Research Article GU J Sci 37(1): 1-14 (2024) DOI: 10.35378/gujs.1191006

Gazi University

Journal of Science

http://dergipark.gov.tr/gujs

Obtaining Efficient Mutant from the Wild Type *Bacillus subtilis* E6-5 by Physical and Chemical Mutagenesis for High Efficiency Protease Production, Optimizing the Mutant's Culture Medium

Busra OZALPAR, Elif DEMIRKAN*, Tuba SEVGI

Bursa Uludağ University, Faculty of Arts and Sciences, Department of Biology, Bursa, Turkey

Highlights

- A high yield protease producer mutant was obtained from *B. subtilis* E6-5 by random mutagenesis
- The mutant's production medium was optimized using different physical and nutritional factors.
- A new modified medium was developed.
- Protease bands of mutant strain were demonstrated by gel electrophoresis and zymogram analysis.

Article Info

Received: 18 Oct 2022
Accepted: 11 Apr 2023

Keywords

Protease
Bacillus
Random mutagenesis
Optimization

Abstract

In this study, to enhance protease production, the wild type of *Bacillus subtilis* E6-5 was mutagenized by random mutagenesis using ultraviolet radiation and ethidium bromide. After combined treatment, several mutants were obtained. Among these mutants, the mutant strain with the largest proteolytic zone diameter (25 mm) was selected and named *Bacillus subtilis* ATA38. The enzyme production capacity of the obtained mutant was tested and the mutant strain (404 IU/mL at 24 hours) produced 6.7 times more enzyme than the parental strain (60 IU/mL at 32 hours). The effects of some important parameters in the growth medium on enzyme production were examined. The best carbon, organic nitrogen and metal ion were obtained with wheat starch (525 IU/mL), meat extract (850 IU/mL) and KCl+$CaCl_2$ (548 IU/mL), respectively. pH 6.0, 37°C, 200 rpm, inoculum age 18 hours and inoculation amount 1% were obtained as the best physical factors.To further increase the yield, the best nutritional and physical parameters were combined to create a new modified medium. It was determined that the enzyme yield with mutant strain increased 2.7 times in the modified medium (1096 IU/mL) compared to the control (404 U/mL). The mutant strain (1096 IU/mL) showed an 18.2-fold increase in production compared to the wild type (60 IU/mL) in the modified medium. Protease enzyme obtained from ATA38 mutant strain may have great potential in industry for different purposes.

1. INTRODUCTION

Enzymes, which have important metabolic activities in cells, have now come into life to be used for a wide variety of purposes. Protease, amylase, lipase, cellulase and phytase etc. are industrially important enzymes. Of these, proteases have a global industrial market of 60-65% [1]. Protease is an economically important enzyme as it has applications in different industries like the leather industry, dairy, silk industry, organic fertilizer, pharmaceutical industries, laundry detergents, skin cleansers, toothpaste and hair care creams [2, 3]. Proteases are produced from a variety of sources like yeast, bacteria and molds. Among bacteria *Bacillus* sp. strains are frequently used in the production of extracellular enzymes in industrial sectors [4,5].

In order to increase enzyme production, hyperactive microbial strains are tried to be obtained by random mutagenesis [6]. For this purpose, physical [Ultraviolet Irradiation (UV)] and chemical mutagens [Ethidium bromide (EtBr), ethyl methyl sulfonate (EMS), nitrogenous acid (HNO_2), N-methyl-N-nitro-N-nitrosoguanidine (NTG)] are frequently used [7].

