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HEAT AND pH STABLE PROTEASE PRODUCED BY A BACTERIUM ISOLATED FROM FIELDS WITH HIGH BORON

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ABSTRACT

In this study, production, optimization and characterization of protease produced by a bacterium isolated from Kırka boron mine distinct were performed.
The proteolytic activities of isolates were screened based on Anson method. The optimal production conditions for protease were determined by using Central Composite Design.
Bacillus sp. DB14 isolate (accession number: KY110868) exhibited the highest activity in terms of protease enzyme (346.15 U/mg) among the other isolates screened. The highest result for protease activity of *Bacillus* sp. DB14 isolate was obtained as 481.76 U/mg according to experimental set. Optimum pH and temperature of *Bacillus* sp. DB14 protease were found as 10.5 and 60 °C, respectively. Interestingly, this enzyme could stand stable at the range of 20-50 °C. However, the enzyme activity started to decrease highly after 50 °C. Besides, pH stability of this enzyme was provided between pH 7 and pH 11. Inhibition of protease was observed in solution with 10 mM Ni^{2+}, Fe^{3+}, Hg^{2+}, Cd^{2+} and B^{3+}. Cu^{2+} ion was found to enhance enzyme activity.
Bacillus sp. DB14 protease exhibited remarkably stability in the temperature range of 20 °C and 50 °C. Proteolytic activity values obtained at the range of pH 7-11 were stable. These results indicated that the alkaline protease from this isolate might be potential for biotechnological applications.

Keywords: Protease, boron-tolerant, characterization

INTRODUCTION

Boron has been proposed as a significant non-metal micronutrient in microorganisms, plants and animals (**Warington, 1923**; **Rowe and Eckhert, 1999**). Apart from cyanobacteria (**Mateo *et al.*, 1986**), boron has been reported to be vital for several bacteria. Furthermore, boron containing molecules have involved in quorum sensing mechanism (**Chen *et al.*, 2002**) and cell signaling mechanisms (**Gonzalez-Fontes *et al.*, 2008**). Also, antibiotics containing boron such as boromycin and tartrolon are known to produce through bacterial synthesis **(Irschic *et al.*, 1995; Kohno *et al.*, 1996**). Nevertheless, boron may be toxic in live organisms such as bacteria and plant (**Korkmaz et al., 2018**). In addition, it has been found that the key role of boron is the cross linking of cell wall constituents such as rhamnogalacturonan-II and pectin in plants (**Kobayashi *et al.*, 1996**). Moreover, boron forms complexes with glycoproteins in membrane structures (**Goldbach and Wimmer, 2007**) and it has been postulated that it is essential for stabilization of molecules with cis-diol groups (**Bolanos, 2004**).
Besides, recently, new bacterial strains from fields containing high boron concentration have been isolated. Ahmed and colleagues isolated the strains required boron for the growth and these strains tolerated more than 450 mM boron (**Ahmed *et al.*, 2007**). Ahmed and Fujiwara isolated some boron tolerant bacteria from soil samples and showed that these isolates belonged to the six genera such as *Bacillus*, *Arthrobacter*, *Algoriphagus*, *Gracilibacillus*, *Lysinibacillus* and *Rhodococcus* (**Ahmed and Fujiwara, 2010**).
Even, the significance of boron is intensely linked to its implications in the prebiotic origins of genetic material (**Scorei, 2012**). According to this argument, the ribose furanosyl borate ester remains possibly the most essential borate sugar in the building process of the "RNA world".
The microbes requiring or tolerating harsh conditions are known as extremophile or extremotolerant. Studies on these microorganisms are essential owing to determination of microbial diversity and their biotechnological applications **(Satyanarayana *et al.*, 2005; Aytar *et al.*, 2013; Aytar *et al.*, 2015**).
Especially, extremophile enzymes called as extremozymes have been stated to be resistant to chaotropic agents, detergents, extreme pH and temperature conditions and organic solvents (**Akkır *et al.*, 2017**). Among these enzymes, proteases are one of the most significant industrial enzymes utilized in various sectors (**Berneman, 2001**).
Proteases (EC 3.4.2124) which breaks the peptide bonds in amino acid residues of other enzymes are member of the class of hydrolases. They are utilized in the large industrial applications such as detergent, leather, pharmaceutical, protein processing, foods (**Hakim et al., 2018; Lakshmi *et al.*, 2018**). The proteases are one of the vital enzymes which account 40%-60% of the total worldwide enzymes sales (**Harer *et al.*, 2018; Hakim *et al.*, 2018**). Microbial enzymes are more efficient compared to enzymes of other organisms such as plant and animal (**Banerjee *et al.*, 1999**). Because microorganisms can grow rapidly in inexpensive culture medium and their enzymes have efficient catalytic activity, stability to extreme temperature or pH and high productivity (**Hasan *et al.*, 2006**).
Indeed, boron may have a main role in controlling certain pathways using serine protease or oxidoreductase, which is an enzyme relating to pyridine and flavin nucleotides. These biocatalysts need to pyridine or flavin nucleotides and by forming transition state analogs or competing for NAD or FAD, boron reversibly inhibits their activities (**Hunt, 1998; Hunt, 2012**).
Although there are several studies on isolating boron-tolerant bacteria and determining bacterial diversity, there has been no study of biotechnological applications of these bacteria in the literature. The target of this study to determine optimum conditions of production of extracellular protease obtained from *Bacillus* sp. DB14 strain from Kırka boron mine distinct.

