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PARTIAL PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYMES FROM NATIVE *Bacillus Cereus* STRAINS

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Current Trends in Natural Sciences

Abstract
Proteases are the main enzymes responsible for the breakdown of protein molecules into amino acids. These enzymes can easily be obtained from plants, animals and microorganisms. However, the easiest, fastest and cheapest source is microorganisms. Bacillus cereus strains B16, B17, B18, B19 and B20 were selected for use in this study. The optimum conditions and other parameters of the strains in the production process of proteases were investigated. The strains were characterized using biochemical methods. Different culture conditions were analyzed for the production efficiency of protease activity. Based on the proteolytic activity analysis, Bacillus cereus B17 strain was selected for detailed studies. Protease purification was done by dialysis, ion exchange chromatography and gel filtration chromatography. It was determined that the growth and the protease activity of Bacillus cereus B17 was greatly affected by parameters such as pH and temperature of the medium, presence of metal ions and inhibitors, and incubation time.

Keywords: Bacillus cereus, Enzyme characterization, Protease, Protease activity.

1. INTRODUCTION

Proteases are the general name given to the largest group of enzymes that break down proteins into amino acids and take part in the catalysis of peptide bonds (Chang and Yang, 2000). These enzymes are of particular importance due to their use in many areas of industry such as food, medicine, textile, feed and cosmetics (Hooper, 2002). Plants and animals are used as protease sources, but the fastest, easiest and most economical method is to obtain them from microorganisms. Because, especially the bacteria are easily obtained and multiply rapidly (Singh et al., 2016). In addition, they have the potential to be genetically modified to change their catalytic properties and production amounts. Production of proteases from various microorganisms can slightly be induced structurally (Rao et al., 1998; Souza et al., 2015).

The industrial enzyme market worldwide is 10 billion dollars and the largest share is microorganism-derived proteases (Benmrad, 2016). The demand for proteases is constantly increasing. Easy and alternative ways are required to meet the production demand. Microbial-derived proteases cover 60% of the global enzyme trade (Zambare et al., 2011; Souza et al, 2015). They are obtained from fungi

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such as *Thermomyces, Rhizopus, Penicillium, Mucor, Humicola, Aspergillus*. If we compare the enzymes of fungal and bacterial origin, fungal proteases show more enzyme diversity, but they have less reaction rate and heat stability compared to bacterial enzymes (Wu, 2006; Souza et al, 2015).
Microbial proteases can be grouped according to several different factors. The first is its catalytic mechanism and origin. Second, they are divided into endopeptidases and exopeptidases according to the active site in the polypeptide groups. Endopeptidases cleave a peptide bond in the polypeptide chain remotely at both ends. Exopeptidases cut these ends closely and are divided into subgroups according to their domains carboxyl or amino as carboxy-peptidases and amino-peptidases. In addition, they are examined in six different groups as threonine, glutamic acid, metallo cysteine, aspartic acid and serine, according to the catalytic excess products in their activity region (Li, 2013).
There are many sources of bacterial proteases used in industry. The genus *Bacillus* is one of them. They can produce higher volumes of neutral and alkaline proteolytic enzymes with different properties, including oxidizing compounds, organic solvents, detergents, high stability to pH and extreme temperatures (Contesini et al., 2018).
The genus *Bacillus* are rod-shaped, gram-positive, endospore-forming bacteria that are members of the phylum Firmucites, containing more than 327 described species. They can act as aerobes or facultative anaerobes (PCB, 2000). Species of the genus *Bacillus* are characterized by a large genome size ranging from 3.35 to 10.0 mega bases, with a higher GC content of 35%-46% (Alcaraz et al., 2008). *Bacillus cereus* (*Bc*), commonly known as a soil bacterium, is also found in food products. It is an anaerobic, facultative, motile, beta-hemolytic rod-shaped gram (+) bacterium (Kenneth and Ray, 2004).
Industrially, bacteria of the genus *Bacillus* have a great potential in the sector. It has areas of use in different sectors such as food, medicine, detergent, medical, leather processing (Gupta et al.,2002; Sumantha et al., 2006). There are some reasons why these bacteria are preferred. They secrete protein in the extracellular environment with relatively short fermentation time (Sewalt et al., 2016). In addition, due to the diversity of the strains, it is possible to obtain different types of enzymes. Proteases, lipases and carbohydrate active enzymes are the main enzymes produced by *Bacillus*. Worldwide, proteolytic enzymes isolated from *Bacillus* are of greater importance among all related enzymes (Schallmey et al., 2004). In addition, in 1958, recombinant protein was first produced by Spizizen using *Bacillus subtilis* (Rahmer et al., 2015,). As the use of recombinant technology is continuously increased and updated, the use of the genus *Bacillus* to produce homologous and heterologous proteins is increasing in parallel (Schallmey et al., 2004).

