g.L^{-1}$): 10 yeast extract, 5 citric acid, 5 sucrose, 1 K_2HPO_4, 0.1 $MgSO_4 \cdot 7H_2O$ and 0.1 $CaCl_2.2H_2O$ at pH 7 and was incubated at 45 °C for 48 h with an agitation rate of 120 rpm.

3.3. PCR Amplification and 16s rDNA Sequencing

Taxonomic characterization of the isolated strain was examined through analysis of 16S rRNA gene sequence. The genomic DNA was extracted using the boiling method by Sambrook *et al*. (10). Universal 16s rRNA forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (5'-AAGGAGGTGGATCCAGCCGCA-3') were used to amplify 16S rRNA genes at Techne FT Gene 2D. Then, the sequencing of purified 16S rRNA was directly conducted by Microsynth (Switzerland). Thereafter, the obtained sequence was aligned using the National Center for Biotechnology Information (NCBI) and Arb- Silva database and ribosomal database project (PRD). At last, MEGA7 software and the neighbor-joining method were used to design a phylogenetic tree.

3.4. Optimization of the Production Medium

3.4.1. The Effect of Carbon and Nitrogen Sources

The effect of various sources of carbon on the growth of bacteria and production of protease was investigated by replacement of sucrose in the medium by one of the carbon sources such as 1% (w/v) glucose, galactose, fructose, maltose, and starch. After 24 h incubation at 37 °C under shaking (120 rpm), the growth and enzyme activity was measured. The mean of three individual experiments was used to report endpoints. To evaluate whether different nitrogen sources affect the growth of bacteria and production of alkaline protease, the yeast extract in the medium was replaced by 0.5% (w/v) from different sources of organic (peptone, tryptone, alkali-soluble casein and skim milk) and inorganic (ammonium sulfate and urea) nitrogen sources. The incubation condition contains 24 h at 37 °C, under shaking conditions of 120 rpm. The maximum amount of activity was considered 100

and the rest were compared based on it.

3.4.2. The Effect of Temperature, pH and Incubation Time

The effect of different times from 24 to 96 h, temperatures 30 to 60 °C, and pH 4 to 10 on the growth of bacteria and the production of protease was tested to achieve the optimal values of each. This was done by the one-factor-at-a-time method: keeping the other parameters constant.

3.5. Protease Enzyme Purification

After 72 h incubation, the medium was centrifuged for 20 min at 15000 ×g. Then, the supernatant fluid was saturated and precipitated with 85% saturated ammonium sulfate. Then, it was dialyzed in 20 mM Tris-HCl buffer for 24 h with 3 changes at 4 °C. The dialyzed pool was loaded onto a DEAE-Sepharose column equilibrated with a Tris buffer (20 mM, pH 8) at a flow rate of 0.5 mL.min^{-1} and eluted with increasing NaCl concentrations (0.0-1.0 M). The measurement of absorbance at 280 nm with a spectrophotometer (Cary 100 bio) was used to evaluate the protein contents of fractions. Electrophoresis of obtained fractions was performed using Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 12.5% separating and 5% stacking gels. Detection of protein bands was performed using silver staining. Albumin serum and carbonic anhydrase with 66 kDa and 29 kDa were used as the standard molecular mass ladders.

3.6. Alkaline Protease Assay

The alkaline proteolytic activity of the enzyme was analyzed against casein substrate as described by Monnet *et al* with small modifications (11). In brief, the enzymatic reaction was prepared with 0.1 mL 2% casein and 0.1 mL aliquot of the enzyme solution were incubated for 10 min at 50 °C which was halted by adding 0.2 mL of 10% (w/v) trichloroacetic acid (TCA) solution. Then, suspended particles were derived out after centrifugation at 10000 rpm for 10 min. Then, 0.1 mL of the supernatant fluid was mixed with 0.5 mL of 0.4 moL.L^{-1} of Na_2CO_3 and 0.1 mL of Lowry reagent (Folin-Ciocalteu's) and incubated at 50 °C for 20 min. Then, the absorbance was measured at 660 nm. One unit of alkaline protease activity was defined as the amount of enzyme that can hydrolyze casein substrate and produced a product comparable to release 1 µg tyrosine per minute (pH 8.0) at 50 °C. The tyrosine standard curve was used to measure the amount of tyrosine in reaction. All the experiments were done in triplicates. Data are expressed as mean ±SD of triplicate experiments.

