**Düzce University**
**Journal of Science & Technology**

*Research Article*

# Isolation, Purification and Characterization of New Cold Active Subtilisin-like Protease from *Bacillus sp. strain EL-GU1*

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DOI : 10.29130/dubited.537340

## ABSTRACT

One of the important hydrolytic enzymes are proteases that slice peptide bonds between amino acid residues. Proteases have various industrial applications including detergent, food, pharmaceutical, leather and diagnostic reagent industries. Among them, the most commercialized enzymes are alkaline proteases in the industry. Due to their potential applications in the detergent industry as cleaning additives, they are of particular interest. In this study, a novel protease from *Bacillus sp. strain EL-GU1* was reported showing highest activity at pH 6 and 20°C. The novel protease was purified by using ammonium sulfate precipitation and identified by 16S rDNA sequencing. Highest activity was observed as 3300 µmol/min$^{-1}$mg$^{-1}$ when casein used as a substrate. Kinetic parameters of the enzyme were determined; $K_M$, $V_{max}$, $k_{cat}$ and catalytic efficiency values were calculated as 1.4 mM, 1 mM/s, 2.10$^{-7}$ s$^{-1}$, 0.14 10$^{-7}$ s$^{-1}$M$^{-1}$, respectively. These results indicated that the novel cold active protease from *Bacillus sp. strain EL- GU1* can be a good candidate for the detergent industry.

***Keywords:*** *Bacillus sp., protease, enzyme characterization, cold- active and alkaline protease*

# Soğuk Aktif ve Alkali Subtilisin Benzeri Proteaz Enziminin *Bacillus sp. strain EL-GU1'* den İzolasyonu, Saflaştırılması ve Karakterizasyonu

## ÖZET

Proteaz, amino asit rezidüleri arasındaki peptid bağlarını parçalayan ve deterjan, gıda, ilaç ve deri gibi çeşitli endüstriyel saha uygulamalarında kullanılan bir enzimdir. Proteaz enzim çeşitleri arasında endüstride en çok ilgi çeken, deterjan endüstrisinde ticari olarak en fazla potansiyel uygulama alanı bulan ve temizlik katkı maddesi olarak yaygın kullanılar alkalin proteazlardır.

Geliş: 11/03/2019, Düzeltme: 28/06/2019, Kabul: 17/07/2019

Bu çalışmada Bacillus sp. EL-*GU1*'den yeni bir alkali proteaz enzimi amonyum sülfat çöktürme metodu kullanılarak saflaştırıldı ve 16s rDNA dizileme yöntemi ile de proteaz olarak tanımlandı. En yüksek enzim aktivitesi, pH 6'da ve 25°C'de kazein substrat olarak kullanıldığında 3300 µmol/ $min^{-1}mg^{-1}$ olarak elde edildi. Sırasıyla enzim kinetik parametreleri olan $K_M$, $V_{max}$, $k_{cat}$ ve katalitik verimlilik değeri
1.4 mM, 1 mM/s, $2.10^{-7}$ $s^{-1}$, 0.14 $10^{-7}$ $s^{-1}M^{-1}$ olarak hesaplandı. Bu sonuçlar bize gösteriyor ki *Bacillus sp. EL-GU1* suşu deterjan endüstrisinde kullanılmak için iyi bir aday olup ürettiği proteaz enzimi yeni ve özgün, düşük sıcaklıklarda da aktivite gösterebilen bir özelliktedir.

***Anahtar Kelimeler:*** *Bacillus sp., proteaz, enzim karakterizasyonu, soğuk aktif ve alkali proteaz*

# I. Introduction

Proteases, hydrolytic enzymes slicing the peptide bonds of amino acid residues [1]. Among the proteases, alkaline proteases, are the most commercialized enzymes in the industry [1, 2]. Alkaline proteases have various industrial applications such as detergents [1, 3], foods, pharmaceuticals, leathers and diagnostic reagents industries [1, 4]. Among the alkaline proteases, serine proteases [EC3.4.21], which are also endopeptidases are industrially more important than the exopeptidases [17]. The other characteristics of the proteases are the usage for the biotransformation of chitinous materials in waste treatment as an alternative to the disposal of shellfish wastes [1, 5, 6]. The extracted chitin and chitosan as an end product of this enzymatical process are being used in many applications in cosmetics and food processing [1]. The industrial demand for highly active proteases with appropriate specificity and stability of pH, temperature encourages the search for new enzymes [1]. Among the bacterial proteases, the most widely used and studied industrial enzymes are produced especially from *Bacillus sp.*, and *Bacillus subtilis* [7]. Subtilisin, a group of serine proteases initiating the cleavage on the amide bond throughout a serine residue. Molecular weights of subtilisin is 25-45 kDa [1]. Bacillus species are well-known producers of subtilisin [1]. There are widely used applications in the industry of laundry and dishwashing detergents, food processing, skin care ointments, and for research purposes in synthetic organic chemistry and so on [1, 2, 8].

The goal of this study was purification and characterization of the novel cold active protease produced by *Bacillus sp. strain EL-GU,* isolated from Ornamental plant soil in Trabzon, Black sea region, Turkey. Moreover, molecular characterization of the strain was performed by using 16S rDNA sequencing method.