In this study, a procedure was developed to optimise production, extraction and stability conditions, to determine the ideal production conditions in terms of Carbon and Nitrogen source, pH and temperature, and finally to find ways to inhibit or restrict the activity of the enzyme with some technical applications on a new cadidate strain *Bc* B17. Protease enzyme was produced and isolated under selected optimum conditions for activity analysis.

2. MATERIALS AND METHODS

2.1. Identification of the Strains

Bacillus cereus strains B16, B17, B18, B19, B20 were obtained from Baghdad University and identified through morphological and biochemical means (Oxidase Test, Catalase Test, Methyl Red Test, Voges-Proskauer Test, Nitrate Reduction Test, Indole Test) using the methods of Collee et al. (1996) and Atlas et al. (1995).

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2.2. Protease Production Studies

2.2.1. Semi-Quantitative Screening

Bacillus cereus strains were inoculated on nitrate agar medium using the smear plate method. It was incubated at 37°C for overnight. Pure colonies were transferred to skimmed milk agar medium and incubated under the same conditions. The presence of a transparent zone appearance around the colonies was evaluated as the ability of the strains to produce proteases (Touka and Hameed, 2014).

2.2.2. Quantitative Screening

Bacterial cultures were centrifuged at 15.000 rpm at 4 °C for 20 min. The supernatant was used for proteolytic activity experiments using the casein digestion method. The mixture containing 800 µl casein solution and 200 µl enzyme solution was incubated at 55 °C for 30 minutes. Then a ml of TCA reagent was added and incubated on ice for 15 min. It was centrifuged again and absorbance of the supernatant was measured at 280 nm. Activity determination was carried out using the following equation (Singh et al., 2011).

$$\textit{The enzyme activity (U/ml)} = (\textit{Absorbance at 280 nm}) \div (0.01 \times 30 \times 0.2)$$

$$\textit{Specific activity (U/mg)} = \textit{Activity (U/ml)} \div \textit{Concentration of protein (mg/ml)}$$

0.01: Constant
0.2: volume of the enzyme (ml)
30: Duration of the reaction

2.3. Optimum Conditions for Protease Production

Parameters for optimum incubation time, temperature, pH, carbon and nitrogen source were investigated for the *Bc* strains. They were incubated at 12, 24, 36, 48, 60 and 72 hours period at 25, 30, 35, 37, 40, 45 and 50°C, and pH values of 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 and 9 seperately. The culture media of the strains were enriched by starch, glucose, galactose, sucrose and maltose seperately at 1% (v/w) concentrations for carbon source.To determine the optimum nitrogen source, meat, yeast, casein, and peptone were added to the media at a concentration of 1.5%. The most suitable parameters were selected and applied for the next steps. After incubation, protein concentrations at each parameter was evaluated for specific activity and protease activity.

2.4. Protease Purification

The strain with the most effective protease activity and stability was determined as *Bacillus cereus* B17 and its proteases were purified using different methods.

2.4.1. Precipitation by Ammonium Sulfate

Amaonium sulfate was gradually added to the bacterial supernatant from 30 to 80% and mixed at 4°C for 20 min. It was then centrifuged at 6000 rpm for 20 minutes at 4°C. The resulting proteins were solubilized with 0.05 M Tris-HCl (pH 8.0) buffer (Sedghi et al., 2011).

2.4.2. Dialysis

Proteins precipitated with ammonium sulfate were treated with buffers overnight at 4°C using a dialysis bag.

2.4.3. Purification by Ion Exchange Chromatography

DEAE cellulose column was prepared according to the method of Whitaker and Bernard (1972). A 20g/L suspension was prepared for the cellulose column. It was then filtered with Whatman No.1. The resin was resuspended with 0.25 M NaOH and NaCl and filtered again using the same method. It was washed several times with 0.25 M HCl and distilled H2O. Equilibrated with 0.05 M Tris-HCl

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buffer (pH 8.0). Enzyme from the dialysis process was added to the column with 50 ml. The column was washed with an equal volume of Tris-HCl buffer and various concentrations of NaCl in the range of 0.2 M and 0.5 M were used gradually. The proteins were gradually eluted. The flow rate was applied as 3 ml/fraction. Then, absorbance was measured at 280 nm with a UV-VIS spectrophotometer (Kanvar et al., 2006).