3.7. Characterization of the Purified Enzyme

3.7.1. The Effect of pH and Temperature on the Activity and Stability of the Protease

The activity of DEM05 protease was examined in a mixed buffer with a wide range of pH from 4 to 11. Also, to examine the thermostability of the purified enzyme, the activity of the enzyme was studied in the presence of casein as substrate in glycine-NaOH buffer (50 mM, pH 10) at 30 to 60 °C temperature range for 60 minutes at 10-minute intervals.

3.7.2. The Effect of Inhibitors and Metal Ions on Protease Activity

To characterize the class of the purified protease, several inhibitors including ethylene diaminetetraacetic acid (EDTA), β-mercaptoethanol (β-ME), and phenylmethanesulfonyl fluoride (PMSF) at concentrations of 5 mM and 10 mM were pre-incubated with DEM05 enzyme for 10 min at 50 °C. Protease activity was conducted in the presence of inhibitors and the absence of inhibitors (control).

To evaluate the influence of metal ions on the activity of DEM05 protease, some ions (5 mM and 10 mM) including Na^+, K^+, Mg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, and Co^{2+} were pre-incubated with the enzyme for 10 min at 50 °C before adding the substrate and remaining protease activity were measured in standard condition. The enzyme activity was compared relative to control in the absence of these compounds.

3.7.3. Protease Activity in Organic Solvents

Several organic solvents including methanol, ethanol, butanol, diethyl ether, chloroform, acetone, toluene, and cyclohexane at the final concentration, 10% and 50% (v/v), were pre-incubated with the enzyme at 50 °C for 10 min (water-immiscible solvents were shaken at 180 rpm). Relative activity was measured in the presence and absence of organic solvents (control).

3.7.4. Protease Activity in Surfactants and Oxidizing Agents

Two most common surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, and an oxidizing agent (hydrogen peroxide) were pre-incubated at the final concentrations of 2% and 5% with purified enzyme for 10 min at 50 °C. The enzyme activity was measured with and without additives (control).

3.8. Substrate Specificity

Enzymatic activity was measured in the presence of a concentration of 2% (w/v) of the casein, SMA, BSA, and gelatin substrates.

4. Results

4.1. Isolation and Characterization of Protease Producing Bacteria

Among 50 isolates, isolate No. 5 with the largest clear zone around it, exhibited high proteolytic activity (**Fig. 1A**). The results showed that the strain was a rod-shaped, gram-positive, catalase-positive, motile bacterium. The results indicated that the isolate. No. 5 is devoted to the *Bacillus* genus. Next, the name *Bacillus* sp. DEM05 was selected for it. As the BLAST results showed, 97% sequence identity was found with the *Bacillus sonorensis* strain NBRC 101234 (NR 113993.1). A phylogenetic tree was made using the neighbor-joining method and MEGA7 software (**Fig. 1B**). The 16S rRNA sequence of strain DEM05 was deposited under accession number MK178495 in GenBank. In this line, inferred from morphological, biochemical, and phylogenetic data, the isolated strain was distinguished as *Bacillus* sp. DEM05.

4.2. Purification of the Purified Protease

The purification steps of the enzyme were summarized in **Table 1**. At the end of the purification steps, a protease with a specific activity of 1075 U.mg^{-1}, a yield of 21%, and 25.62-fold purification were obtained. As shown in **Figure 2**, the gel revealed a single band of about 30 kDa corresponding to the purified enzyme.