# II. Material And Methods

## *A. SCREENING MEDIUM AND BACTERIAL ISOLATION*

22 different soil samples were collected from different cities from Turkey. Soil samples were stored at dry places in sterile tubes. Collected samples were plated onto selective agar plates for screening of protease (+) bacterium. The selective agar plates contained (g/l): skim milk (Merck, 115363) 7 g; LB

Broth 17.5 g; agar 10.5 g; in 300 ml, pH 7.0. Incubation was done for 48 hours at 37 ˚C. Observing a clear zone on the skim-milk indicated the presence of a bacterial strain which produces protease enzyme. Individual colonies were purified by triplet repeats of streak inoculation on fresh agar plates. Purity of the colonies were determined according to the gram staining results. Glycerol stock and agar stocks were prepared for all pure protease (+) colonies. Solid agar plates and glycerol stocks were stored at 4°C and -80°C, respectively. The diameter of the colony (DC) was divided by the diameter of the clear zone (DZ) [(DZ/DC)], which gives us the enzymatic index (EZ) for all isolates.

The most protease producer strains were selected according to their enzymatic index value. Top five distinct protease (+) isolates were chosen by looking their enzymatic indexes to perform bacterial DNA isolation.

*B. MOLECULAR IDENTIFICATION OF PROTEASE (+) ISOLATES*

Five isolates with considerable enzymatic index values were identified with 16S rDNA sequencing (Sentromer & Macrogen).

*C. EXTRACT PREPARATION OF CRUDE ENZYME*

All protease (+) bacteria were cultivated overnight in Studier medium [9]. That special medium contains: yeast extract (2,5 g), peptone (10 g), sodium dihydrogen phosphate dehydrates (3 g) sodium fluoride (2,5g) potassium phosphate dibasic (1,5g), 10% glucose solution (2,5 ml), 8% lactose solution (12,5 ml), 50% glycerol solution (6 ml) in 500 ml $dH_2O$. All isolates were inoculated in 50 ml Studier medium and cultivated overnight at 37°C. Two types of the samples were prepared for each isolate including supernatant and cell free extracts of the enzyme. Obtained cultures were centrifuged for 30 min at 0°C with 10.000 rpm in order to get supernatant consisting of extracellular enzymes. The supernatants were collected into fresh tubes. After getting supernatant, pellets of each sample were suspended with Tampon A solution. Then 100 µl PMSF was added. After adding powder of lysozyme waited 5-6 min at 37$^{o}$C. The mixture was sunk into dry-ice for 30 minutes. Cell lysis was accomplished by sonication with vibration severity of 70% for 15 seconds applies. Then taking a break to the vibration step for 45 seconds on the ice, per one sonication (Bandelin Electronic sonicator). This step repeated six times simultaneously. Cell free extract, cleared of other cellular waste molecules, were collected at the final step by centrifugation at 10.000 rpm for 30 minutes. Both supernatants and cell free enzyme extracts were stocked at -20°C to perform protease activity assay and enzyme purification steps.

*D. PROTEASE ACTIVITY ASSAY*

Protease enzyme activity was measured by following procedure by Kunitz (1947) with some modifications on the method: the amount of enzyme required to liberate 1 mole tyrosine/mL per minute was defined as one unit of protease activity [10]. Spectrophotometric method was used for measurements of proteolytic enzyme activities. The enzyme activity was based on the number of peptides released, as monitored by absorbance at 280 nm. 50 µl enzyme fraction for 1% casein substrate solution was prepared for each reaction. 1 g casein was dissolved in 0.1 M Sodium phosphate buffer for preparation of 1% casein substrate. All activity reactions were performed in triplets. The incubation of substrate solution and mixture of enzyme was done for 30 seconds at 30˚C with shaking at 100 rpm for each reaction. Immediately 5% TCA (1700 µl) was added to block the enzyme reaction.

After stopping the reaction, the mixture was poured into 10 ml of distilled water then the absorbance at $A_{280}$ nm was measured. For each measurement, the blank was measured by using mixture of substrate and TCA without enzyme extract under the same conditions. In another word, distilled water was added instead of enzyme for reads of blank values.

### *E. PARTIAL PURIFICATION OF PROTEASES*

Both cell free and supernatant enzyme extracts were partially purified by ammonium sulfate precipitation method. Further purification steps weren't performed. In this method, proteins were precipitated by adding appropriate amount of ammonium sulfate to get the intended concentration of $(NH_4)_2SO_4$ in the range of (%40-100) at 4°C [11]. After precipitation of the proteins, all precipitants were collected by centrifugation at 10.000 rpm for 10 min. Pellets were dissolved in 5 ml of 100 mM PBS buffer (Phosphate buffer saline). Dialysis was performed in 50 mM, 500 ml PBS Buffer at 4°C in order to discard the ammonium sulfate salts from protein mixture placed in dialysis bag (Bio Basic Dialysis Bag). All partial purified enzyme fractions (in each precipitation step 40%, 60%, 80%, and 100%) were checked with spectrophotometric method in terms of protease enzyme activity. To determine the molecular weight of the enzyme, fractions containing protease enzymes were run in the sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to see the separation of the proteins.

## III. CHARACTERIZATION OF PROTEASES

### *A. EFFECT OF pH ON THE ACTIVITY OF PROTEASE*

The optimum pH of the serine protease was determined with casein 1% (w/v) as substrate dissolved in different buffers. Following buffers (20 mM) were used: glycine-HCI (pH:2-3), sodium acetate (pH:4-5), sodium phosphate (pH:6-7), tris-HCI (pH:8-10). The pH stability of alkaline protease was determined by pre-incubating enzyme in different buffers for 10 minutes at 30 °C [12]**.**

### *B. EFFECT OF TEMPERATURE AND STABILITY*

The optimum temperature of protease activity was determined with reaction mixture incubated at different temperature in a range from 0 to 35°C. For determination of thermostability, 0.1 ml of enzyme was pre-incubated with 0.4 ml of 20 mM sodium phosphate buffer pH:6 (from results of optimum pH assay) for 10 min at each temperature from 0 to 35 °C. After 10 min incubation, protease activity was determined by the method described relatively.