2.4.4. Purification by Gel Filtration Chromatography.

Sephadex G150 (1.5x40cm) was equilibrated with 0.05M Na2HPO4 buffer at a flow rate of 30 ml/hr. Samples purified by ion exchange were treated with 0.01 MTris-HCl (pH:7.0) buffer. Elution was performed with the same buffer at a flow rate of 3 ml/hr. Protease activities were then measured at 280 nm (Gurumurthy and Neelagund, 2012).

2.5. Protease Characterization

2.5.1. pH on protease activity and stability

Buffer solutions at determined pH values were adjusted and 0.9 ml solution was mixed with 0.1 ml of partially purified protease. Enzyme activity was estimated according to the formula given in 2.2.2. Then, incubated for 30 minutes at room temperature determining the stability.

2.5.3. Determination of The Ideal Temperature for the Activity and Thermal Stability

At determined temperature parameture ranges (30-80 °C), 50 µl of enzyme solution waz mixed with 950 µl of 0.05 M Tris-HCl (pH7.0) buffer and held for 30 minutes. The mixture was placed on ice and then activity was measured. The highest yield was accepted as 100% and the compared with the temperature. Protease activity at temperatures 30, 40, 50, 60, 70 and 80°C were evaluated for both activity and stability. Enzymatic activity was compared with each other at the temperatures applied.

2.5.5. Determination of The Effect of Metal Ions and Inhibitor on the Activity of Protease Enzyme

Partially purified protease samples were treated seperately in 2mM of MgCl2, ZnSO4, CaCl2, MnCl2 and EDTA (1:1v/v) for 30 min and activity was evaluated.

3. RESULTS AND DISCUSSIONS

3.1. Identification of Strains

The results of several biochemical tests to confirm that they are *Bacillus cereus* are as in Table 3.1. A transparent zone was seen in the materials obtained using skim milk agar, indicating protein degradation by the protease enzyme (Figure 3.1).

Table 3.1. Biochemical Test results for Identification of Bacillus cereus B17.

Tests	Results
Gram stain	Positive
Shape cells	Rods shape
Blood agar	β -hemolysis
Oxidase	Negative
Catalase	Positive
Indole	Positive
Methyl red	Positive
Voges Proskauer	Positive
Nitrate Reduction	Variable

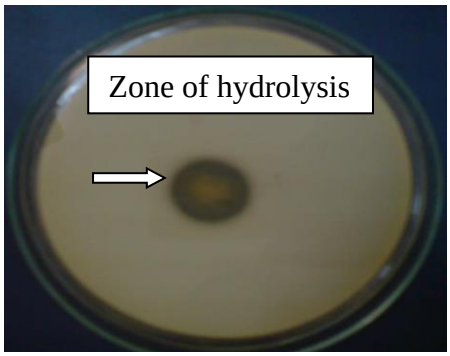

Figure 3.1. Proteolytic activity of Bacillus cereus B17 strain incubated on skim milk agar.

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3.2. Proteolytic Activity Screening

As a result of the protein activity determination using the casein digestion method, it was revealed that all *Bacillus cereus* strains produced protease. Among the *Bacillus cereus* B16**,** B17, B18, B19, B20 strains, B17 was the most effective. Results are between 36.4 U/mg in crude filtrate and 23-27 U/mg in samples. According to these results, B17 strain was selected to improve protease production.

3.3. Optimum Conditions Determined for Protease Production from *Bacillus cereus*

3.3.1. Optimum Culture Conditions on Protease Production of *Bacillus cereus*

Differences were observed in the activity of proteases produced from strain B17 grown in environments containing different carbon and nitrogen sources. Lactose and glucose appeared to be the most effective sources of carbon. As a nitrogen source, the use of yeast extract and peptone together was determined as the best source (Table 3.2 and 3.3).

In a study conducted by Fereshteh et al., (2003), it was reported that the amount of glucose supports enzyme production but suppresses it when it is more than 1%. Silva et al., (2007) reported that *Bacillus cereus* uses various carbon sources together and the most suitable sources are lactose and glucose.

Table 3.2. Effect of carbon sources on protease enzyme produced by Bacillus cereus B17.

Sources of carbon (1%)	Specific activity (U/mg protein)
Lactose	**183**
Glucose	**125**
Maltose	100
Starch	100
Galactose	66

Table 3.3. Effect of nitrogen sources on protease enzyme produced by Bacillus cereus B17.