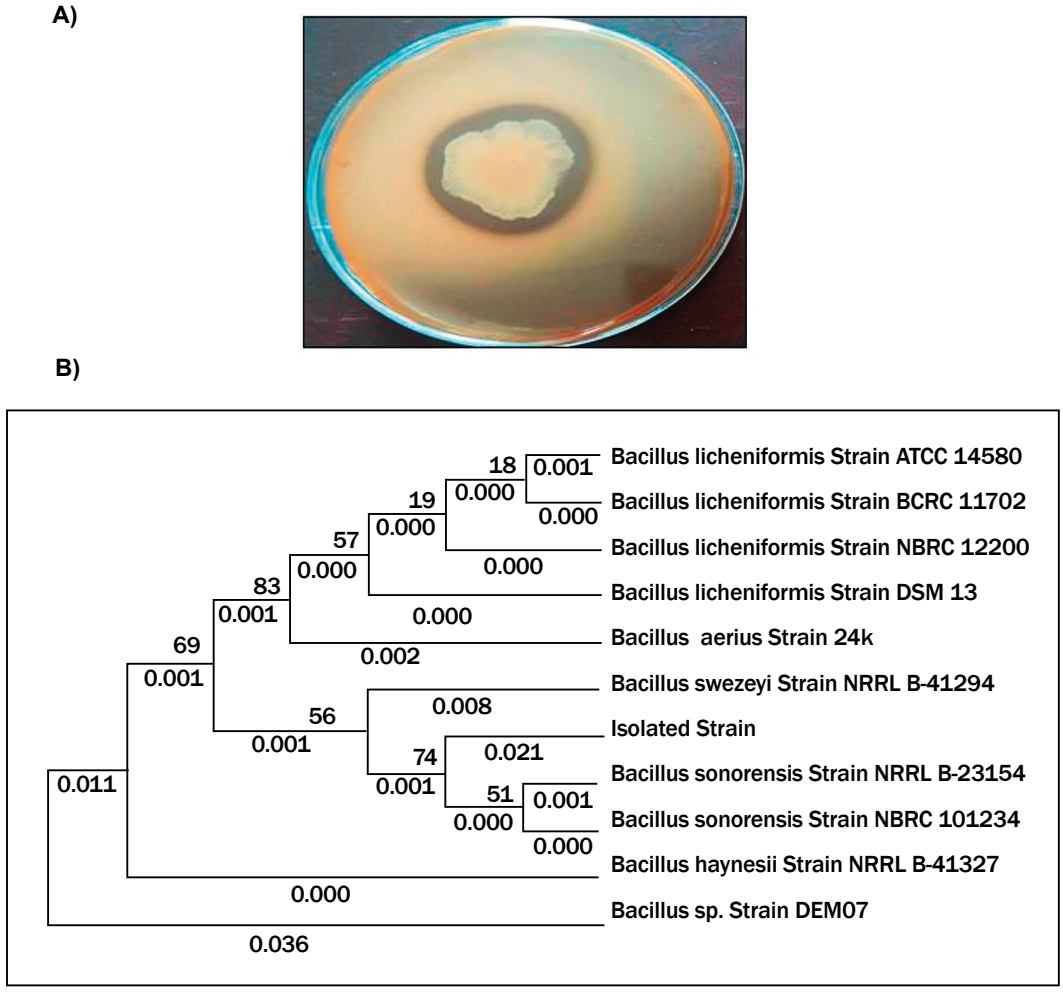

Figure 1. A) Clear hydrolytic zone due to proteolysis of skim milk by *Bacillus* sp. DEM05. **B)** Maximum likelihood analysis to determine phylogenetic relationship of *Bacillus* sp. DEM05 and related *Bacillus* taxa using gene sequence analysis of 16S rRNA. All sequences were retrieved from GenBank.

Table 1. Summary of the purification of extracellular protease from Bacillus sp. DEM05.

Purification steps	Total activity ($U.mL^{-1}$)	Total protein ($mg.mL^{-1}$)	Specific activity ($U.mg^{-1}$)	Yield (%)	Purification fold
Culture supernatant	3995	95.2	41.96	100	1
$(NH_4)_2SO_4$ (85%) precipitation	2960	32.5	91.08	74	2.17
DEAE-sepharose column	860	0.8	1075	21	25.62

Each value represents the means of three experiments and the error bar indicates ±SD.

