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# *Isolation and Purification of Proteolytic Enzymes, Produced by Strains of Genus Bacillus*

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**Abstract.** One hundred sixty-six bacterial strains belonging to genus *Bacillus* were tested for protease production. Ninety percent of the studied strains demonstrated protease activity on nutrient gelatin and milk agar. A strain *Bacillus thuringiensis* was selected as the most promising for enzyme production based on its initial enzyme activity of 9.2 U/ml. The nutrient medium composition and cultivating conditions were optimized aiming better yields. The highest protease activity of 15 U/ml was achieved on the following conditions: inoculation of the medium with 5% inoculum (6.0 McF), followed by 16 hours of cultivation in liquid medium containing 0.5% glucose, 0.55% Bacto Peptone, 50mM phosphate buffer and 0.2% magnesium ions. The produced enzymes were partially purified 5.6 fold by ultrafiltration and size-exclusion chromatography on Sephadex G-75 and had a specific activity of 17.7 U/mg. The approximate molecular weights were determined by SDS-PAGE to be between 45 and 66 kDa.

**Key words:** *Bacillus*, protease, enzyme production, SDS-PAGE, zymography.

## Introduction

A vast variety of organisms, such as plants, animals, and microorganisms produce proteolytic enzymes. It is estimated that more than 40% of the proteases, which are sold worldwide, are of a microbial origin (GODFREY & WEST, 1996). Microorganisms are attractive sources of proteases because they can be cultivated in artificial conditions and in large quantities for a relatively short time by established fermentation methods. Screening and characterization of proteases from different sources has many advantages both from an environmental and industrial point of view (MIENDA *et al.*, 2014). In addition, microbial proteins have a long shelf life and can be stored for weeks under normal conditions without losing their activity (GUPTA *et al.*, 2002). Bacteria are the best choice for producers of enzymes. They are preferred to these with plant or animal origin because they have almost all desirable qualities for application in biotechnology (HAMZA, 2017).

The species of genus *Bacillus* are one of the most important groups of protease producers. This is mainly due to their high protein secretion capacity of over 20 g/l protein (HARWOOD & CRANENBURGH, 2008) and rapid growth rate with short fermentation cycles (CONTESINI *et al.*, 2017). This is also the reason for the existence of a large number of patents and "commercial" proteases from strains of *Bacillus* species (VETTER *et al.*, 1993; MERKEL *et al.*, 2007). The

secreted proteases from bacilli are three different types from which alkaline, neutral proteases are produced in the highest amounts, and esterases, which have weak proteolytic activity, are secreted in minimal quantity (MÄNTSÄLÄ & ZALKIN, 1980; REHMAN *et al.*, 2017; ANANDHARAJ *et al.*, 2016). Proteases, secreted from *Bacillus* species, have some unique properties, which explain their application in different industries. They have a broad pH and temperature activity and stability range. This makes them suitable as ingredients for the production of detergents. The addition of enzymes in the detergents lowers the need for high temperatures of the water, reduces the need for extensive mixing and prolongs the life of the clothes. Preparations with proteases are ideal for the removal of blood stains and stains from food with high protein content (CONTESINI *et al.*, 2017). Some *Bacillus* strains, used as enzyme producers, have GRAS status, which means that they are safe and their use in the food production is allowed. Their proteolytic enzymes can be used for the ripening of cheese, production of fermented sauces, and for production of bioactive peptides (CONTESINI *et al.*, 2017; OZCANE & KURDAL, 2012). *Bacillus* proteases are also stable in organic solvents, which makes them ideal for use in organic synthesis reactions (FABIANO *et al.*, 2017).

Proteases are produced from *Bacillus* species mainly during stationary phase and the synthesis is regulated by the source and quantity of carbon and nitrogen, agitation, dissolved oxygen and other parameters (FABIANO *et al.*, 2017). In *Bacillus thuringiensis*, the synthesis and secretion of proteases start during the late exponential phase and stationary phase of growth. They secrete them to hydrolyze the remaining polypeptides in the medium to satisfy the cell needs for nitrogen (BRAR *et al.*, 2007). Proteases are also a crucial factor for the insecticidal properties of this species. These enzymes hydrolyze the prototoxins to form active insecticidal crystals (ANDREWS *et al.*, 1985). Bt proteases have also some other favorable properties. They are stable and remain active in the range of pH 7-10 and temperatures of 30-80 °C (ZOUARI *et al.*, 1999). After the fermentation process, the medium contains not only proteases but also δ-endotoxins. The produced toxins are used in the agriculture for pest control. Theoretically, proteases and endotoxins can be produced in the same time and can be separately purified, giving two products of great importance and making the production process cost-effective (ZOUARI *et al.*, 1999). In the field of medicine, proteases are used for digestive aids, for the treatment of skin problems and they are even used against inflammation (CRAIK *et al.*, 2011).

The aim of the current study was to examine the extracellular protease production capabilities of *Bacillus* strains and to optimize the cultivation conditions for the enzyme synthesis for achieving higher yields.

## Materials and Methods

*Bacterial strains*

Strains of genus *Bacillus* (166 strains) from the microbial collection of the Department of Biochemistry and microbiology, Faculty of Biology, University of Plovdiv, Bulgaria were examined for proteolytic activity. Cultures of the organisms were maintained on nutrient agar medium at 4°C for routine laboratory use. For long-term use, the strains were maintained in nutrient agar under paraffin layer at 4°C. The initial screening was based on the ability of the strains to hydrolyze gelatin and milk casein.

*Hydrolysis of gelatin*

Test tubes, containing soluble gelatin medium (peptone – 10 g/l; NaCl – 2.5 g/l; gelatin – 120 g/l; pH 6.8) were inoculated with the test strains. After 7 to 14 days of incubation at 37°C, the test tubes were cooled to 4°C. The liquefaction of the medium is considered as a positive result.

*Hydrolysis of casein*

Each strain was inoculated on a petri dish containing solid milk agar medium (skim milk – 30 g/l; Nutrient broth

(HiMedia, India) – 13 g/l; agar – 15 g/l; pH 7.2) and incubated for 24 to 48 hours at 37°C. The casein hydrolysis zone appears as a transparent circle around the colony, which diameter is measured.

*Fermentation conditions and separation of culture filtrates*

A subculture was prepared prior to the fermentation by inoculation of the strain in nutrient broth and incubation for 8h on a rotary shaker at 37°C, 120 rpm. The cell density of the culture was then diluted to 6.0 McF units. Erlenmeyer flasks with 300 ml volume, containing 30 ml growth medium (casein hydrolysate – 5.5 g/l; Bacto Peptone – 5 g/l; glucose – 5 g/l; $Na_2CO_3$ – 5 g/l; $MgSO_4$ – 2 g/l; pH 8; sterilized for 15 minutes at 121°C) were inoculated with 5% (6.0 McF) subculture. The inoculated flasks were placed in a rotary shaker at 37°C, 120 rpm for 12 hours. For the separation of the cells, the culture medium was centrifuged at 4°C, 14 000 rpm for 20 minutes. The supernatant was collected for further analysis.