Sources of nitrogen (1%)	Specific activity (U/mg protein)
Casein	185
Yeast extract	**122**
Meat extract	120
Peptone	**110**
Sodium nitrate	107

3.3.2. Optimum Temperature for The Production of Protease

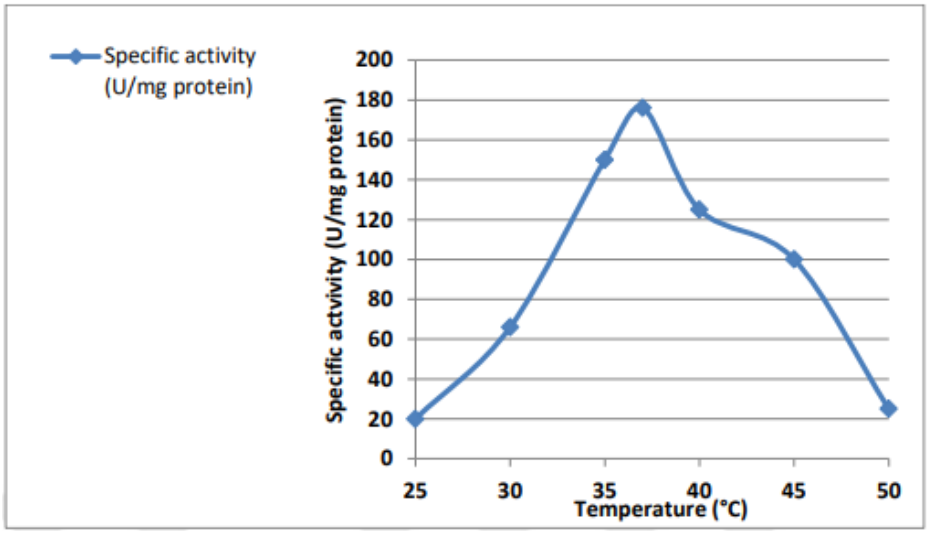

Figure 3.2. Effect of temperature on protease enzyme produced by Bacillus cereus B17

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The most effective production temperature of the protease enzyme produced from *Bacillus cereus* B17 strain was determined as 37°C. It caused a decrease in enzyme production as the lower and upper temperatures approached 25 and 50 °C. The effect of incubation temperature on enzyme production is given in Figure 3.2.

3.3.3. Optimum pH for Protease Production by *Bacillus cereus* B17

The activity-pH graph for protease enzymes produced from B17 strains grown in environments with pH values ranging from 4.0 to 9.0 is given in Figure 3.3. No change in specific activity was observed in the pH 4-6.5 range. However, as the pH increased to 8.5, the activity also increased. The highest value was measured as 155 U/mg at pH 8.5. A decrease in activity was observed as the pH approached 9.0. In another study, the ideal pH value of proteases was determined as 8.5 (Panuwan et al., 2003; Baron et al., 2019).