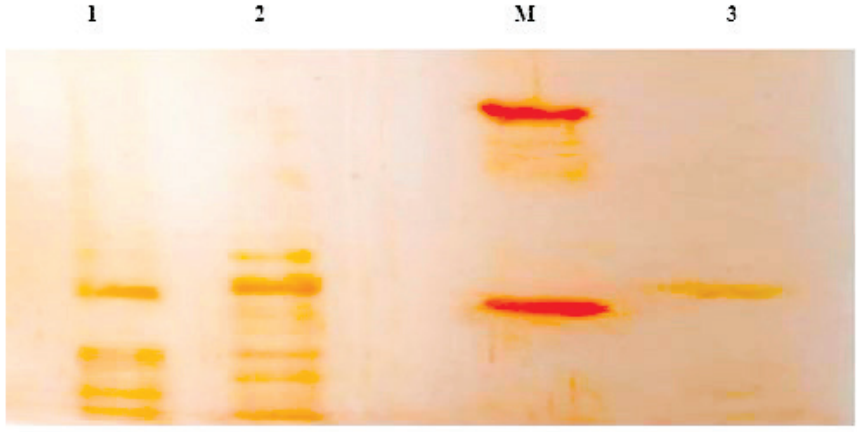

Figure 2. The SDS-PAGE analysis of the purification steps of DEM05 protease on 12.5 % SDS–polyacrylamide. Lane **1** and Lane **2**: DEAE-sepharose column (0.4 M NaCl),
lane **M**: Protein marker (serum albumin with 66.2 kDa and carbonic anhydrase with 29 kDa), lane **3**: purified DEM05 protease (DEAE-sepharose column (0.8 M NaCl)). Protein bands were detected by silver staining.

4.3. Optimization of Cultural Conditions

According to the experiments, starch and sucrose were identified as the best sources of carbon. Also, almost all tested nitrogen sources had a good effect on protease production by DEM05 protease. The maximum bacterial growth and protease production were achieved at 72 h incubation, pH 7, and 37 °C (see the supplementary data).

4.4. Characterization of the Protease

4.4.1. Effect of pH and Temperature on Activity and Stability of the DEM05 Protease

As indicated in **Figure 3A**, DEM05 protease was active over a wide range of pH from 4 to11: optimum activity at pH 10. Pre-incubation of the enzyme in mix buffers with different values of pH showed that the enzyme was stable at pH 7.0 for 1 h (**Fig. 3B**). Substantially, the protease was stable through an extensive range of pH from 4 to 11.

Pursuing the enzyme activity in the wide range of temperature from 20 to 70 °C showed that the optimum activity of the protease was at 50 °C. In addition, nearly 60% of the original protease activity was retained at 60 and 70 °C (**Fig. 3C**). Thermostability studies showed that the DEM05 protease was hardly stable up to 40 °C even after incubation for 60 min. The enzyme was significantly stable up to 50 °C for 30 min. Moreover, protease retained above 25% of its initial activity at 50 °C after 50 min. It should be noted that at 60 °C, it still reserved almost 40% of its primary activity after 30 minutes referred that the protease that could be employed under soft or rough thermal conditions (**Fig. 3D**).