MATERIALS AND METHODS

Isolation of microorganisms

The isolation procedure of these microorganisms was previously explained in our paper (**Nural Yaman *et al.*, 2019**). The soil and water samples from boron mine fields in Kırka, Eskişehir were collected. Soil (10 g) or water (10 ml) sample were suspended in 0.9% NaCl solutions (90 ml) and a set of serial dilutions was performed. Soil or water dilutions were plated onto Luria Bertani (LB) agar (pH 7.0) including 50 and 100 mM boric acid and incubated at 30 °C for 72 h . Purified isolates were stored at the refrigerator in glycerol stock at -20 °C.

Protease production medium and screening of proteolytic activity

All isolates were transferred to basal culture medium to produce protease, which involved (g/l) glucose, 10; peptone, 5; yeast extract, 5; calcium chloride 0.1; sodium chloride, 0.5; potassium dihydrogen phosphate, 0.4; dipotassium hydrogen phosphate, 0.3 and magnesium sulphate, 0.1. After the pH was adjusted to 7.5, 1% (v/v) of seed culture was inoculated to the medium, and then incubated at 30 °C for 24 h. The culture was centrifuged at 10000 rpm for 5 minutes, and protease activity was determined in the supernatant.
The investigation of protease activity was performed by the Anson method (**Takami *et al.*, 1989**). Protein concentrations were determined through the Bradford method (Bradford 1976). The amount of the enzyme resultant from the release of 1 mg of tyrosine each minute at 30 °C is determined as one unit of enzyme activity (**Özçelik *et al.*, 2013; Akkır *et al.*, 2017**).
The strain exhibiting maximum proteolytic activity was chosen for other experiments.

Experimental design

Among the isolates, DB14 isolate showing higher protease activity was chosen for enzyme production. In this study, pH, incubation time, inoculum amount and their interactions which are contemplated to be efficient parameters on the enzyme specific activity (U/mg) were defined with mathematical models utilizing Central Composite Design method via Trial version of Design Expert 10.0.6 software for statistical design in the production of protease under controlled temperature (30 °C). The α value in the calculation of axial points was taken as 1 through the software for orthogonal quadratic design. Dependent parameters and their levels for this work are shown in Table 1. To define the impacts of three parameters shown in this table, 20 tests were performed with axial points. For the period of the model formation from the experiment results, the terms to be included in the model were chosen by using backwards elimination method due to the existence of quadratic terms. Besides, the interactions of these parameters were investigated.