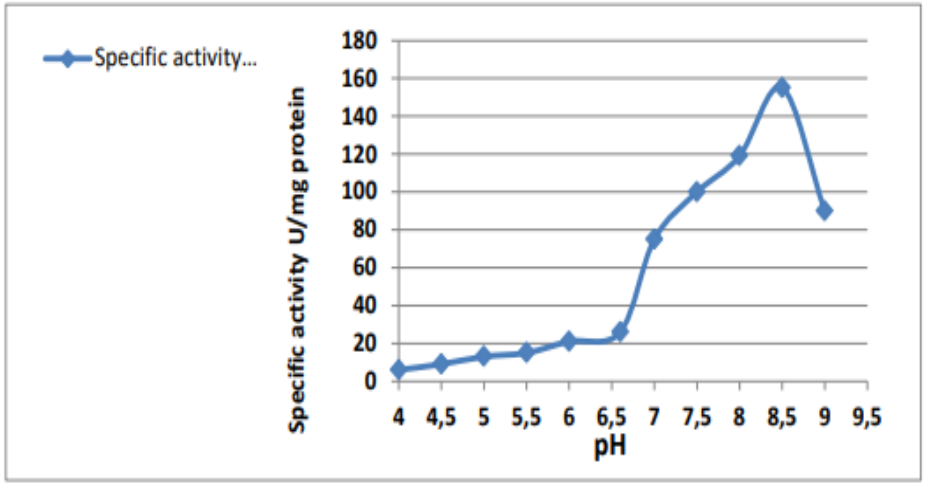

Figure 3.3. Effect of pH on protease activity of Bacillus cereus B17

3.3.4. Optimal Incubation Time for Protease Production

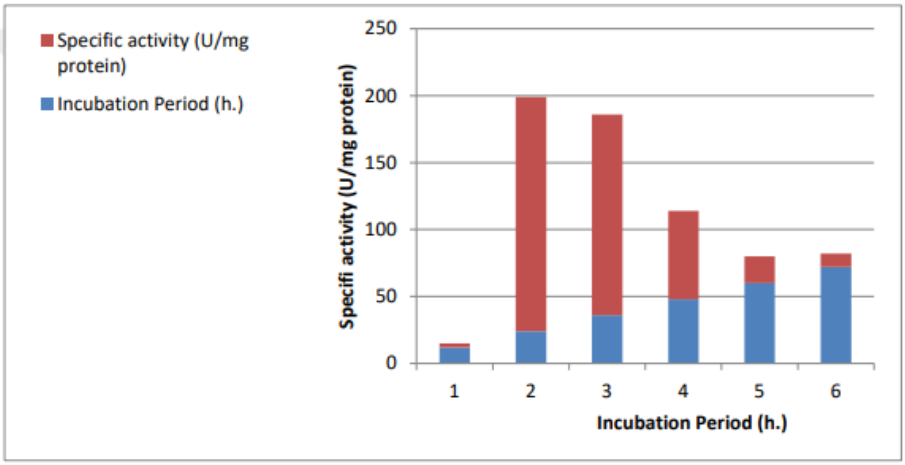

Figure 3.4. Effect of incubation times on protease activity Bacillus cereus B17

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The effect of incubation time on protease production is given in Figure 3.4. Different incubation times of 12, 24, 36, 48, 60 and 72 hours were applied and were coded from 1 to 6, respectively. According to the results of this application, the activity was determined as 175 U/mg protein when incubated for 24 hours. As the application time increased, the activity decreased.
In parallel with this study, 175 U/mg protein was obtained from *Bacillus* sp. within 24 hours (Silva et al.2007).

3.4.1. Ammonium Sulfate Precipitation

The activity of the proteins precipitated using 70% saturated ammonium sulfate gave the highest result as 192.3 U/mg. As a result of the process performed with the dialysis method, the activity was measured as 287.8 U/mg protein. The results are given in Table 3.4.

Table 3.4. Purification steps of neutral protease produced from Bacillus cereus B17

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	100	185	1	185	18500	1	100
Ammonium sulphate precipitation 70%	65	250	1.3	192.3	16250	1.03	80
Dialysis	50	251	0.9	287.8	12550	1.5	67.8
DEAE-cellulose	45	260	0.12	2166	11700	11.7	60
Sephadex G150	39	265	0.1	2650	10335	14.3	52

3.4.2. Gel filtration chromatography

Fractions (26-32) collected from Bacitracin-silica column chromatography according to chromatographic analyzes were added to that previously equilibrated with 20 mM Tris-HCl buffer (pH 7) Sephadex G150 (1.5x 40cm). The results of gel filtration chromatography are given in Figure 3.5. The results showed that there was a single peak representing maximum protease activity. The second column purification resulted in a specific activity of 265 U/mg and a yield of 52%.

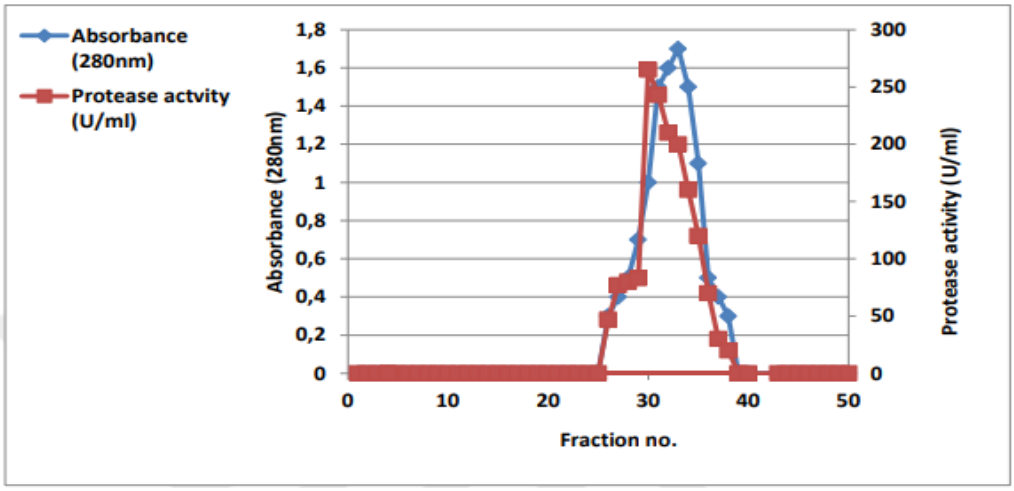

Figure 3.5. Gel filtration chromatography of proteases from Bacillus cereus B17 strain.