### *C. EFFECT OF METAL IONS AND INHIBITORS ON ENZYME ACTIVITY*

In order to determine the effect of metal ions and inhibitors on protease enzyme activity, casein (1%) was used as the substrate for enzymatic incubation with metal ions in given concentrations in Table 4 within 20 mM sodium phosphate buffer, at optimum pH: 6 and temperature 25 $^{o}$C. Incubation period was set up as 30 minutes [13]. Control group was assigned to the samples which did not include any metal ions, with relative activity value of 100%.

### *D. SUBSTRATE SPECIFICITY OF PROTEASE*

Different substrate effects on the protease activity were determined by 30 minutes incubation of enzyme extracts with 1% BSA (Bovine serum albumin) and 1% casein in 20 mM sodium phosphate buffer solution at pH:6 and 25$^{o}$C [12]. Then after 30 minutes, enzyme activity was measured by spectrophotometer, simultaneously.

### *E. DETERMINATION OF PROTEIN CONCENTRATION*

Total protein amount was calculated with Bradford Assay by using BSA as the standard measurement and it was determined as 1,976 mg/ml. Linear regression of the BSA standardized concentration was calculated as $y = \beta_0 + \beta_1.x$, where $\beta_0$ is the intercept of the line, equals to 0.0256, and $\beta_1$, is the slope of the line, equals to 0.5994.

### *F. CATALYTIC EFFICIENCY OF PROTEASE*

Experiment was carried out using different concentrations of the solution for casein substrate which are in the range of 0.1 mM to 8 mM, under optimum pH and temperature. Based on their spectrophotometric reads, the activities were determined. Using these activity indicator values, Michaelis-Menten graph was plotted to estimate kinetic parameters ($K_m$ and $V_{max}$).

## IV. RESULTS

### *A. SCREENING, ISOLATION AND IDENTIFICATION OF PROTEASE (+) BACTERIA*

Protease producer bacteria was isolated from the soil which were collected from Istanbul, Bursa and Trabzon [Table 1].

***Table 1.*** *Soil samples and their locations.*

| **Sample Name** | **Sample Number** | **Location (Sample Collected from)** |
|---|---|---|
| Farm field soil | **1** | Trabzon, Black Sea |
| Soil Under walnut tree | **3** | Trabzon, Black Sea |
| Ornamental plant soil | **5** | Trabzon, Black Sea |

***Table 1 (continue).*** *Soil samples and their locations.*

| | | |
|---|---|---|
| Soil under oak tree | **11** | Bursa, Nilüfer Tahtalı Village |
| Lake sample | **12** | Istanbul, Büyükçekmece Lake |
| Soil under *Robinia pseudoaccia L.* (top acacia) tree | **22** | İstanbul Atatürk Orman Çiftliği |

Isolation of the protease (+) bacteria was performed by using plate assay technique. The selective agar media was prepared with a skim milk, used as the substrate for protease. According to the enzymatic index results, five isolates were characterized using gram staining and 16S rDNA Sequencing (Figure 1 and Table 2). 16S rDNA gene amplified by PCR using universal primers and 16S rDNA sequence similarities were compared with other organisms through BLAST database.

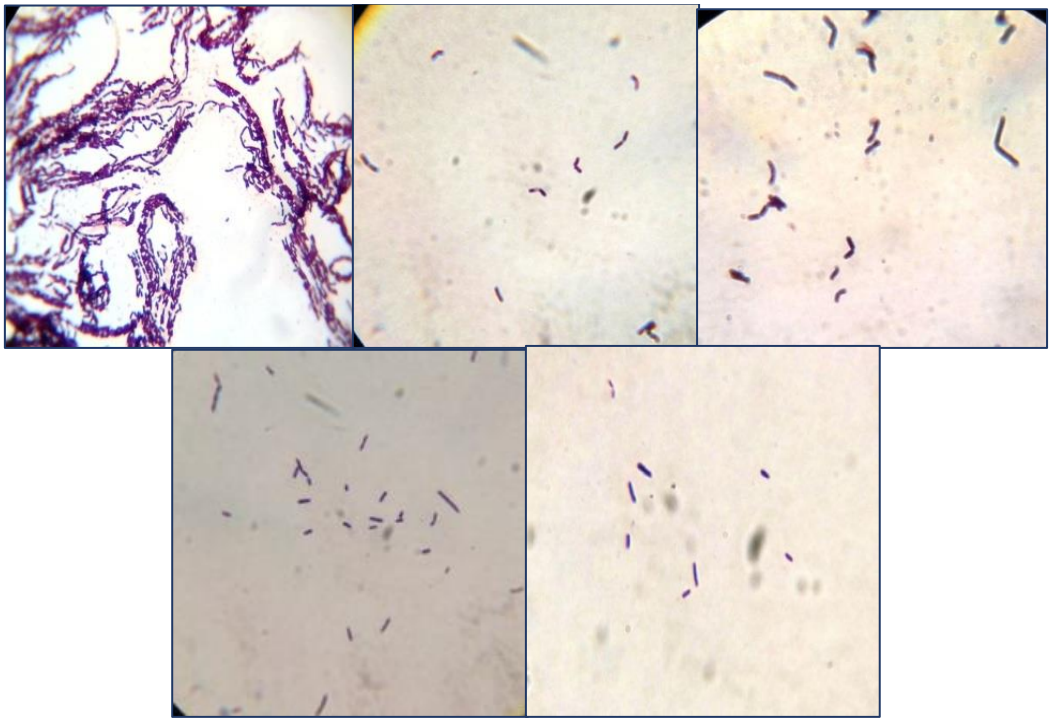

***Figure 1.*** *Gram staining results of protease (+) bacteria (Left to right: PR1, PR2, PR3, PR4, PR5).*

***Table 2.*** *Identification of Protease (+) isolates by 16S rDNA sequencing.*

| | | **Most closely related organism** | | |
|---|---|---|---|---|
| **Isolate** | **Length of 16S rDNA gene sequenced** | **Species** | **GenBank accession no.** | **Gene identity %** |
| **PR1** | 1460 | *Bacillus sp. CRh17* | KF322024.1 | 99 |
| **PR2** | 1479 | *Bacillus sp. RD_DACAR_05* | KU597568.1 | 99 |