Enzyme characterization

Determination of optimum temperature and pH

To determine the optimum pH and temperature for proteolytic activity, casein was dissolved in 50 mM of glycine–NaOH buffer at pH and temperature values ranging from 8.0 to 12.0 and from 20 to 80 °C, respectively.

Determination of thermostability and pH stability

The maximum activity of the raw protease was expressed as 100%. Based on this phrase, the relative activity of the protease for all experiments was calculated. Thermal stability was examined by pre-incubating the culture supernatant at different temperatures: 20-60 °C for 1h before measuring protease activity, which was measured as described above. The protease pH stability was defined for various pH values ranging from 7.0 to 12.5 for 1 h at 30 °C.

Impact of different metals, metalloid and denaturants on proteolytic activity

To investigate the impact of heavy metals and metalloid as well as $NiCl_2$, $FeCl_3$, $HgCl_2$, $CdCl_2$, H_3BO_3 and $CuSO_4$, the proteolytic enzyme was incubated for 20 min separately with each of these metals at a final concentration of 10 mM.
The protease was incubated with SDS and EDTA at a final concentration of 0.5 and 1.0 mM. Furthermore, the same enzyme was incubated with urea at a final concentration of 0.5-1.0-5.0-10.0-100.0 mM to define the enzyme stability. Control solution prepared without any denaturant was utilized in each of the tests and investigated for comparison.
The relative activity was calculated utilized crude protease. Control solution prepared without any metal and metalloid ions was applied in the tests and investigated for comparison.

Table 1 The results of screening experiments

Isolate	Specific activity (U/mg)
B1	53.82
B2	151.12
B3	118.37
B4	142.93
B5	92.55
B6	104.60
B7	136.68
B8	133.58
B10	96.20
B11	94.49
B12	115.49
B13	126.77
B14	79.01
B15	133.49
B16	118.58
B17	137.81
DB1	134.27
DB3	267.84
DB4	118.51
DB14	346.15
DB23	242.61
DB24	120.09

RESULTS AND DISCUSSION

Screening of isolates for protease production

In our previous study, nine of the isolates were isolated on LB agar with 100 mM boric acid and the others were isolated on LB agar with 50 mM boric acid **(Nural Yaman *et al.*, 2019**). After all isolates were transferred to basal medium to produce proteolytic activity, the specific activities of 23 isolates obtained from the Kırka boron mine distinct in Eskişehir Province were determined by measuring the amount of total protein and protease activity. *Bacillus* sp. DB14 isolate (accession number: KY110868) exhibited the highest activity in terms of protease enzyme (346.15 U/mg) among the other isolates, and this isolate was therefore chosen for further studies. The specific activity values of all of the tested strains are given in Table 1.

Effects of parameters on specific protease activity

The production of proteolytic enzyme by *Bacillus* sp. DB14 isolate was indicated in Table 2. Variance analyses of three response variables were carried out and the regression models were represented utilizing Design Expert program. The graphs were utilized to define the main effects of parameters such as their interactions on the response variables. As shown in Table 2, the highest result for protease activity was reached to 481.76 U/mg. The reduced result of variance analysis (ANOVA) of specific activity was indicated in Table 3. This model was found to be statistically significant because their p-values (<0.0001) (for all response variables) are less than 0.05. Predicted specific activity value represents approximately 90% of actual activity. Experimental error was not obtained to be statistically significant because the p-value of lack of fit (0.67) was more than 0.05 for enzyme specific activity value.
Regression coefficient value calculated based on the coded values of the terms of the model formed after statistical analysis was shown in the same table. By means of these variance analyses, the regression model was given in Eq. (1).