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Protease purification was performed by using Sephadex G150 (1.5x 40cm) column and Bacitracin-silica affinity column (Van Den Burg et al., 1989). In another study, proteases were purified from *Bacillus cereus* by gel filtration, Qhiper D chromatography and lysine affinity chromatography, and a 27% yield increase was observed (Singh et al., 2016).

3.4.3. Effect of pH on Enzyme Activity and Stability

The results of the research carried out in the pH 5-10 range are shown in Figure 3.6. The optimum pH level was between 7-8 and the lowest enzyme activity was observed at Ph 5.0 and 10.0. As a result of the analysis performed at room conditions with buffer at different pH levels (5-10), the higest enzyme activity was measured at pH 7-8. The protease enzyme was also active in the pH range of 6-9 (Figure 3.6). Sookkheo et al., (2000) observed the optimum pH of protease enzymes from *Bacillus* sp. at the range of 7.0-8.5 (Sookkheo et al., 2000). Segel et al., (1976) reported that the activity decreased by 66%, 18% and 51%, respectively, in protease samples obtained from *Bacillus* sp., which they incubated at different pH (5.5, 8.0, 9.0) for one day (Segel et al., 1976).

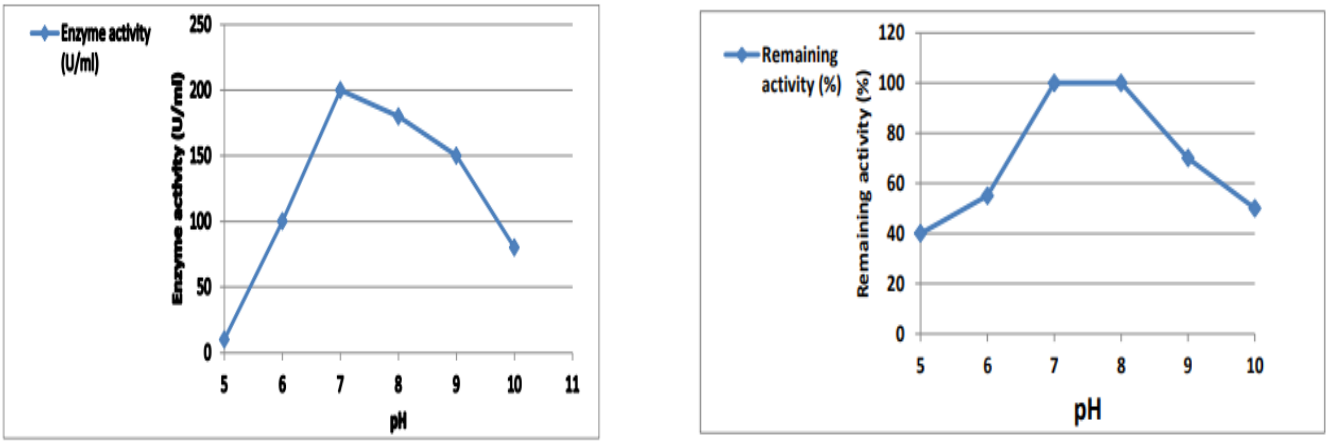

Figure 3.6. Effect of pH on purified protease activity and stability from Bacillus cereus B17 strain

3.4.4. Effect of Temperature on Protease Activity and Stability

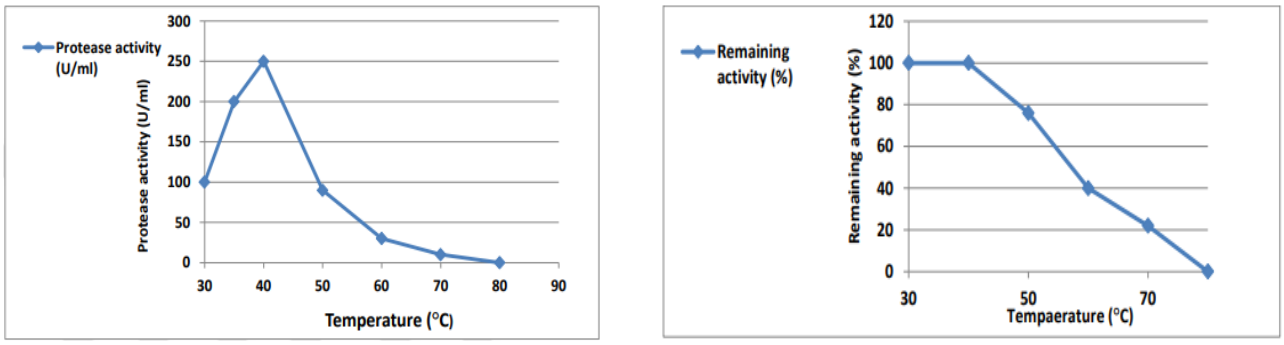

Figure 3.7. Effect of temperature on purified protease activity and stability from Bacillus cereus B17 strain