***Table 2 (continue).*** *Identification of Protease (+) isolates by 16S rDNA sequencing*

| | | | | |
|---|---|---|---|---|
| **PR3** | 1479 | *Bacillus sp. strain SAP02_1* | JN872500.1 | 99 |
| **PR4** | 1460 | *Pseudomonas aeruginosa strain SV1* | KF322024.1 | 99 |
| **PR5** | 1516 | *Bacillus altitudinis strain KUDC1739* | KC414718.1 | 99 |

Among different strains and their proteolytic activities, the sample labelled as PR3 was selected according to its high enzymatic index value. PR3 isolate showed 99% identity with *Bacillus sp. strain SAP02_1*, according to 16S rRNA sequence comparison within BLAST database. The phylogenetic tree was constructed and given in Figure 2. The new isolate, PR3 was named as *Bacillus sp. strain EL-GU1*.

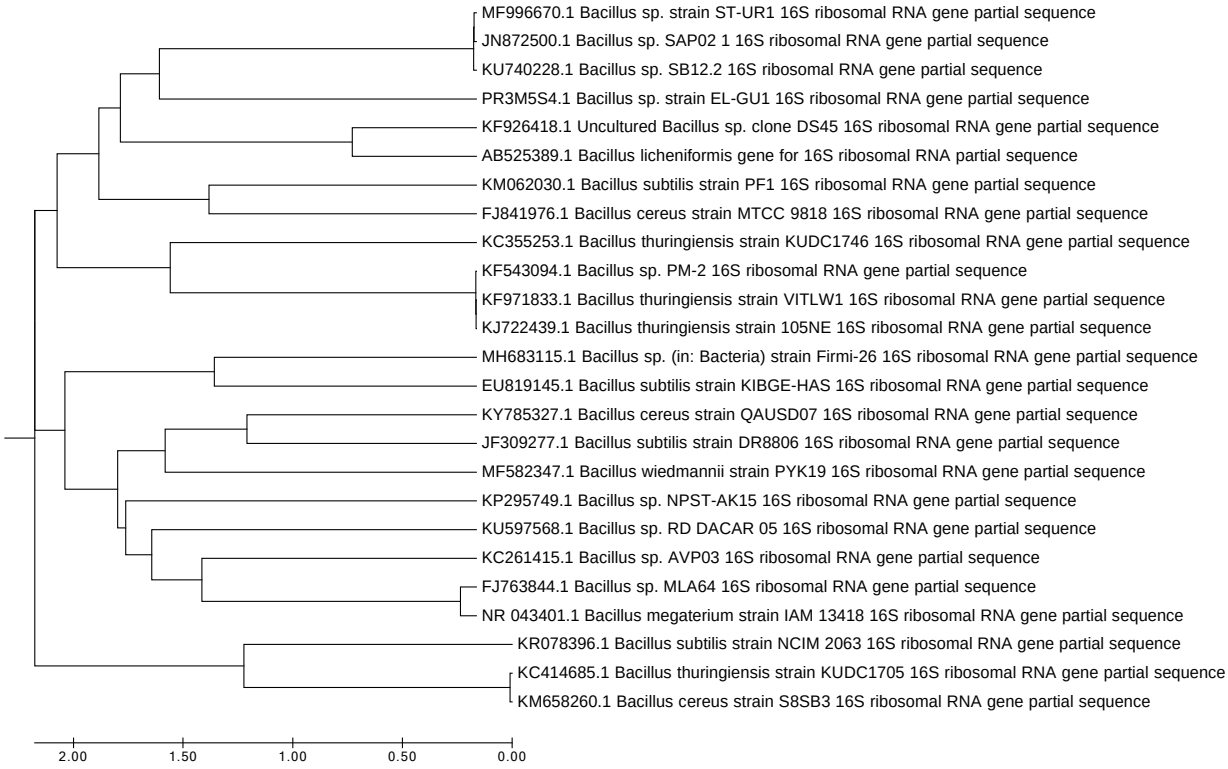


***Figure 2****. Phylogenetic tree of isolate PR3 based on 16S rDNA Sequencing.*
*Sequences were aligned by Muscle. The UPGMA method used to infer the evolutionary history [36]. Sum of branch length for optimum tree is 28.92684829 is shown. Inference of the phylogenetic tree carried out by using the evolutionary distances with branch lengths in the same units. Maximum Composite Likelihood method was used to compute the evolutionary distances [37] and are in the units of the number of base substitutions per site. 25 nucleotide sequences were involved in the analysis. Included codon positions were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. A total of 371 positions were in the final dataset. MEGA7 was used to conduct Evolutionary analyses [38].*

## *B. PRODUCTION AND PARTIALLY PURIFICATION OF SUBTILISIN-LIKE PROTEASE*

Cell free and supernatant enzyme extracts were used for protease spectrophotometric activity assay. According to the protease activity assay results, the highest protease activity was determined in an intracellular enzyme extract from *Bacillus sp. strain EL-GU1* [Table 3]. Due to its highest protease activity, *Bacillus sp. strain EL-GU1 was* chosen for purification and characterization of protease. In addition to this finding, earlier enzyme characterization paper of our lab reported that our *Bacillus sp. strain EL-GU1* had also laccase enzyme activity of 21±3 %, as it was identified the isolate La-4 in this paper, relative to the other candidates [39].