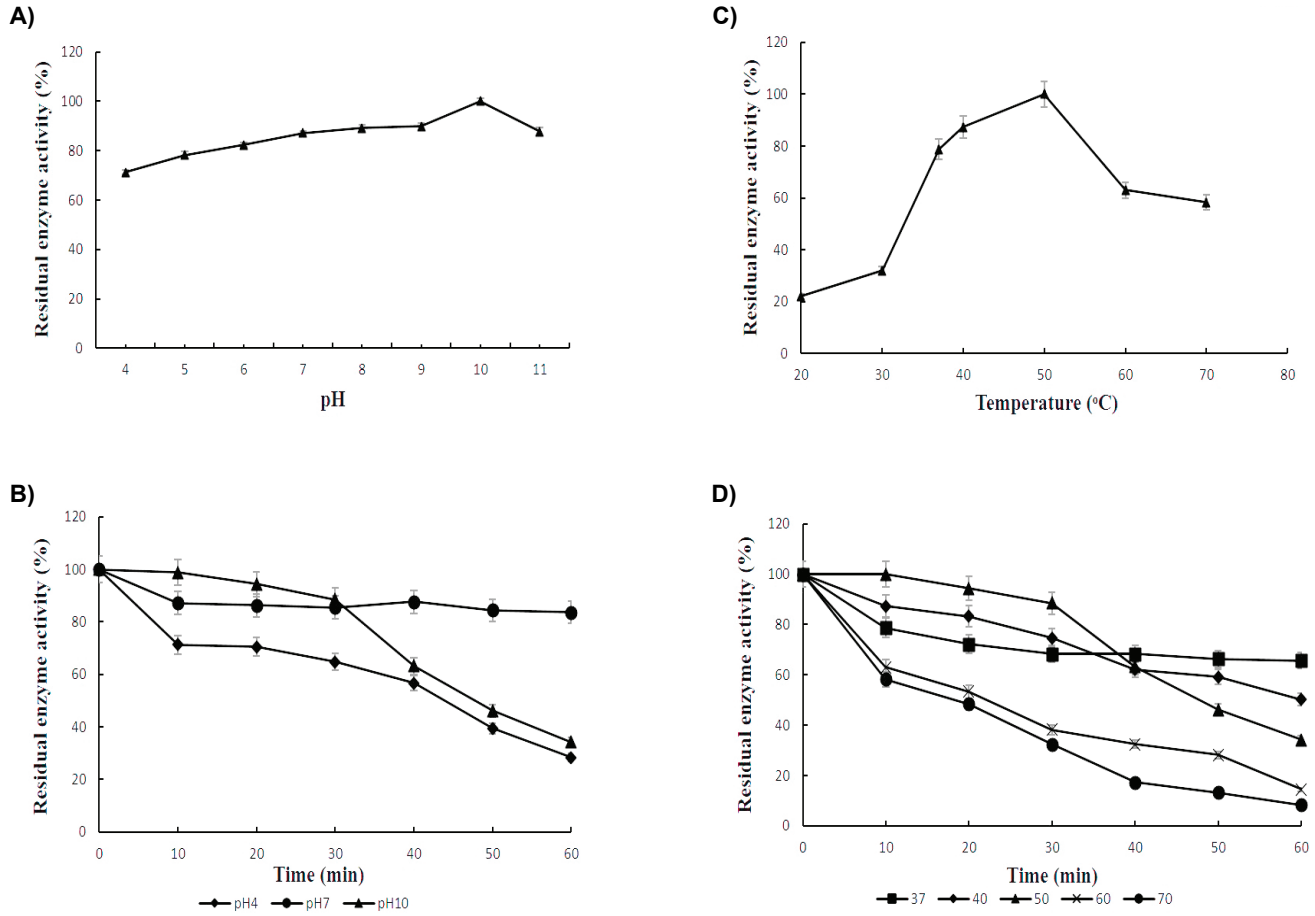

Figure 3. Effect of pH on activity **(A)** and stability **(B)** of the DEM05 protease. Protease activities were measured at 50 °C in a mixed buffer (phosphate buffer, pH 6.0–7.0; Tris–HCl buffer, pH 8.0–9.0; carbonate buffer, pH 10.0–11.0) at 50 mM. Effect of temperature on the activity **(C)** and stability **(D)** of protease. The maximal activity of the protease was taken as 100%. Data are represented at least three independent experiments. (the error bars indicate ±SD)

4.4.2. The Effect of Inhibitors and Metal Ions

The enzyme activity remained unchanged in the presence of PMSF at 5 and 10 mM). So, it doesn't belong to the serine proteases. Also, in the case of β-mercaptoethanol did not observe any inhibitory effect on protease activity, rather, this reducing agent improved the enzyme activity by 10% of its primary activity. The enzyme activity was fully inhibited by 10 mM EDTA, suggesting it can thus be classified as metalloprotease (**Table 2**).

The influences of various metal ions, at 5 and 10 mM, on the protease activity were also investigated at 50 °C and pH 10.0. As indicated, the activity of DMEM05 protease was increased with both concentrations tested for all metal ions but decreased only by 2% and 12% at 10 mM concentrations of sodium and calcium ions, respectively. The purified protease was strongly influenced by Cu^{2+}, Co^{2+}, and Zn^{2+} ions and its activity increased significantly to 250%, 305%, and 410%, respectively (**Table 2**).

4.4.3. Effects of Organic Solvents

It can be observed that DEM05 protease has good stability in most of the solvents used in this experiment, especially at a concentration of 10 % (v/v). Even, the protease activity was mildly increased with diethyl ether, toluene, ethanol, and methanol. Moreover, the enzyme was resistant to higher solvent concentrations (50 %, v/v), ethanol, diethyl ether, cyclohexane, acetone, and toluene, which retained 75.3–90 % of its initial activity (**Table 3**).