*Optimization of the culture conditions for enzyme production*

Optimum protease production was studied at different incubation periods (0 – 24 h), inoculum volume (1 – 10%), buffering agents, carbon sources (mannose, fructose, maltose and ribose), concentrations of the carbon source (0.2 – 0.8%), and different metal ions ($Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Ca^{2+}$).

*Total protein and Enzyme activity analysis*

The protein concentration was determined by the Bradford (BRADFORD *et al.*, 1976) and Hartree (HARTREE, 1972) methods using BSA as standard.

*Protease assay*

Proteolytic activity was determined by the modified Anson method using a solution of 0.65% casein as substrate as described by CUPP-ENYARD (2008). In brief the reaction was carried out in a reaction mixture containing 2 ml of 0.65% casein (Sigma-Aldrich, New Zeland) and 1 ml adequately diluted supernatant by incubation at 37°C for 30 minutes. The reaction was stopped by the addition of 5 ml of 110 mM trichloroacetic acid. The quantity of the hydrolyzed casein was determined spectrophotometrically at $A_{650}$ against casein treated with inactive enzyme as blank. A tyrosine standard curve was used for the expression of the enzyme activity. One unit of protease activity was defined as the amount of enzyme liberating 1 µg of tyrosine per minute.

*Purification of protease*

*Ultrafiltration*

The bacterial supernatant was subjected to ultrafiltration by Millipore`s Amicon Stirred cell UFSC20001 (200 ml), using membranes made of regenerated cellulose with dimensions of the pores of 10 kDa. A constant pressure of 0.3 Mpa was generated using a pressurized bottle with argon gas.

*Sephadex G-75 chromatography*

The concentrate obtained by ultrafiltration was loaded onto a Sephadex G-75 column (2.5 x 94 cm ), pre-equilibrated with 50 mM Tris-HCl (pH 7.5), at a flow rate of 20 ml/h. Fractions of 7 ml were collected and those having proteolytic activity were subjected to electrophoretic separation by SDS-PAGE.

*Electrophoretic separation*

*Denaturating SDS-PAGE*

The samples were mixed with reducing sample buffer (0.5 ml 1M Tris-HCl pH 6.8 buffer, 2 g glycerol, 0.4 g SDS, 0,3 ml mercaptoethanol, 4 mg Bromophenol blue, 8.5 ml distilled water) in 1.5 ml tube. The tubes were heat treated at 100°C for 90 seconds and cooled in ice. A 40 µl per sample was loaded on 10% polyacrylamide gel and separated at 120 V for 4h in SDS-Tris-glycine buffer, pH 8.05. The gel was stained with Coomassie blue R-250 (Merck, USA) for 1h and distained using a solution of water, methanol and acetic acid. The molecular weight of the proteins was determined using protein ladder (Amersham Low Molecular Weight Calibration Kit).

*Zymography*

Ten percent polyacrylamide gels were co-polymerised with 0.1% gelatin. The

samples were mixed with non-reducing sample buffer (0.5 ml 1M Tris-HCl pH 6.8 buffer, 2 g glycerol, 0.4 g SDS, 4 mg Bromophenol blue, 8.5 ml distilled water) and incubated for 5 min at 25°C before being loaded into gels. The following electrophoresis at 120 V at 4°C. After the electrophoretic separation, the gels were incubated for 30 min in wash buffer (2.5% Triton X-100 in $H_2O$). Then left in developing buffer at 37 for 16 h (10 mM Tris pH 7.5 with 5 mM $CaCl_2$, 1 M $ZnCl_2$). Gels were stained with 30% methanol and 10% acetic acid, containing 0.5% Coomassie brilliant blue R-250 (Merck, USA). De-staining was performed with 50% methanol and 10% acetic acid. Gelatinase activity was visualized as unstained bands on a blue background, representing areas of proteolysis of the substrate protein (MCKENNA *et al.*, 2017).

*Statistical analysis*

Data analysis was performed using Statistica™ (StatSoft). The results were presented as mean ± 95% confidence interval.

**Results and Discussion**

In the present study, 166 strains of genus *Bacillus* were screened for proteolytic activity using milk agar and gelatin medium. Ninety percent of the tested strains showed proteolytic activity, their taxonomic distribution is shown on Figure 1 a. Sixteen strains with hydrolysis zone with radius, larger than 5 mm, were selected for further quantitative determination of their extracellular protease activity. The strains demonstrated highly variable enzyme activity, but the highest activity was established for *B. cereus* №67, (8.04 U/ml), *B. cereus* №88 (7.74 U/ml), *B. thuringiensis* №14 (9.20 U/ml) and *B. thuringiensis* №4 (8.13 U/ml) (Fig. 1 b). Further analyses were carried out with *B. thuringiensis №14* as the most productive strain. Other researchers reported for producers, whose proteolytic activities ranged from 2.85 U/ml (NABRDALIK *et al.*, 2010) to 20.67 U/ml (BADHE *et al.*, 2016), and even higher values (BHUNIA *et al.*, 2011).

*Dynamics of the enzyme production of B. thuringiensis №14*

For the determination of the optimal duration of the fermentation process aiming high enzyme activity, the studied strain was cultivated for 24 hours and on every 2 hours, a sample from the culture was taken for analysis. The pH of the culture and the extracellular proteolytic activity were determined (Fig. 2 a).

The protease secretion began at the 4-th hour of the cultivation and gradually increased until it reached its maximum activity of 9.79 U/ml at the 16-th hour. The starting pH of the medium was pH 8, and during the cultivation, it slowly alkalized reaching pH 8.7. The highest protease activity was established at pH 7.1. The optimal cultivation periods is a strain-specific property and can vary – 24 h, 48 h, etc. The optimal pH level of the medium can also vary from pH 6 to pH 11 (CONTESINI *et al.*, 2017; BADHE *et al.*, 2016).

*Optimization of the production of proteolytic enzymes*

One of the most important components of the medium is the buffering agent. At first, it was buffered with 0.5% $Na_2C0_3$, as described in the literature (PANT *et al.*, 2015). During the experimental work, the carbonate-buffering system proved to be insufficient for maintaining a constant pH when cultivating *B. thuringiensis* №14. Phosphate buffer with varying concentrations (from 50 mM to 200 mM) was tested as a substitute buffering system. The highest activity was achieved when the medium was buffered with 50 mM phosphate buffer - 11.82 ± 0.74 U/ml (Fig. 2 b). Higher phosphate concentrations of 100 mM reduced the enzyme activity to 4.16 ± 0.63 U/ml, 3.22 ± 0.83 U/ml at 150 mM and 1.53 ± 0.47 U/ml at 200 mM (Fig. 2 b).