Purification of subtilisin-like protease was performed by using ammonium sulfate precipitation method. All fractions obtained from ammonium sulfate precipitation were (40-60-80-100%-the last supernatant) used for protease activity assay. Highest activity was observed at 60% fraction. Amount of protein after purification was calculated as 1,976 mg/ml by using BSA as a standard.

***Table 3.*** *Relative activities of proteases from different isolates.*

| **Isolate** | **Organism** | **Cell Free**<br>**Relative activity (%)** | **Supernatant**<br>**Relative activity (%)** |
|---|---|---|---|
| **PR1** | *Bacillus sp.* | 0 | 0 |
| **PR2** | *Bacillus sp.* | 89 ± 6,7 | 0 |
| **PR3** | *Bacillus sp. strain SAP02_1* | 100 ± 3,3 | 1 ± 0,8 |
| **PR4** | *Pseudomonas aeruginosa* | 0 | 0 |
| **PR5** | *Bacillus altitudinis strain* | 87 ± 3,4 | 100 ± 3,8 |

## *C. CHARACTERIZATION OF PROTEASE SUBTILISIN-LIKE PROTEASE*

### *C.1 EFFECT OF pH ON ACTIVITY OF PROTEASE*

By using different buffers prepared in wide pH range of 2 to 10 with casein as the substrate for protease, pH effect on the protease activity was determined. There was no protease activity at pH 2 and pH 3. Interestingly, relative protease activity seen at pH 4 and 5 were relative activity of 48% and 58%, respectively. The highest protease activity was observed at pH 6. In neutral condition (pH 7), protease activity dramatically decreased to 33%. On the other hand, in alkaline conditions (pH 8-10), protease performed very poor in terms of activity (i.e. at pH 8, relative activity of 3%) [Figure 3].

*C.2 EFFECT OF TEMPERATURE ON ACTIVITY AND STABILITY OF PROTEASE*

The effect of a temperature on protease activity was determined at different temperatures in range of 0 to 35°C with the interval of 5°C. There was no protease activity up to 20°C. However, the maximum protease activity was observed at 20°C and it slightly decreases to 81%, when the temperature reached at 25°C. On the other hand, the protease activity dramatically decreases to 62% at 30°C. At 35°C point, protease activity was completely lost [Figure 4].

Temperature stability of the protease was determined at 25°C after incubation of protease at optimum pH:6, for 30 minutes. After the arranged time of incubation at 25°C, the activity of protease decreased to 63% from 100% [Figure 5]. As a conclusion, it keeps the stability of the enzyme at that optimum temperature quite well.