Table 2. Effect of different factors on DEM05 protease activity

	Residual activity (%)	
Inhibitors	**5 mM**	**10 mM**
Control	100	100
2-Mercaptoethanol	103 ± 1.18	110.5 ± 0.28
PMSF	104 ± 1.44	100.6 ± 0.99
EDTA	39.2 ± 0.76	1.4 ± 0.38
Metal ions	**5mM**	**10mM**
Ca^{2+}	105.1 ± 0.76	88.2 ± 1.63
Co^{2+}	305.4 ± 1.28	153.6 ± 1.24
Zn^{2+}	410 ± 2.22	140.8 ± 1.39
Na^{+}	110.7 ± 0.47	98.8 ± 0.23
K^{+}	138 ± 0.55	119.4 ± 0.94
Cu^{2+}	250.6 ± 1.58	190.3 ± 0.73
Fe^{2+}	150 ± 1.18	138.4 ± 0.66
Mg^{2+}	114 ± 1.08	110.7 ± 0.31

Table 3. Effect of organic solvents on DEM05 protease activity

Organic solvents	**10% (v/v)**	**50% (v/v)**
Toluene	108.1 ± 0.41	90 ± 0.64
Cyclohexane	99.2 ± 0.91	79.2 ± 0.85
Diethyl ether	107 ± 0.83	77.1 ± 0.59
Ethanol	109 ± 0.67	75.3 ± 0.63
Methanol	107.4 ± 0.45	50.6 ± 0.59
Butanol	94.8 ± 0.61	42.8 ± 0.38
Chloroform	100.8 ± 0.83	38 ± 0.39
Acetone	88.1 ± 0.33	81.3 ± 0.22
Surfactants/ Oxidizing agent	**2%**	**5%**
H_2O_2	172.7 ± 0.58	116.2 ± 0.64
SDS	187 ± 0.27	132.6 ± 0.98
Triton X-100	255.1 ± 0.97	236.8 ± 0.84
Substrates	**2%**	
Casein	100	
SMA	97.5 ± 0.51	
BSA	56 ± 0.73	
Gelatin	23 ± 0.91	

4.4.4. The Effect of Surfactants and/ or Detergents and H_2O_2 on the Activity of Protease

As presented in **Table 3**, the enzyme was considerably stable in the presence of selected compounds. Incubation of the enzyme with 5% of Triton X-100, SDS, and H_2O_2 substantially augmented the protease activity by 136%, 32%, and 16% respectively.

4.4.5. Substrate Specificity

As outlined in **Table 3**, in the same concentration (2%) of the casein, SMA, BSA, and gelatin substrates, the enzyme exhibited the maximum activity in the presence of casein substrate, which was considered 100%. In the presence of SMA as a substrate, the activity of the protease reached 97.5% that is slightly reduced compared to the casein.

5. Discussion

In the final purification step, we used the DEAE-Sepharose ion-exchange chromatography method as described earlier. After SDS-PAGE, the molecular mass of the DEM05 protease was determined to be 30 kDa. Joo and Choi (2012) (12) used the same method to purify an alkaline protease from *Bacillus horikoshii* with a molecular mass of 28 kDa and a yield of 34.1%. Similarly, ion exchange chromatography was used in another study to purify an alkaline protease from *Bacillus circulans* MTCC7942 with the molecular mass of 43 kDa, resulting in a yield of 39% (13).

The optimum activity of DEM05 protease was at pH 10. Interestingly, detergent-matchable enzymes require alkaline conditions for their catalysis. Similarly, the earlier study illustrated that proteases from *B. licheniformis* NH1 and *B. mojavensis* showed the maximum activity at pH of 10 and 10.5, respectively (14, 15). The enzyme was also most active at 50 °C. DEM05 protease can survive up to 50 °C indicating that this isolate was a heat-resistant *Bacillus* species. Similarly, the optimal temperature of the protease from *Bacillus* cereus TKU022 was 50 °C (16). However, many proteases have been isolated from Bacillus species with different optimum temperatures, for example, the optimum temperature of an alkaline protease purified by Patil *et al* was 60 °C (13).