The volume of inoculum is an essential factor that creates a balance between biomass and available resources, which ensures optimal enzymatic production (SANDHYA *et al.*, 2005). To determine the optimal volume of inoculum, equal volumes of culture medium

were inoculated with a 1%, 2.5%, 5%, 7.5% and 10% 8-hour subculture with a cell density of 6.0 McF ($1.8 \times 10^9$). The highest enzyme production was achieved when 5% inoculum was used - 13.80 ± 2.85 U/ml (Fig. 3 a). When inoculum with volume lower and higher than 5% was used, the examined strain produced a reduced quantity of the proteolytic enzymes (Fig. 3 a). ABUSHAM *et al.* (2009) and GEORGE-OKAFOR *et al.* (2012) described the same results. In the first case, this can be explained by the insufficient number of active cells of the strain producer, in the second case when the cell density is too high, the free surface to volume ratio is reduced.

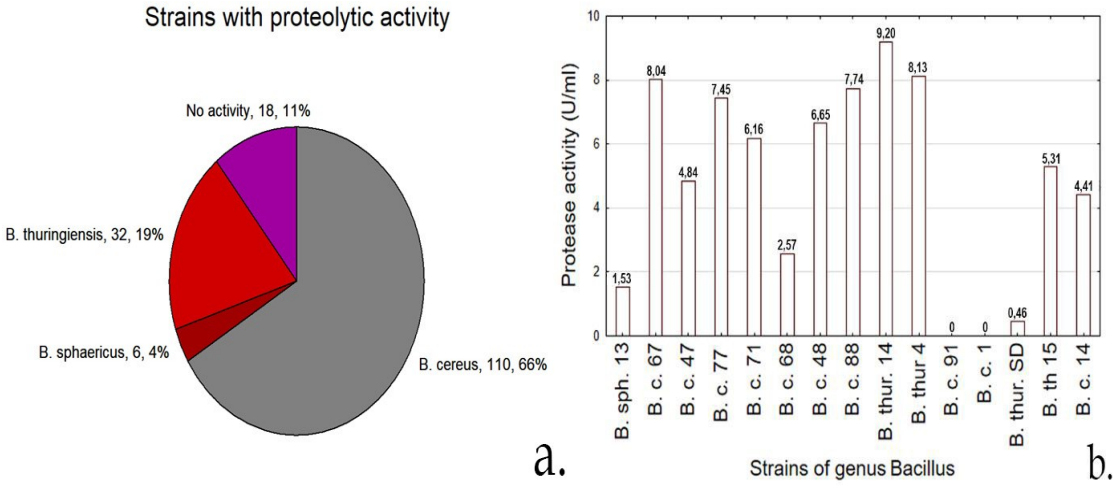


**Fig. 1.** Protease activity of strains of genus *Bacillus* – qualitative (a) and quantitative (b) analysis.

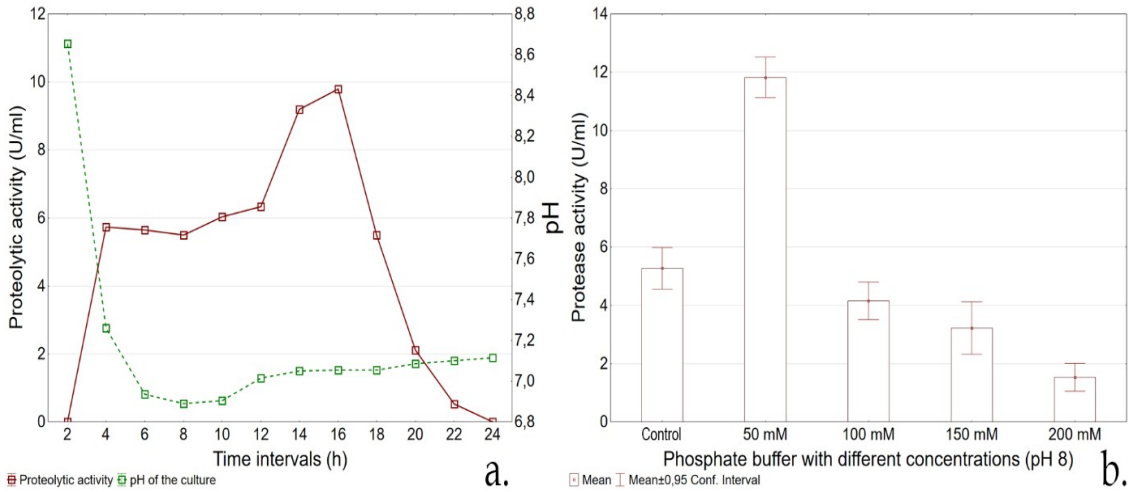


**Fig. 2.** a) Dynamics of the protease production; b) Influence of the buffering system on the protease production. *$Na_2CO_3$ was used as control (K) buffering agent.