$$\boldsymbol{Specific\ activity}\left(\frac{U}{mg}\right) = 345.58 - 33.56 * A + 18.30 * B - 3.29 * C - 10.52 * A * B + 21.13 * A * C + 32.06 * B^2 + 14.38 * C^2$$

Equation 1

Table 2 Design matrix and results of experiments

	Parameters			
Standard order	**A:pH**	**B:Incubation time (day)**	**C:Inoculum amount (%)**	**Response: Specific activity (U/mg)**
1	6.5	1	1	422.20
2	8.5	1	1	340.39
3	6.5	5	1	481.76
4	8.5	5	1	329.41
5	6.5	1	5	383.52
6	8.5	1	5	357.77
7	6.5	5	5	430.87
8	8.5	5	5	391.49
9	6.5	3	3	377.27
10	8.5	3	3	340.95
11	7.5	1	3	350.31
12	7.5	5	3	403.67
13	7.5	3	1	370.71
14	7.5	3	5	347.92
15	7.5	3	3	326.85
16	7.5	3	3	333.64
17	7.5	3	3	345.14
18	7.5	3	3	332.13
19	7.5	3	3	340.58
20	7.5	3	3	369.35

For enzyme specific activity (U/mg), Fig. 1a and Fig. 1b graphically indicate the AB, and AC interactions, respectively. According to Fig. 1a; increasing the pH during the low incubation period slightly reduced the specific activity, while the pH increases during the high incubation period resulted in much more reduction in the specific activity. As shown in Fig. 1b, increasing the pH at a low inoculum amount significantly reduced specific activity, while increasing the pH at high inoculum amounts led to a slight decrease in specific activity.

As may be indicated from the ANOVA table (Table 3) and graphs (Fig. 1a-1b), the most effective parameter for specific activity is the pH, which is in interaction with both the incubation time and the inoculum amount. After all the data are investigated, the amount of inoculum was an insignificant effect on investigated levels in this work for all three response variables.

Table 3 ANOVA for enzyme specific activity

	Specific activity	
Source	p value	Model coefficients for coded values
Model	<0.0001	+345.58
A (pH)	<0.0001	-33.56
B (incubation time-day)	0.0036	+18.30
C (inoculum amount-%)	0.5298	-3.29
AB	0.0889	-10.52
AC	0.0029	+21.13
B^2	0.0039	+32.06
C^2	0.1356	+14.38
Lack of fit		0.4329
R^2		0.9006
Adj.R^2		0.8427
Pred. R^2		0.6774

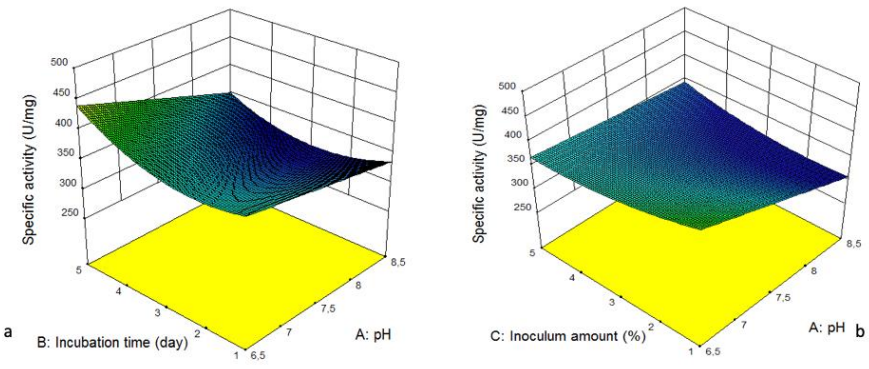

Figure 1 The response surface of double interaction of pH, incubation time and inoculum amount: (a and b) for protease specific activity

Prediction and optimization

The optimum value of response variables such as protease specific activity was predicted from the regression models by using numerical optimization algorithm. In the optimization, experimental and predicted maximum specific activity values are shown in Table 4. Predicted parameter levels are also indicated in Table 4 for protease activity. Confidence interval for response variable is also given at 95% in this table. Moreover, the test result of verification experiment performed at predicted parameter levels is represented in this table. As it can be indicated from this table, verification test result was found to be very close to predicted ones and fell between confidence intervals at 95%.