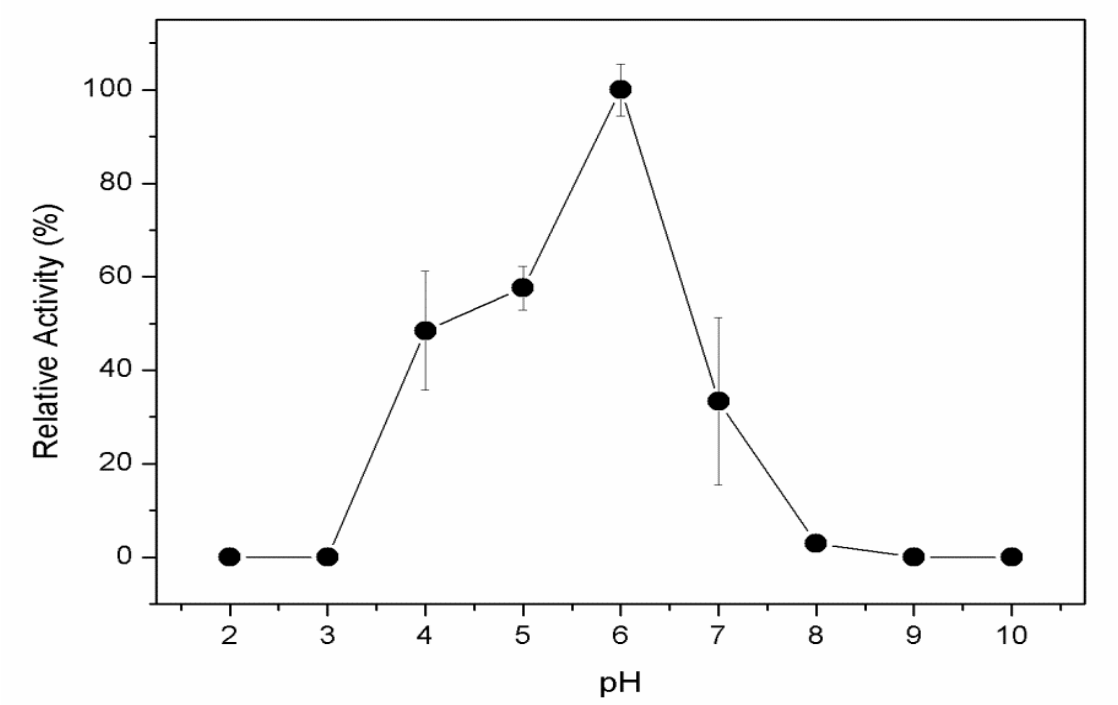


***Figure 3****. Effect of pH on the activity of protease from Bacillus sp. strain EL-GU1*.

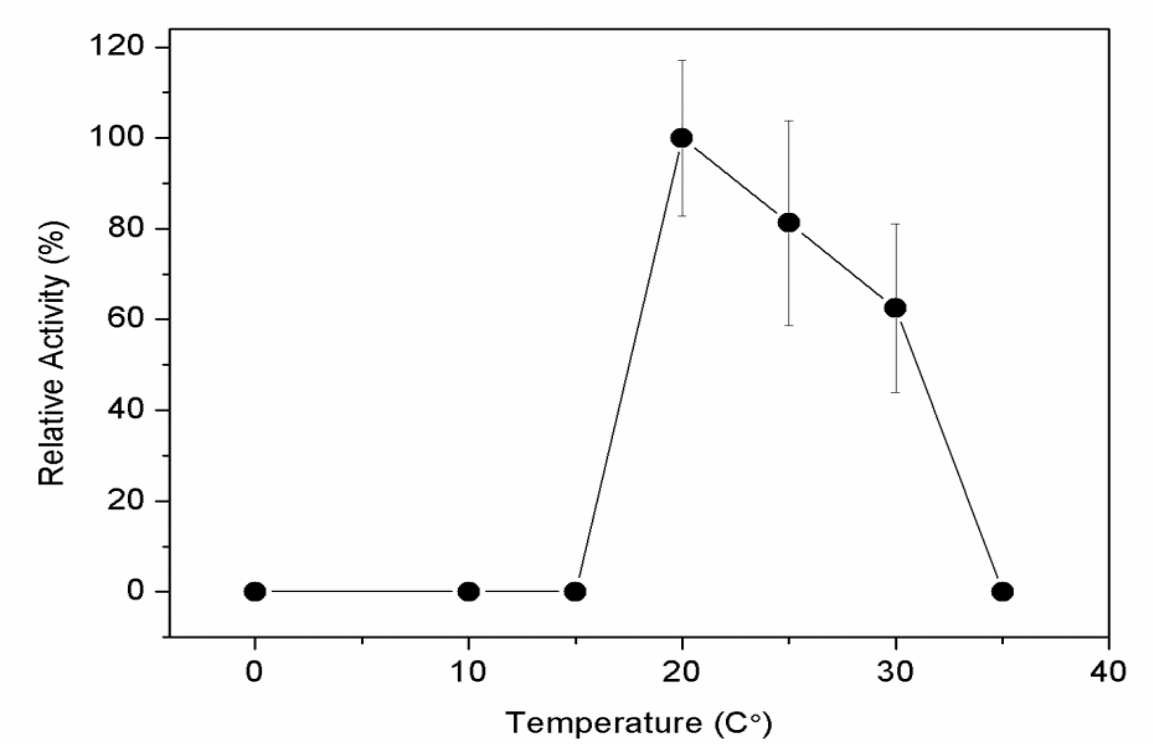


***Figure 4***. *Effect of temperature on the activity of protease from Bacillus sp. strain EL-GU1.*

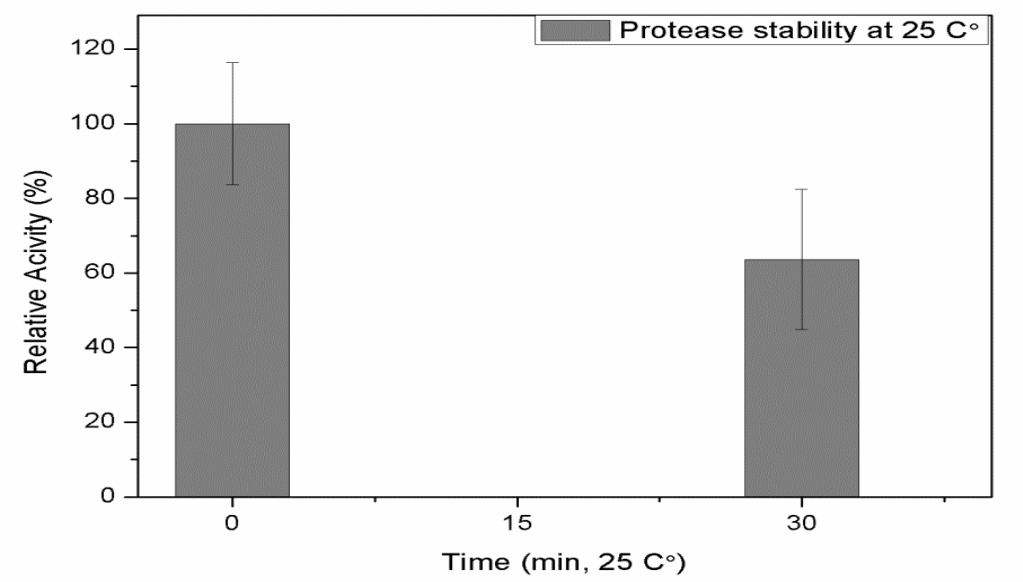


***Figure 5***. *Effect of temperature on the stability of protease from Bacillus sp. strain EL-GU1 at 25 °C.*

## *C.3 EFFECT OF METAL IONS AND INHIBITORS ON PROTEASE ACTIVITY*

Effect of inhibitors and metal ions on the protease activity was determined by using PMSF-β-mercapto-ethanol as an inhibitor and $MgSO_4$ -$FeSO_4$ solutions as metal ions. Protease activity was completely inhibited in the presence of both PMSF and β-mercaptoethanol. Similar with the inhibitors, $MgSO_4$ also completely inhibited the activity of enzyme. There was only one condition that activity of protease increased about 2-fold, which reaches up to 2 u/mg in the presence of $FeSO_4$ [Table 4].