The catalytic mechanism of the protease, as well as active site catalytic residue and its cofactor requirements of the enzyme, could be recognized by inhibition studies (17). Incitement of proteolytic activity by β-mercaptoethanol may be due to thiol-disulfide conversion that likely stimulates the protease activity (12, 14-18). The enzyme activity was fully inhibited by 10 mM of EDTA which is an inhibitor of metalloproteases. According to these results, the DEM05 protease belongs to the family of the metalloproteases. Saminathan and Narayanan (2015) (19) showed that the protease activity was strongly stopped by 1mM of EDTA (by 94.2%). In one research by Sookkheo *et al*, it was found that 10 mM concentrations of EDTA and 1,10-phenanthroline chelating agents had a robust inhibitory effect on enzyme activity (20).

Alkaline proteases have been shown to require a divalent cation such as Ca^{2+} and Mn^{2+}, or a mixture of these metal cations, to maximize their activity. These cations protect the enzyme against thermal denaturation and play an important role in maintaining the active structure of the enzyme at high temperatures (21,22). The fact that the activity of DEM05 protease has significantly increased in the presence of zinc, cobalt, and copper ions at a concentration of 5 mM is noticeable among other proteases. Similarly, in one study, protease activity by copper, iron, and cobalt ions increased by 82%, 37%, and 31%, respectively, relative to its initial activity, but decreased slightly in the presence of zinc and potassium (23). One study was indicated that $CoCl_2$ could mildly enhance the enzyme activity (24). In another study, the protease activity increased in the presence of 10 mM metal ions namely Mn^{2+}, Mg^{2+}, Cu^{2+}, and Co^{2+} (25). However, $CaCl_2$ (10 mM) slightly inhibited the DEM05 protease activity, so this enzyme appears to be a calcium-dependent metalloprotease. In this line, Kazan *et al.* (26) reported that Ca^{2+} had a slow inhibitory effect on protease activity. Farhan *et al.* (27) also showed that calcium ions reduced the activity of the metalloprotease by 57%.

Peptide and ester synthesis reactions by protease enzymes have the greatest outcome under non-aqueous circumstances. Due to this potential, the recruitment of proteases in organic media has attracted much attention in current decades (28). Owing to this preference, investigations on the stability of the purified protease in the presence of various organic solvents were performed. In the presence of 10% concentrations of toluene, ethanol, methanol, and diethyl ether, we observed almost a slight increase in enzyme activity. Shah *et al.* reported that water-immiscible solvents, namely, decane, hexadecane, hexane, cyclooctane, toluene, and benzene could enhance the activity of protease in contrast to the water-miscible solvents. Meanwhile, water-miscible solvents such as ethanol and DMSO reduced the activity of the purified enzymes to 70% and 80%, respectively (29).

Surfactants and oxidizing agents have been shown to

modulate the activity of the enzyme ingredient. In this line, the efficacy of the enzyme in such an environment depends on the resistance to these reagents. In general, the selected surfactants and bleaching agents not only couldn't decline the DEM05 protease activity but also enhanced it. This characteristic implies the remarkable adaptability of the purified enzyme with detergent contents. In one study, Mardina and Yousef (30) showed that the metalloprotease was stable in 1% Triton X-100, while its activity increased by 82.7% in the presence of SDS.

The DEM05 protease was able to proteolysis all of the chosen substrates with different levels of specificity. The ability of proteases in hydrolyzing the broad range of the substrate is one of the essential features considered for industrial applications (31). As literature stated, the highest activity of the alkaline proteases has been observed toward casein compared to BSA and gelatin (27-29).

6. Conclusions

Generally, industries require enzymes that can tolerate a wide range of extreme conditions such as high temperatures and pH. Besides, high activity and stability were dependent on the types of surfactants, organic solvents, and bleaching agents. Hence, considering compatibility with a broad range of pH and temperature as well as a various number of detergents and organic solvents, the DEM05 protease seems to pave the way to use in applicable industries such detergent, leather, pharmaceuticals, and synthesis of biomolecules.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author Contribution Statement

All authors have made substantial contributions to the following: the conception and design of the study, analysis, interpretation of data, drafting the article and final approval of the version to be submitted.

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