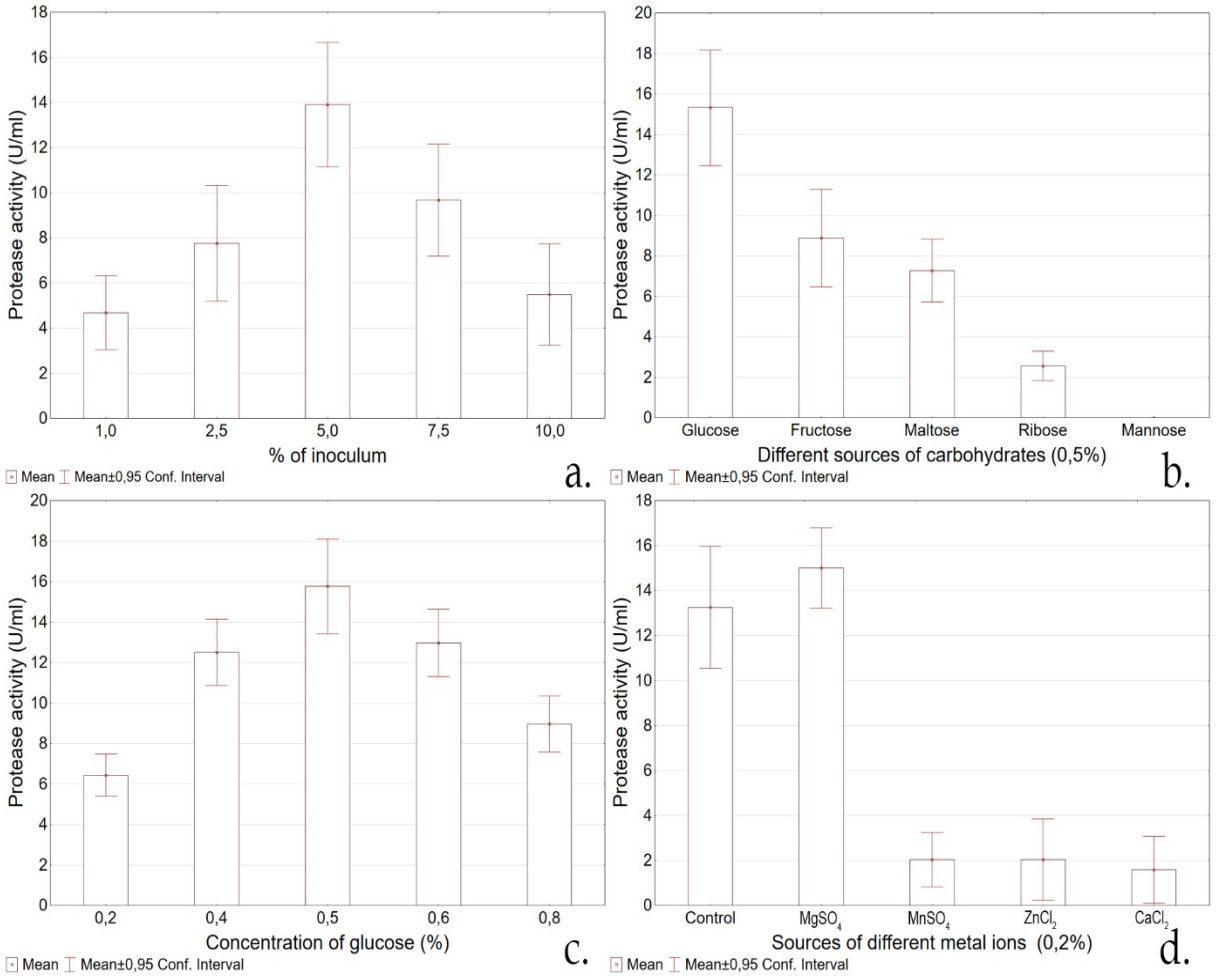


**Fig. 3.** Influence of a) the volume of the inoculum; b) different carbohydrates; c) different concentrations of glucose; d) different metal ions on the protease production.

The production of protease also depends on the specific preference of the carbon source. To determine the most appropriate carbon source for the synthesis of protease enzymes four carbohydrates with a concentration of 0.5% were examined as substitutes of the glucose – fructose, maltose, ribose, and mannose. The highest protease production was achieved on the initial medium with glucose as a sole carbon source - 15.32 ± 2.86 U/ml (Fig. 3 b). When fructose was used the activity was 8.89 ± 2.41 U/ml, when maltose was used - 7.27 ± 1.55 U/ml and ribose - 2.57 ± 0.72 U/ml There was no extracellular protease activity on medium containing mannose. The lack of some key enzymes may prevent the absorption of mannose, so the strain develops at the expense of the pepton available in the medium. At the same time, the high-unchanged monosaccharide concentration in it inhibits the synthesis of proteases (KOLE *et al.*, 1988). BADHE *et al.* (2016) studied the influence of various monosaccharides and polysaccharides and found that the highest stimulation of protease activity is observed with the addition of glucose. Similar findings are made by other authors (SEN & SATYANARAYANA, 1993; PASTOR *et al.*, 2001; SANTHI, 2014), which are reporting that the highest production is observed when glucose or starch is added to the culture medium.

Another crucial factor for the protease production is the concentration of the carbon source. Lower concentrations may be insufficient to sustain a large bacterial

population, while higher concentrations may have an inhibitory effect over the protease synthesis (KOLE *et al.*, 2007). The effect of the different glucose concentrations on the enzyme production was determined (Fig. 3 c). Our results confirm that high protease production is achieved when glucose is used as a carbon source in the medium at a concentration of 0.5% - 15.76 ± 2.33 U/ml. When glucose is added to the medium at concentrations lower or higher than 0.5%, the enzyme production is reduced. Our data differs from the findings of other authors. SUGUMARAN *et al.* (2012) have found that their strain produces a maximal quantity of proteases when the carbon source is 0.6%. ASHA *et al.* (2018) report that the optimal concentration of the carbon source for their strain is above 1% and MHAMDI *et al.*, (2014) report for preference to even higher concentrations of 6%. NADEEM *et al.* (2008) report that increasing the concentration of glucose to above 0.5% results in increased protease production, but our results don`t support that observation.

The expression of proteases and the regulation of their activity depends on the presence of some metal ions (ADINARAYANA *et al.*, 2003). Sources of metal ions ($MgSO_4$, $MnSO_4$, $ZnCl_2$, $CaCl_2$) were added to the culture medium to evaluate their effect on the extracellular protease activity. The addition of $Mg^{2+}$ enhanced the enzyme activity up to 15.02 ± 1.77 U/ml (Fig 3 d). Manganese, zinc and calcium ions had rather inhibitory effects over the synthesis or the activity of the proteolytic enzymes. These findings are consistent with the work of other authors (SHARMA *et al.*, 2012; VEERAPANDIAN *et al.*, 2016; KHAJURIA *et al.*, 2015; YILMAZ *et al.*, 2016; SAYEM *et al.*, 2006). Different metal compounds can stimulate the protease production, and this is strain specific property. Some authors describe that manganese and calcium ions can separately stimulate the production (SHARMA *et al.*, 2012; VEERAPANDIAN *et al.*, 2016; KHAJURIA *et al.*, 2015; CHITTOOR *et al.*, 2016). Others have shown that the stimulation is achieved by the synergetic influence of calcium and magnesium ions (NASCIMENTO *et al.*, 2004). The observed inhibition of protease activity in the present study is demonstrating the absence of metalloproteases in the supernatant of *B. thuringiensis № 14*. Many authors had found a similar inhibitory effect on the synthesis and activity of proteases (SHARMA *et al.*, 2012; VEERAPANDIAN *et al.*, 2016; LAILI *et al.*, 2017; NASCIMENTO *et al.*, 2004; SAYEM *et al.*, 2006) with the inclusion of zinc, cobalt, mercury and copper ions in the medium. In some cases, calcium and magnesium may also have an inhibitory effect (LAILI *et al.*, 2017; LIU *et al.*, 2013).