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The effects of temperature application in the range of 30-80 °C on activity and stability are given in Figure 3.7. It was observed that the optimum temperature value for enzyme activity was 40°C. As the temperature increased, the activity decreased and the enzyme was not active at 80°C. Considering the thermostability of the enzyme, stability was observed in the range of 30-70 °C for 30 minutes. The highest stability was measured in the range of 30-40 °C. (Figure 3.7)

3.4.5. Effect on Inhibitor and Metal Ion on Protease Activity

The results of the analysis in which the effects of selected inhibitors EDTA and metal ions on protease activity were examined are given in Table 3.5. While the EDTA greatly decreased the activity, it was increased in the presence of Cu^{+2}, Zn^{+2} and Mg^{+2}, Mn^{+2} ions compared with control. In parallel with these results, researchers noted that the activity of protease enzyme obtained from *B. subtilis* and *B. megaterium* was reset in the presence of EDTA (Adinarayana et al., 2003; Yossan et al., 2006).

Table 3.5. Effects of inhibitor and metal ions on protease activity from Bacillus cereus B17 strain.

Reagent	concaentration (mM)	Remaining activity(%)
Control (Enzyme)		100
CuCl2	2	105
ZnSO4	2	103
MgCl2	2	115
MnCl2	2	100
EDTA	2	0

4. CONCLUSIONS

Optimum production conditions for proteases from *Bacillus cereus* were determined and the most effective carbon source was determined to be lactose and glucose, and the most effective nitrogen source was casein. The most effective temperature value was determined as 37°C, as in most enzymes, with the thermostability between 30-40°C . Also the best incubation period was 24 hours. In addition, ideal saturation was identified as 70% in protein precipitation with amaonium sulfate.
When the effects of temperature, pH, metal ions and inhibitors on the obtained protease activity from *Bacillus cereus* B17 were examined; the best pH value was between 7.0 and 8.0; Inclusion of EDTA as an inhibitor significantly decreased the enzyme activity; Cu^{+2}, Zn^{+2} and Mg^{+2}, and Mn^{+2} metal ions resulted in increase in enzyme activity.

5. REFERENCES

Adinarayana, K., Ellaiah, P., Prasad, D.S. (2003). Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. Aaps Pharmscitech, 4(4), 440-448.

Alcaraz, L. D., Olmedo, G., Bonilla, G., Cerritos, R., Hernández, G., Cruz, A., López, V. (2008). The genome *of Bacillus coahuilensis* reveals adaptations essential for survival in the relic of an ancient marine environment. Proceedings of the National Academy of Sciences, 105(15), 5803-5808.

Atlas, A., Parks, M. Brown C. (1995). Laboratory Manual of Experimental Microbiology. Mosby –Year –Book, Inc., USA.

Baron, N.C., Rigobelo, E.C., Zied, D.C. (2019). Filamentous fungi in biological control: current status and future perspectives. Chilean journal of agricultural research, 79(2), 307-315.

Benmrad, M. O., Moujehed, E., Elhoul, M. B., Jaouadi, N. Z., Mechri, S., Rekik, H., Bejar, S. (2016). A novel organic solvent-and detergent-stable serine alkaline protease from Trametes cingulata strain CTM10101. International journal of biological macromolecules, 91, 961-972.

*Corresponding author, E-mail address: ylmazsemh@yahoo.com

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Chang, H.Y., Yang, X. (2000). Proteases for cell suicide: functions and regulation of caspases. Microbiology and molecular biology reviews, 64(4), 821-846.

Collee, B., Miles, J. G. Watt, R.S. (1996). Tests for the identification of bacteria. In: Mackie and MacCartnary. Practical and Simmons, A. Churchill. Livingstone.

Contesini, F.J., Melo, R., Sato H. (2018). An overview of *Bacillus* proteases: from production to application. Critical reviews in biotechnology, 38(3), 321-334.

Fereshteh, F., Jamsheed, E. and Mehrzad, F. (2003). "Isolation and identification of an alkaline protease producing *Bacillus* from soil. Iranian," J. biotechnol, p.1(3): 183–185.

Gupta, R., Beg, Q., Lorenz, P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Applied microbiology and biotechnology, 59(1), 15-32.

Hooper N.M. (2002). Proteases in biology and medicine, London porttand Press.

Kanvar, S.S., Ghazi, I.A., Chimni S.S., Joshi G.K., Rao G.V., Kaushal R.K., Gupta R., Punj V. (2006). Purification and properties of a novel extra-cellular thermotolerant metallolipase of Bacillus coagulans MTCC-6375 isolate. Protein Expression and Purification, 46(2), 421-428.