Table 4 Predicted and verification test result for specific activity value.

Response variable: Specific activity

Conditions			Predicted value			Verification test
A (pH)	B (incubation time-day)	D (inoculum amount-%)	%95 CI low	Predicted value	%95 CI max	Experimental result
6.5	5.0	1.0	450.304	478.817	507.33	480.543

Characterization of crude protease

Determination of optimum temperature and pH

The temperature profile of protease activity is presented on Fig. 2. The maximum enzyme activity was obtained at 60 °C. Similar reports were obtained in the literature. Jaouadi and coworkers reported that the optimum temperature of proteolytic enzyme from *Bacillus pumilus* CBS was 65 °C (**Jaouadi *et al.*, 2008**). The optimum temperature of proteolytic enzyme from *Bacillus* sp. was found as 40 °C by Josephine and his friends (**Josephine *et al.*, 2017**). Harer and coworkers described the optimum temperature of protease from *Bacillus thuringiensis*-SH-II-1A to be 45 °C (**Harer *et al.*, 2018**). Lakshmi and colleagues defined the optimum temperature of protease from *Bacillus cereus* strain S8 as 70 °C (**Lakshmi *et al.*, 2018**).

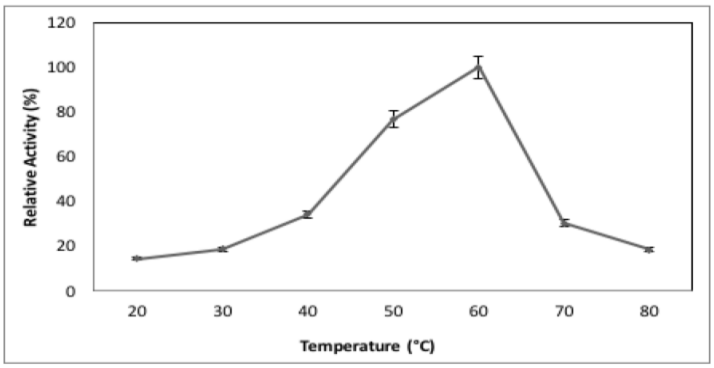

Figure 2 Optimal reaction temperature of the protease

The optimum pH of proteolytic activity was found to be 10.5 (Fig. 3). Similar results were described for several *Bacillus* strains in the literature. Harer *et al.* (2015) reported optimum pH for protease of *Bacillus thuringiensis*-SH-II-1A to be pH 10-11. Hadjidj *et al.* (2018) reported optimum pH of protease from *Bacillus licheniformis* K7A being as pH 10. In the same way, Lakshmi *et al.* (2018) observed optimum activity of protease from *Bacillus cereus* strain S8 when pH was 10.