*Partial purification of proteolytic enzymes from Bacillus thuringiensis №14*

The strain was cultivated at the optimal conditions, after which the liquid culture was centrifuged at 14 000 rpm, 4°C for 20 minutes. The collected supernatant subjected to ultrafiltration using Millipore`s Amicon® Stirred Cell with filter membranes made of regenerated cellulose with 10 kDa pores under pressure (0.3 atm) using argon gas. Using this method, the volume was reduced 6.66-fold. The final concentrate showed protease activity of 15.66 U/ml. The enzyme purification was 1.74-fold times, the yield based on the activity was 50.7%, and the total protein was reduced 3.43-fold compared with the supernatant (Fig. 4.).

The contained proteolytic enzymes were partially purified using size-exclusion chromatography. Ten milliliters of the concentrate were applied to a column (94 x 2.5 cm) filled with Sephadex G-75. Forty-one fractions with 7 ml volume were collected. The proteolytic enzymes were eluted in 13 fractions from 172 to 263 ml. The total volume collected was 91 ml (Fig. 4, Table 1).

After the gel filtration with Sephadex G-75, the achieved purification was 5.62 compared with the specific activity of the supernatant. The amount of the total protein was reduced 11.56-fold and the yield according to the total activity was 48.61% compared with the supernatant. Three

separated peaks of activity were detected on the chromatogram corresponding to fractions – 19 (2.56 U/ml), 24 (1.36 U/ml) and 27 (3.77 U/ml), which were eluted respectively at the 186 – 193 ml; 221 – 228 ml; 242 – 249 ml. Our results suggest that the strain can produce more than one extracellular protease enzyme, which molecular weight varies in a broad range.

Using this method of chromatographic separation AHMETOGLU *et al.* (2015) achieved 13-fold purification of the protease with a yield of 23%. Other researchers achieved a 23.78-fold purification and a 40.9% yield of enzymes produced by a strain of *Bacillus megaterium RRM2* using a Sephadex G-100 chromatographic column (RAJKUMAR *et al.*, 2011). Some researchers are carrying out a direct purification with ion-exchange chromatography of the resulting preparation after. For example, BADHE *et al.* (2016) achieved 2.94-fold purification of a *Bacillus subtilis* preparation by ion exchange chromatography with DEAE-cellulose. The cited data confirmed the high susceptibility of the enzymes to the separation methods used, associated with loss of activity and low-end yields.

*Electrophoretic separation and visualization*

Samples of the supernatant, the concentrated supernatant after ultrafiltration and samples of the most active fractions obtained after size-exclusion chromatography were analyzed using SDS-PAGE electrophoresis for the determination of the molecular weight. The SDS-PAGE results clearly showed that the supernatant and its concentrate had high protein content with a broad range of molecular weight (Fig. 5). The protein content in the fractions was reduced thanks to the chromatographic purification. In fractions, 27 and 28 were detected three identical bends with a molecular weight between 45 and 66 kDa. In fraction 19, there was established a fourth band with lower molecular weight. The size of the isolated proteases varied between 45 – 66 kDa. Although many researchers are describing proteases with a molecular weight of 40 kDa (JALKUTE *et al.*, 2017), 40 and 60 kDa (SELIM *et al.*, 2015) their weight can vary in a broad range between 15 kDa to 130 kDa (OZTÜRK *et al.*, 2009).

The same samples (from the supernatant, concentrated supernatant, and the most active fractions) were analyzed for proteolytic activity by zymography (Fig. 6). The method allowed the identification of gelatinase activity in the analyzed samples using SDS polyacrylamide gels, which were impregnated with gelatin. The results showed that *Bacillus thuringiensis №14* produces several extracellular protease enzymes with different molecular weight. Two clearly visible signals were detected in all studied samples, while in fraction 19, there were additional signals with lower molecular weight.

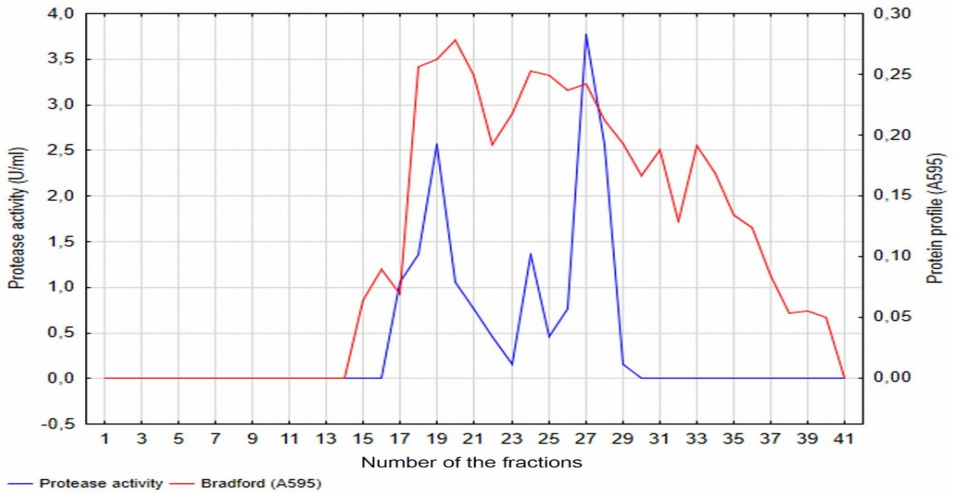


**Fig. 4.** Sephadex G-75 chromatography of protease enzymes produced by *B. thuringiensis* No 14.

**Table 1.** Purification of proteolytic enzymes produced by *B. thuringiensis No 14.*

| Step | Volume (ml) | Activity (U) | Protein (mg) | Spec. activity (U/ mg) | Purification (fold) | Yields (%) |
|---|---|---|---|---|---|---|
| Supernatant | 67.00 | 308.87 | 97.82 | 3.16 | - | 100 |
| Ultrafiltrate | 10.00 | 156.60 | 28.50 | 5.49 | 1.74 | 50.70 |
| Sephadex G75 | 91.00 | 150.15 | 8.46 | 17.74 | 5.62 | 48.61 |