***Table 4.*** *Effect of different metal ions on protease activity.*

| Metal ions | Final Concentration (mM) | Relative activity (%) |
|---|---|---|
| **Control** | NI | 100.0 |
| **PMSF** | 5 mM | 0 |
| **Beta-Mercaptoethanol** | 5 mM | 0 |
| **FeSO4** | 5 mM | 220.0 |
| **MgSO4** | 5 mM | 0 |

NI: No Metal Ion

## *C.4 SUBSTRATE SPECIFICITY*

BSA (Bovine Serum Albumin) and casein were used as substrates to determine substrate specificity of the protease. Protease showed higher affinity to BSA (1.1 Unit/mg) when compared with casein (0.6 Unit/mg) [Table 5].

***Table 5.*** *Substrate profile of Protease from Bacillus sp. strain EL-GU1.*

| Substrate | ε ($M^{-1}$ $cm^{-1}$) | $\lambda_{max}$ (nm) | Relative activity (%) |
|---|---|---|---|
| **BSA** | 43824 | 280 | 100±0.4 |
| **Casein** | NI | 280 | 54.5±0 |

NI: No information
Values represent mean ± SD (n=3)

## *C.5 CATALYTIC EFFICIENCY OF PROTEASE*

The catalytic efficiency of the protease was determined by calculating $K_M$, $V_{max}$ and $k_{cat}$, values. Casein was used as the substrate in different concentrations between 0.1 to 8 mM. Optimum activity of protease was calculated as 3300 μmol/ $min^{-1}mg^{-1}$ in pH 6 and 20°C.

$K_M$, $V_{max}$ and $k_{cat}$ values for the hydrolysis of casein were calculated from the Michaelis Menten plot as 1.4 mM, 1 mM/s and $2.10^{-7}$ $s^{-1}$, respectively. The catalytic activity ($k_{cat}/K_M$) was calculated as 0.14. $10^{-7}$ $s^{-1}M^{-1}$. Kinetic parameters of the protease from *Bacillus licheniformis UV-9* were estimated by Lineweaver-Burk Plot that the $K_M$ and $V_{max}$ values were determined as 5 mg/ml ($10^{-2}$mM) and 61.58 μM/ml/min, respectively [14]. Kinetic parameters of the protease from *Bacillus subtilis KIBGE-HAS* was determined by using different concentrations of casein, ranging from 0.1% to 1.5%. $K_M$ and $V_{max}$ for the protease from *Bacillus subtilis KIBGE-HAS* were determined as 130 mg/dl ($2.6\ 10^{-3}$ mM) and 113 U/ml/min respectively [15].

# V.CONCLUSION

Protease is among the most important enzymes in industrial applications due to their ability to be used in diverse applications in different industries [16, 17]. Microorganisms have particular importance because of their potential to produce proteases that allows them to adopt extreme conditions such as high temperature or pH. Bacteria especially *Bacillus* species are known as one of the most valuable protease sources. There are several studies which show basically the proteases from different Bacillus species have valuable features that make them suitable for industrial applications such as stability in alkaline conditions, for instance *Bacillus sp JB-99*, *Bacillus sp NPST-AK15* and *Bacillus subtilis megatherium* are very good at that [18, 19, 20].

In recent studies, cold-active proteases are taken particular interest due to their ability to be used in textile and detergent industry. Cold-active proteases can be used to remove macromolecular stains from fabric at low temperature [17]. Completing all procedure at low temperature, allows clothes to be stayed without worn out for long time from the exposure to high temperature. Cold-active proteases bring us unique and new way for clearance of new fabrics those constructed from the synthetic fibers which cannot tolerate temperatures above 50-60 °C [17].

Many cold active protease producer bacteria have been reported in recent years, such as *Bacillus licheniformis RKK-04* [24], *Bacillus subtilis AP-MSU6* [21], *Bacillus cereus strain AT* [22], *Bacillus subtilis DR8806* [20]. Most cold-active proteases show activity at around 30-40 °C and alkaline conditions around pH 8-11.

Importance of cold-active proteases for the industry gives a raise of explorations for many new protease producer bacteria isolates from different sources. In a study, six protease (+) isolates were collected from farm soil in the garden of the Mishra Institute of Medical & Technical Sciences in India. *Bacillus sp.* isolate was identified as the best protease producer and optimum temperature and pH were determined as 60°C and pH 7 [23]. In another study, from various lands of the Karachi city, fifty-three alkaliphile bacterial isolates were collected, 25 isolates were determined as alkaline protease producers. *Bacillus brevis SSA1* was selected due to its highest activity and optimum protease activity observed in pH 8 and 60°C [24].

*Bacillus sp. strain EL-GU1* was chosen for further purification and characterization studies due to their higher protease activity when it was compared with other isolates. After isolation of pure colony by streak plate method, the isolate *Bacillus sp. strain EL-GU1* was inoculated in Studier medium and cultivated overnight 37°C. Crude enzyme extract was purified by using ammonium sulfate precipitation. Molecular weight of the protease from *Bacillus sp. strain EL-GU1* was determined as 15 kDa. Inhibition of its enzyme activity by PMSF indicates that it was a serine protease [17]. Subtilases, a group of serine proteases [endopeptidases], are one of the most used proteases. Subtilisins, typically have molecular weights of about 15–45 kDa like protease from *Bacillus subtilis AP-MSU6* (18 kDa) [21], *Bacillus subtilis NCIM No.64* (28 kDa) [25], *Bacillus sp MLA64* (24 kDa) [26], *Bacillus subtilis DR8806* (37 kDa) [27]. According to the molecular weight, a new protease can be classified as subtilisin-like proteases. As it's described earlier, purified protease was used in characterization studies. Optimum pH, optimum temperature, stability of temperature, effects of metal ions, substrate specifity and catalytic efficiency were determined as characterization parameters. Effect of pH on the protease activity was determined at wide range of pH (2-10). Optimum pH for protease was found as pH:6. Despite our results, many proteases from *Bacillus* shows maximum activity at alkaline

conditions such as *Bacillus pumilus* (pH 11.5) [28], *Bacillus sp NPST-AK15* (pH 11) [19], *Bacillus sp.* (pH 9.6) [29]. There are only a few studies reported that acid tolerant proteases were produced from *Bacillus* species. Similar to our results, protease from *Bacillus subtilis JM-3* and *Bacillus megaterium KLP-98* showed optimum activity at pH 5.5 [30, 31].