Kenneth J.R. and Ray C.G. (2004). Sherris Medical Microbiology (4th ed.). McGraw Hill.

Li, Q., Yi, L., Marek, P., & Iverson, B.L. (2013). Commercial proteases: present and future. FEBS letters, 587(8), 1155-1163.

Panuwan L., Ekhachai CH, Anchalee CH and et al.(2003). Screening and isolation of *Bacillus subtilis* exhibiting protease activity from Thuo Nao, Sci. Asia, 28: 241–245.

PCB T. (2000). Bacillus. In Baron S; et al. (eds.). Bacillus. In: Barron's Medical Microbiology (4th ed.).," Univ Texas Med. Branch. ISBN, pp. 978-0-9631172-1–2.

Rahmer, R., Morabbi Heravi, K., Altenbuchner, J. (2015). Construction of a supercompetent *Bacillus subtilis* 168 using the PmtlA-comKS inducible cassette. Frontiers in microbiology, 6, 1431.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., Deshpande, V.V. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and molecular biology reviews, 62(3), 597-635.

Schallmey, M., Singh A., Ward, O. (2004). Developments in the use of *Bacillus* species for industrial production. Canadian journal of microbiology, 50(1),1-17.

Sedghi, M., Naderi-Manesh, H., Naderi, M. (2011). Ammonium sulfate precipitation assay as a method for albumin depletion of human aqueous humor in proteomic analysis. Clinical Biochemistry, 44(13), 313-314

Segel J.J. (1976). Biochemical Calculation. 2nd edition. John Wiley and Sons., New York.

Sewalt, V., Shanahan, D., Gregg, L., La Marta, J., Carrillo, R. (2016). The Generally Recognized as Safe (GRAS) process for industrial microbial enzymes. Industrial Biotechnology, 12(5), 295-302.

Silva, C., Delatorre, A., Martins, M. (2007). Effect of the culture conditions on the production of an extracellular protease by thermophilic *Bacillus* sp and some properties of the enzymatic activity. Brazilian Journal of Microbiology, 38(2), 253-258.

Singh, R., Kumar, M., Mittal, A., Mehta, P. K. (2016). Microbial enzymes: industrial progress in 21st century. 3 Biotech, 6(2), 174.

Singh, S. K., Tripathi, V. R., Garg, S. K. (2011). An oxidant, detergent and salt stable alkaline protease from *Bacillus cereus* SIU1. African journal of Biotechnology, 10(57), 12257-12261.

Sookkheo, B., Sinchaikul, S., Phutrakul, S., Chen, S.T. (2000). Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. Protein expression and purification, 20(2), 142-151.

Souza, P., Bittencourt, M., Caprara, C., Freitas, M., Almeida, R., Silveira, D., Magalhães, P. (2015). A biotechnology perspective of fungal proteases. Brazilian Journal of Microbiology, 46(2), 337 346.

Sumantha, A., Larroche, C., Pandey, A. (2006). Microbiology and industrial biotechnology of food-grade proteases: a perspective. Food Technology and Biotechnology, 44(2), 211.

Touka H.A., and Hameed M.J. (2014). Detection of Virulence Factors of Local Isolates of Proteus mirabilis Investigation of virulence factors of Proteus mirabilis isolated locally. Journal of Al-Nahrain University Science, 71(4), 731-743.

Van Den Burg, B., Eijsink, V. G., Stulp, B. K., Venema, G. (1989). One-step affinity purification of *Bacillus* neutral proteases using bacitracinsilica.Journal of biochemical and biophysical methods, 18(3), 209-219.

Whitaker, J. R., and Bernard, R. A. (1972). Experiments for: An Introduction for Enzymology, the Whiber Press Davis.2:12-14

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Wu, T.Y., Mohammad, A.W., Jahim, J. M., Anuar, N. (2006). Investigations on protease production by a wild-type Aspergillus terreus strain using diluted retentate of pre-filtered palm oil mill effluent (POME) as substrate. Enzyme and Microbial Technology, 39(6), 1223-1229.

Yossan, S., Reungsang, A., Yasuda, M. (2006). Purification and characterization of alkaline protease from *Bacillus megaterium* isolated from Thai fish sauce fermentation process. Science Asia, 32(4), 377-383.

Zambare, V., Nilegaonkar, S., Kanekar, P. (2011). A novel extracellular protease from Pseudomonas aeruginosa MCM B-327: enzyme production and its partial characterization. New biotechnology, 28(2), 173-181.

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