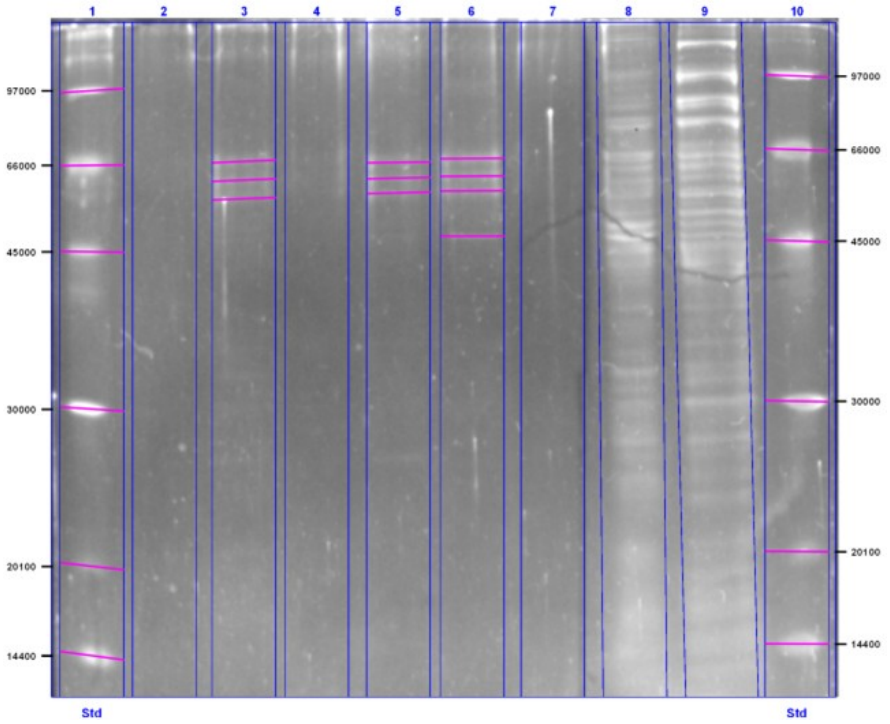


**Fig. 5.** SDS gel electrophoresis of samples of the supernatant (9), ultrafiltrate (8) and the three most active fractions – 19 (6), 27 (5) and 28 (3).

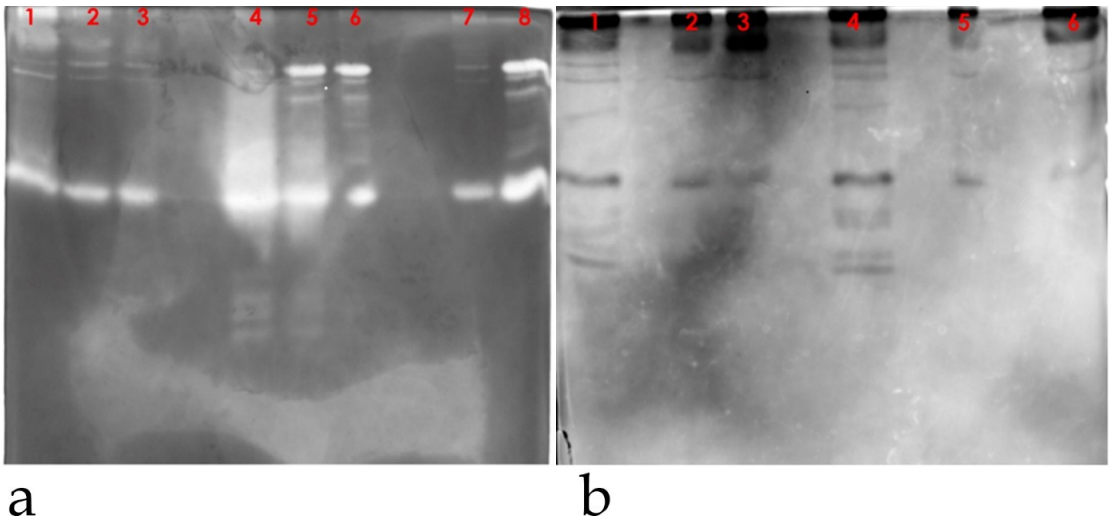


**Fig. 6.** Zymography of the supernatant (a – 1, 2, 4 and 7), the ultrafiltrate (a – 4, 5, 6 and 8) and the three most active fractions 19 (b – 1, 4), 27 (b – 2, 5) and 28 (b – 3, 6).

**Conclusions**

The studied *B. thuringiensis* strain showed consistent extracellular protease activity. The highest activity was recorded at the end of the exponential phase of growth. Changes of the buffering agent and the addition of $Mg^{2+}$ to the culture medium lead to enhancement of the activity up to 15 U/ml. The stable protease production makes the strain *B. thuringiensis* №14 a promising producer. The partial purification showed that the strain is producing at least two separate proteases with a molecular weight between 45 and 66 kDa. Their presence was confirmed by zymography. Further studies are needed in other to separate and characterize the proteases.

**References**

ABUSHAM A., N. RHAMAN, B. SALLEH, M. BASRI. 2009. Optimization of physical factors affecting production of thermostable organic solvent tolerant protease from a newly isolated halotolerant *Bacillus subtilis* strain Rand. - *Microbial Cell Factories,* 8(20). [DOI].

ADINARAYANA K., P. ELLAIAH, D. PRASAD. 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. - *AAPS PharmSciTech*. 4:1-9. [DOI].

AHMETOGLU N., B. MATPAN, A. FATMA, K. GUVEN. 2015. Production, purification and characterisation of thermostable metallo-protease from newly isolated *Bacillus* sp. KG5. - *Eurasian Journal of Biociences,* 9:1-11.

ANANDHARAJ M., B. SIVASANKARI, S. NAGARAJAN, R. RANI. 2016. Production, Purification, and Biochemical Characterization of Thermostable Metallo-Protease from Novel *Bacillus alkalitelluris* TWI3 Isolated from Tannery Waste. - *Applied biochemistry and biotechnology,* 178(8): 1666-1686. [DOI].

ANDREWS R., BIBILOS M., BULLA L. 1985. Protease activation of the entomocidal protoxin of *Bacillus thuringiensis* subsp. *kurstaki*. - *Applied Environmental Microbiology*. 50(4): 737-742.

ASHA B., PALANISWAMY M. 2018. Optimization of alkaline protease production by Bacillus cereus FT 1 isolated from soil. - *Journal of Applied Pharmaceutical Science,* 8(02): 119-127. [DOI].

BACH E., V. SANT'ANNA, D. DAROIT, A. CORRÊAA, J. SEGALIN, A. BRANDELLI. 2012. Production, one-step purification, and characterization of a keratinolytic protease from *Serratia marcescens* P3. - *Process Biochemistry,* 47: 2455-2462. [DOI].

BADHE P., M. JOSHI, R. ADIVAREKAR. 2016. Optimized production of extracellular proteases by *Bacillus subtilis* from degraded abattoir waste. - *Journal of Bioscience and Biotechnology*, 5(1): 29-36.

BELMESSIKH A., H. BOUKHALFA, A. MECHAKRA-MAZA, Z. GHERIBI-AOULMI, A. AMRANE. 2013. Statistical optimization of culture medium for neutral protease production by *Aspergillus oryzae*. Comparative study between solid and submerged fermentations on tomato pomace. - *Journal of the Taiwan Institute of Chemical Engineers*, 44: 377–385. [DOI].