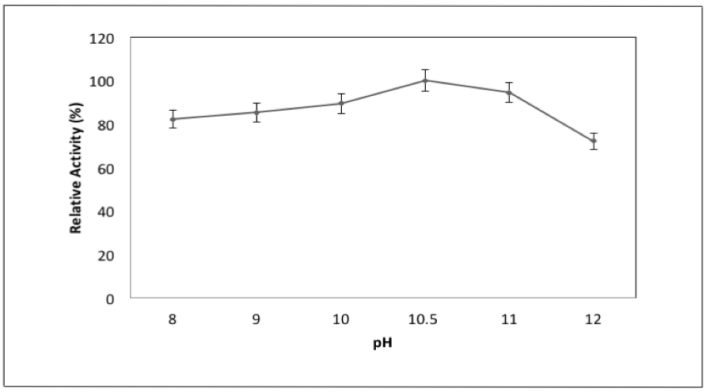

Figure 3 Optimal reaction pH of the protease

Determination of thermostability and pH stability

Protease activity obtained at 50 °C was accepted as 100 %. Relative activity was calculated based on this phrase. *Bacillus* sp. DB14 protease lost around 67% of activity at 60 °C (Fig. 4) after1 h incubation. Although the optimum temperature was 60 °C, *Bacillus* sp. DB14 protease exhibited interestingly stability in the temperature range of 20 °C and 50 °C. Similarly, Lakshmi *et al.* (2018) reported thermostability of protease from *Bacillus cereus* strain S8 exhibiting in the temperature range of 30-80 °C, while Hakim *et al.* (2018) showed that *Bacillus subtilis* AKAL7 protease was stable from 30 °C to 50 °C. Jaouadi *et al.* (2008) determined that the protease of *Bacillus pumilus* CBS was stable in the temperature range 30-55 °C.

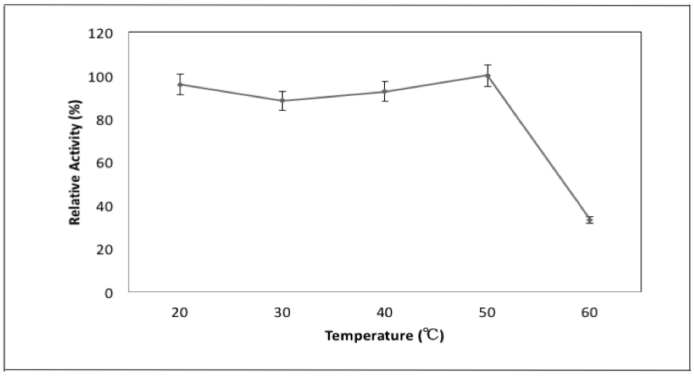

Figure 4 Effect of incubation temperature on protease stability

Protease activity obtained at pH 10.5 was accepted as 100 % and relative activity was calculated based on this phrase. Protease activities obtained at the range of pH 7-11 were stable (Fig. 5). *Bacillus* sp. DB14 protease lost around 69% of activity at pH 12.5, after 1 h incubation. Similar results were reported in the literature. The protease from *Bacillus pumilus* CBS showed stability from pH 7.0 to pH 10.6 (**Jaouadi *et al.*, 2008**).

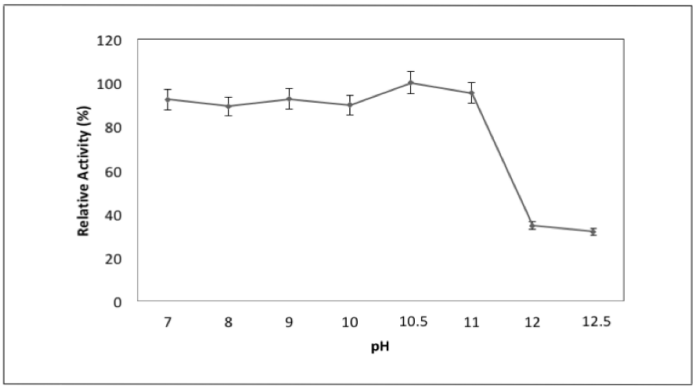

Figure 5 Effect of incubation pH on protease stability

Impact of different metals, metalloid and denaturants on protease activity

Inhibition of enzymatic activity was detected for 10 mM Ni^{2+} (80.44% of relative activity), Fe^{3+} (69.91% of relative activity), Hg^{2+} (34.34% of relative activity), Cd^{2+} (60.79% of relative activity) and B^{3+} (65.65% of relative activity) (Fig. 6). Cu^{2+} ion enhanced protease stability, in 1.25 times, during 20 minutes at 30 °C. Cu^{2+} ions may be important for catalysis. It is reported that the activity of alkaline protease from *Bacillus* sp., *Bacillus subtilis, a*nd *Bacillus megaterium* RRM2 was decreased by Fe^{3+}, Hg^{2+}, Zn^{2+} and Co^{2+} (**Venugopal and Saramma, 2007; Olajuyigbe and Falade, 2014; Hakim *et al.*, 2018**).