Optimum temperature of protease was found as 20°C. Many cold-active proteases were reported in previous studies [Table 6] but only few of them have ability to keep their activity in around 20°C. Protease from *Bacillus cereus MTCC 6840* showed activity at low temperature from 15°C to relatively high temperatures up to 55°C but optimum activity was observed at 20°C [32]. In addition, there are some studies reported that most of the proteases from Bacillus species shows maximum activity at temperature around 30-40°C. (*Bacillus sp SB12* (37°C) [43], *Bacillus pumilus (30*°C*)* [28], *Bacillus sp.* (30°C) [29].

***Table 6.*** *Properties of protease enzyme produced by Bacillus species from different studies.*

| Species | Molecular weight (kDa) | Optimum pH | Optimum temperature (°C) | Reference |
|---|---|---|---|---|
| ***Bacillus sp. strain EL-GU1*** | 15 | 6 | 20 | |
| ***Bacillus sp.*** | | 7 | 60 | 23 |
| ***Bacillus sp P-2*** | | 9.6 | 30 | 28 |
| ***Bacillus sp JB-99*** | | 11 | 70 | 18 |
| ***Bacillus sp NPST-AK15*** | | 11 | 40 | 19 |
| ***Bacillus sp SB12*** | 41 | 9 | 37 | 43 |
| ***Bacillus spp.*** | | 10.5-11 | 40 | 40 |
| ***Bacillus subtilis AP-MSU6*** | 18.3 | 9 | 40 | 21 |
| ***Bacillus subtilis megatherium*** | 45 | 9.1 | 60 | 20 |
| ***Bacillus subtilis DR8806*** | 37 | 8 | 45 | 27 |
| ***Bacillus amyloliquefaciencs S94*** | 45 | 10 | 45 | 41 |
| ***Bacillus cereus*** | | 9 | 20 | 31 |
| ***Bacillus cereus strain AT*** | 46 | 8 | 50 | 22 |
| ***Bacillus cereus MTCC 6840*** | | 9 | 20 | 32 |
| ***Bacillus licheniformis RKK-04*** | 31 | 10 | 50 | 42 |
| ***Bacillus pumilus*** | | 11.5 | 30 | 27 |
| ***Bacillus brevis SSA1*** | | 8 | 60 | 24 |

In our study, the temperature stability of protease was determined by spectrophotometric activity assay after 30 minutes of incubation at 25°C. 63% of the protease activity was kept [Figure 5]. The analysis regarding to the thermostability of an alkaline protease from *Bacillus sp. SB12* showed that the enzyme completely kept its activity after pre-incubating for 120 min at a range of 25°C-50°C [33]. Activity of protease was decreased at 60°C up to 50% of its activity after 60 min pre-incubation [33]. Thermal stability of protease from *Bacillus subtilis DR8806* was examined after incubation at different temperatures for more than 1 hour. Enzyme kept its activity about 100% of the initial activity at 45°C and it stayed active at temperature up to 70◦C. The residual activity of the enzyme was 70%, 50% and 30% at 50°C, 60°C and 70°C, respectively [27].

Effects of both metal ions and inhibitors on protease activity were also determined in this study. PMSF, β-mercaptaethanol and $MgSO_4$ completely inhibited protease activity. Protease activity was increased %100 in the presence of $FeSO_4$. Similar with our results, there are different studies that prove the inhibitor effect of PMSF and β-mercaptoethanol on the enzyme activity of protease. Protease enzyme activity from *Bacillus subtilis PF1* decreased up to 36% and 28% when two different concentrations (0.1% and 0.5%) were used. In the same study, inhibitor effect of PMSF was reported at 10 mM concentration. Protease activity was strongly inhibited in presence of PMSF from 100% to 97% [34]. In another study, effect of $Fe^{+2}$ determined that increased *Bacillus sp. SB12* protease enzymatic activity from 129 (U/ml/min) to 180 (U/ml/min) [32]. Despite our results, negative effect of $Fe^{+2}$ was reported on protease from *Bacillus cereus strain AT* that decreased enzymatic activity up to 59% [22].

In the manner of substrate specificity of protease, our study stated like 100% and 54% relative activity for BSA and casein, respectively. Similar with our results, protease from *Bacillus brevis MWB-01* was showed more activity on casein when it compared with BSA. Activity of protease decreased from 100% to 74% when BSA used as a substrate for this protease [34]. Protease activity of *Bacillus subtilis BUU1* on casein and BSA also measured as 100% and 22%, relatively [13].

Considering optimum pH of 6 and enhancer metal ion as $Fe^{+2}$ of protease enzyme that was obtained from *Bacillus sp. strain EL-GU1*, it makes a lot of sense because soil source of our extracted Bacillus which is Trabzon-Black-sea region in Turkey has pH range of 6 to 7 and it is rich in terms of $Fe^{+2}$ ions [44]. It might be an evidence that our cold active protease enzyme from *Bacillus sp. strain EL-GU1* is well adapted to the soil of the region that they grew up.

## VI. References


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