BHUNIA B., D. DUTTA, S. CHAUDHURI. 2011. Extracellular alkaline protease from *Bacillus licheniformis* NCIM−2042: Improving enzyme activity assay and characterization. - *Engineering in Life Sciences,* 11(2): 207-215. [DOI].

BRADFORD M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - *Analytical Biochemistry*, 72: 248-254.

BRAR S., VERMA, M., TYAGI, R., SURAMPALLI, R., BARNABÉ, S., VALÉRO, J. 2007a. Bacillus thuringiensis proteases: Production and role in growth, sporulation and synergism. - *Process Biochemistry.* 42(5): 773–790. [DOI].

BRAR S., VERMA, M., TYAGI, R., SURAMPALLI, R., BARNABÉ, S., VALÉRO, J. 2007b. *Bacillus thuringiensis* proteases: Production and role in growth, sporulation and synergism. - *Process Biochemistry,* 42(5): 773-790. [DOI].

CHI Z., C. MA, P. WANG, F. LI. 2007. Optimization of medium and cultivation conditions for alkaline protease production by the marine yeast *Aureobasidium pullulans*. - *Bioresource Technology*, 98(3): 534-538. [DOI].

CHITTOOR J., L. BALAJI, G. JAYARAMAN. 2016. Optimization of Parameters that Affect the Activity of the Alkaline Protease from Halotolerant Bacterium, *Bacillus acquimaris* VITP4, by the Application of Response Surface Methodology and Evaluation of the Storage Stability of the Enzyme. - *Iranian Journal of Biotechnology*, 14(1): 23-32. [DOI].

CONTESINI J., R. DE MELO, H. SATO. 2017. An overview of *Bacillus* proteases: from production to application. - *Critical Reviews in Biotechnology*, 38: 321-334. [DOI].

CRAIK C., M. PAGE, E. MADISON. 2011. Proteases as therapeutics. - *Biochemical Journal*, 435(1): 1-16. [DOI].

CUPP-ENYARD C. 2008. Sigma's Non-specific Protease Activity Assay - Casein as a Substrate. - *Journal of Visualized Experiments*, 19: 899. [DOI].

DAUNER M., T. STORNI, U. SAUER. 2001. *Bacillus subtilis* Metabolism and Energetics in Carbon-Limited and Excess-Carbon Chemostat Culture. - *Journal of Bacteriology*, 183(24): 7308–7317. [DOI].

FABIANO F. J., R. DE MELO., H. SATO. 2017. An overview of *Bacillus* proteases: from production to application. - *Critical Reviews in Biotechnology*, 38(3): 321-334. [DOI]

GEORGE-OKAFOR O., E. MIKE-ANOSIKE. 2012. Screening and Optimal Protease Production by Bacillus sp. Sw-2 Using Low Cost Substrate Medium. - *Research Journal of Microbiology*, 7: 327-336. [DOI].

GODFREY T., S. WEST. 1996. Industrial enzymology, 2nd ed., p. 3. *Macmillan Publishers Inc*., New York, N.Y.

GOLDMAN M., H. BLUMENTHAL. 1963. Pathways of glucose catabolism in *Bacillus subtilis*. - *Journal of Bacteriology*, 86(2): 303–311.

GRASSMANN W., H. DYCKERHOFF. 1928. Uber die Proteinase und € die polypeptidase der hefe. - *Hoppe-Seyler's Z Physiol Chem*. 179: 41–78.

GUPTA R., K. BEG, S. KHAN, B. CHAUHAN. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. - *Applied Microbiology and Biotechnology*, 60(4): 381-95. [DOI].

HAMZA A. 2017. Isolation of Protease Producing Bacteria from Soil for Polyester and Silver Recovery from Waste X-ray Film. - *American Journal of BioScience*, 5(5): 74-79. [DOI].

HARTREE F. 1972. Determination of protein: a modification of the Lowry method that gives a linear photometric response. - *Analytical Biochemistry*, 48(2): 422-427. [DOI].

HARWOOD R., R. CRANENBURGH. 2008. *Bacillus* protein secretion: an unfolding story. - *Trends in Microbiology*, 16(2): 73-79. [DOI].

HELAL I., H. AMER, N. ABDELWAHED, M. GHOBASHY. 2012. Physiological and microbiological studies on production of alkaline protease from locally isolated *Bacillus subtilis*. - *Australian Journal of Basic and Applied Sciences*, 6:193–203. [DOI].

JALKUTE C., S. WAGHMARE, N. NADAF, J. DHANAVADE, B. JADHAV, I. PENDHARI, S. PATIL, D. SONAWANE. 2017. Purification and characterization of SDS stable protease from *Bacillus safensis* strain CK. - *Biocatalysis and Agricultural Biotechnology*, 10: 91-95. [DOI].

KHAJURIA V., K. SHARMA, P. SLATHIA, K. RAZDAN, S. SINGH, B. BAJAJ. 2015. Production of a detergent-compatible alkaline protease from *Bacillus*. - *Journal of Materials and Environmental Science*, 6(8): 2089-2096.

KOLE M., I. DRAPER, D. GERSON. 1988. Protease production by *Bacillus subtilis* in oxygen-controlled, glucose fed-batch fermentations. - *Applied Microbiology and Biotechnology*, 28:404-440. [DOI].

KOLE M., I. DRAPER, D. GERSON. 2007. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. - *Journal of Chemical Technology & Biotechnology*, 41(3), 197–206. [DOI].

LAILI N., S. ANTONIUS. 2017. Production and Characterization of Extracellular Protease from *Bacillus* sp. 140-B Isolated from Pineapple Plantation in Lampung, Indonesia. - *KnE Life Sciences*, 170-176. [DOI].

LIU Q., S. SUN, M. PIAO, J. YANG. 2013. Purification and Characterization of a Protease Produced by a *Planomicrobium* sp. L-2 from Gut of *Octopus vulgaris*. - *Preventive Nutrition and Food Science*. 18(4): 273-279. [DOI].

MÄNTSÄLÄ P, H. ZALKIN. 1980. Extracellular and membrane-bound proteases from *Bacillus subtilis*. - *Journal of Bacteriology*, 141(2): 493-501.

MCKENNA O., G. POSSELT, P. BRIZA, P. LACKNER, A. SCHMITT, G. GADERMAIER, S. WESSLER, F. FERREIRA. 2017. Multi-Approach Analysis for the Identification of Proteases within Birch Pollen. - *International Journal of Molecular Sciences,* 4;18(7). [DOI].

MERKEL M., P. SIEGERT, S. WIELAND, K. MAURER, C. BESSLER. 2007. *Subtilisin from Bacillus pumilus and washing and cleaning agents containing said novel subtilisin.* WO/ 2007/131656.