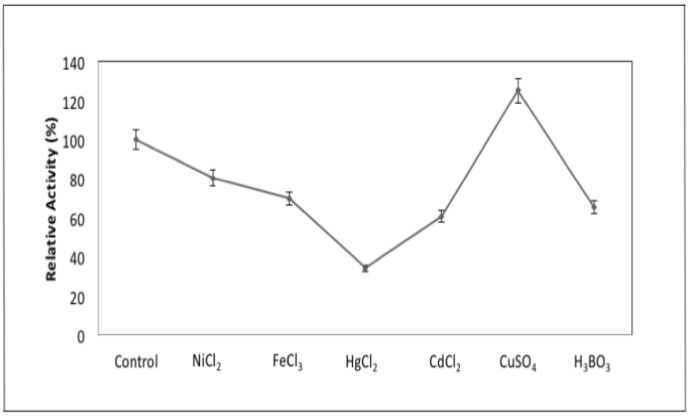

Figure 6 Effect of some metals and metalloids on enzyme stability

Approximately 25 % loss was detected in enzyme activity after adding 0,5 mM and 1 mM SDS (Fig. 7) to reaction medium. Other concentrations of denaturants did not cause a significant reduction in enzyme activity.

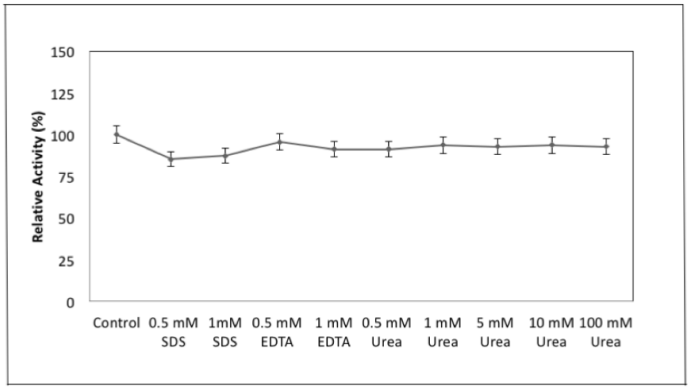

Figure 7 Effect of some denaturants on enzyme stability

In our study, boron ion inhibited protease activity. Boron toxicity may lead to inhibit protease activity. Uluisik *et al*. recommended a possible association between boron toxicity and translational control in yeast according to the observation that mutants showing boron resistance were all lacking genes that play a role in uridine base modifications at the wobble position of tRNAs throughout translation (**Uluisik *et al.*, 2011a**). The same team hypothesized that boron exerted its toxic effect by activation of the overall amino acid control system and then inhibition of protein synthesis. Because the general amino acid control pathway is conserved among eukaryotes, the mechanism of boron toxicity may be of common significance (**Uluisik *et al.*, 2011b**).

CONCLUSION

23 bacterial strains were isolated from Kırka boron mine distinct and these isolates were screened in terms of proteolytic activity. Among these isolates, *Bacillus* sp. DB14 had the highest activity. For optimization of enzyme production, Central Composite Design was utilized. *Bacillus* sp. DB14 protease exhibited interestingly stability in the temperature range of 20 °C and 50 °C. Protease activity values obtained at the range of pH 7- 11 were stable. Furthermore, this enzyme indicated tolerance to different denaturants including urea, EDTA, SDS. These results revealed that the alkaline protease from *Bacillus* DB14 might be potential for biotechnological applications. Therefore, this enzyme with stability in the wide range of temperature and pH may find wide usage areas in different industries such as detergent and leather.

Conflict of interest : The authors declare that they have no conflict of interest.

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