MHAMDI S., A. HADDAR, I. MNIF, F. FRIKHA, M. NASRI, A. KAMOUN. 2014. Optimization of Protease Production by Bacillus mojavensis A21 on Chickpea and Faba Bean. - Advances in Bioscience and Biotechnology. 5, 1049-1060. [DOI].

MIENDA B., A. YAHYA, I. GALADIMA, M. SHAMSIR. 2014. An Overview of Microbial Proteases for Industrial Applications. - *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, 5: 388-396.

NADEEM M., J. QAZI, S. BAIG, Q. SYED. 2008. Effect of Medium Composition on Commercially Important Alkaline Protease Production by Bacillus licheniformis N-2. - *Food Technology and Biotechnology,* 46(4): 388–394.

NARBRDALIK M., K. GRATA, A. LATALA. 2010. Proteolytic activity of *Bacillus cereus* strains. - *Proceedings of ECOpole*, 4:2.

NASCIMENTO W., M. MARTINS. 2004. Production and properties of an extracellular protease from thermophilic *Bacillus* sp. - *Brazilian Journal of Microbiology,* 35: 1-2

OZCAN T, E. KURDAL. 2012. The effects of using a starter culture, lipase, and protease enzymes on ripening of Mihalic cheese. - *International Journal of Dairy Technology.* 65: 585–593. [DOI].

ÖZTÜRK S., M. ÖZEREN-MORGAN, A. SALMAN, A. DENIZCI, B. ARIKAN, D. KAZAN. 2009. Alkaline serine protease from halatoerant *Bacillus licheniformis* BA17. - *Annals of Microbiology,* 59:83-90. [DOI].

PANT G., A. PRAKASH, J. PAVANI, S. BERA, G. DEVIRAM, A. KUMAR, M. PANCHPURI, R. PRASUNA. 2015. Production, optimization and partial purification of protease from *Bacillus subtilis*. - *Journal of Taibah University for Science,* 9(1):20-55. [DOI].

PASTOR M., G. LORDA, A. BALATTI. 2001. Protease obtention using *Bacillus subtilis* 3411 and Amaranth seed meal medium of different aeration rate. - *Brazilian Journal of Microbiology,* 32(1): 6-9.

RAJKUMAR R., K. JAYAPPRIYAN, R. RENGASAMY. 2011. Purification and characterization of a protease produced by *Bacillus megaterium* RRM2: application in detergent and dehairing industries. - *Journal of Basic Microbiology,* 51(6): 614-624. [DOI].

RAO M., A. TANKSALE, M. GHATGE, V. DESHPANDE. 1998. Molecular and biotechnological aspects of microbial proteases. - *Microbiology and Molecular Biology Reviews,* 62: 597-635.

REHMAN R., M. AHMED, A. SIDDIQUE, F. HASAN, A. HAMEED. 2017. Catalytic role of thermostable metalloproteases from *Bacillus subtilis* KT004404 as dehairing and destaining agent. - *Applied Biochemistry and Biotechnology,* 181:434–450. [DOI].

RUSSELL J., G. COOK. 1995. Energetics of Bacterial Growth: Balance of Anabolic and Catabolic Reactions. - *Microbiological reviews,* 59(1): 48-62.

SANDHYA, C., A. SUMANTHA, G. SZAKACS, A. PANDEY. 2005. COMPARATIVE EVALUATION OF NEUTRAL PROTEASE PRODUCTION BY *ASPERGILLUS ORYZAE* IN SUBMERGED AND SOLID-STATE FERMENTATION. - *PROCESS BIOCHEMISTRY*, 40: 2689– 2694. [DOI].

SANTHI R. 2014. Microbial production of protease by *Bacillus cereus* using cassava waste water. - *European Journal of Experimental Biology*, 4(2): 19-24.

SAURABH S., I. JASMINE, G. PRITESH, S. RAJENDRA KUMAR. 2007. Enhanced productivity of serine alkaline protease by *Bacillus sp*. using soybean as substrate. - *Malaysian Journal of Microbiology*. 3(1): 1-6. [DOI].

SAYEM S. 2006. Effect of temperature, pH, and metal ions on the activity and stability of alkaline protease from novel *Bacillus licheniformis* MZK03. - *Proceedings of the Pakistan Academy of Sciences*, 43: 257-262.

SELIM S. 2015. Thermostable Alkaline Protease Production by *Bacillus aryabhattai* J4. - *FASEB Journal*, 29(1).

SEN S., T. SATYANARAYANA. 1993. Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. - *Indian Journal of Microbiology*, 33(1): 43-47. [DOI].

SHARMA S., K. ARUNA. 2012. Optimization of Protease Activity of Thermophilic *Bacillus subtilis* WIFD5 Isolated from Milk Powder. - *Biomedical and Pharmacology Journal*, 5(1).

SINGH R., M. KUMAR, A. MITTAL, P. MEHTA. 2016. Microbial enzymes: industrial progress in 21st century. - *Microbiological Reviews*, 6:174. [DOI].

SUGUMARAN K., V. PONNUSAMI, D. GOWDHAMAN, V. GUNASEKAR, S. SRIVASTAVA. 2012. Thermo stable alkaline protease production from *Bacillus thuringiensis* MTCC 1953: Optimisation and kinetic studies. - *International Journal of ChemTech Research*, 4(1): 198-202.

SUN T., J. ALTENBUCHNER. 2010. Characterization of a mannose utilization system in *Bacillus subtilis*. - *Journal of Bacteriology*, 192 (8): 2128-39. [DOI].

VEERAPANDIAN B., V. PONNUSAMI, K. SUGUMARAN. 2016. Enhanced Thermo-stability of Bacterial Alkaline protease by calcium ions. - *Der Pharmacia Lettre*, 8(18): 192-196.

VETTER R., D. WILKE, B. MOLLER, M. MUELLER, I. MUECKE, M. TAKENBERG, G. KONIECZNY-JANDA. 1993. Alkalische proteasen aus *Bacillus pumilus*. EP 0572992 A1. Solvay Enzymes GMBH & CO KG, 31582 Nienburg, DE DE4218448

WHITEHURST, R., M. VAN OORT. 2010. *Enzymes in food technology*, 2nd ed. pp. 212-213.

YILMAZ B., M. BALTACI, M. SISECIOGLU, A. ADIGUZEL. 2016. Thermotolerant alkaline protease enzyme from *Bacillus licheniformis* A10: purification, characterization, effects of surfactants and organic solvents. - *Journal of Enzyme Inhibition and Medicinal Chemistry*. 31(6): 1241–1247. [DOI].

ZOUARI N., JAOUA S. 1999. Production and characterization of metalloproteases synthesized concomitantly with d-endotoxin by *Bacillus thuringiensis* subsp. kurstaki strain grown on gruel-based media. - *Enzyme and Microbial Technology*. 25: 364–71. [DOI].

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