*Corresponding author, e-mail: edemirkan@uludag.edu.tr

Mutagenic agents can be used alone or in combination. Combined physical and chemical mutagens are more effective to obtain efficient strains [8]. Mutants overproducing industrially important enzymes such as protease, lipase, pectinase, amylase, catalase and cellulase were obtained by random mutagenesis using these agents [9, 10]. UV causes the formation of highly pyrimidine dimers in DNA and includes all types of base pair substitutions [11]. Ethidium bromide (EtBr), the most common chemical mutagen used, acts as intercalate and deforms the DNA. EtBr is a large basic molecule that may resemble a DNA base pair [12]. They are molecules that get between DNA bases, causing the helix to stretch and DNA polymerase to mistakenly add extra nucleotides. As a result, frameshift mutation occurs and this effects biological processes of DNA such as replication and transcription [13].

On the other hand, nutrition and physical parameters also have an important effect on enzyme production from bacteria. In particular, nitrogen and carbon sources, inorganic salts, and other growth factors in the nutrient medium. On the other hand, parameters such as temperature, pH, enzyme concentration, substrate concentration, time, inoculum size, inoculum age, and agitation can affect enzyme production as physical factors [14].

In this study, it was aimed to obtain a productive strain by using combined mutagens (UV and EtBr) for high protease production from the wild type (parental strain) *Bacillus subtilis* E6-5. Cultural conditions such as nutrition and physical parameters for mutant strain were optimized.

2. MATERIAL METHOD

2.1. Microorganism

The wild type *Bacillus subtilis* E6-5 (GenBank accession number OM004560), previously isolated from the soil and named at the species level, was used in the study [15].

2.2. Mutagenesis by Ultraviolet Irradiation

Overnight bacterial suspensions were prepared with 1 mL of 10^{-9} dilution with sterilized saline and then inoculated onto skim milk agar plates. Mutagenesis of UV irradiations was carried out using 5, 10 and 15 cm distances and time intervals between 1 and 120 minutes. The UV-exposed Petri dishes were incubated at 37°C for 24 hours, after which the number of colonies in each Petri dish was noted. Based on the appearance of clear areas around mutant bacterial colonies, proteolytic activity was detected. The diameters of the clear regions were evaluated by measuring with a millimeter ruler. The UV mutant strain that showed the widest proteolytic zone on skimmed milk agar was selected, and used for Ethidium Bromide (EtBr) mutagenesis studies. The mutation lethality (death) rate due to UV light was calculated with the following Equation [16]; U, indicates the total number of colonies in the non-UV-exposed petri dish and T, indicates the total number of colonies in the UV-exposed petri dish

$$\text{Lethality rate} = \frac{(U-T)}{U} \times 100\%. \quad (1)$$

In UV mutagenesis studies, an ultraviolet lamp [UltraViole 254 nm, Intensity (WW/cm^2), 60, Lamp-VL-130.G, 1x30 W, Germination Lamp-Vilber] was used. All UV studies were performed in a dark medium to inhibit photoreactivation.

2.3. Mutagenesis Using Ethidium Bromide

The most productive UV mutant strain was subjected to chemical mutagenesis. EtBr (Sigma E7637) was used as a chemical mutagen. Different concentrations (between 10-500 mg/mL and 0.005-0.1 µg/mL) were used to determine the EtBr concentration, which caused 99% lethality for EtBr mutagenesis of the UV mutant strain.

For the second time in mutation studies, the UV mutant strain was grown overnight to the density of 10^8 CFU/mL. Under aseptic conditions, different concentrations of EtBr were added to these grown cultures, and cultures were incubated for 18 h at 150 rpm, 37°C. Bacterial samples were taken every two hours for centrifugation (10 min at 6000 rpm), and the pellets were washed twice with sterile physiological saline to remove traces of EtBr. Appropriate dilutions were prepared with saline solution and inoculated into Petri dishes with agar containing skim milk powder and incubated at 37°C for 24 h. Bacteria that formed a zone around the colony after incubation were evaluated as protease positive. The diameters of the clear regions were evaluated by measuring with a millimeter ruler. The mutant strain with the widest proteolytic zone compared to the parental type was selected, and used in further studies. The lethality rates due to EtBr were calculated with the above Equation (1) [16].

2.4. Protease Production

In the production of protease from mutant bacteria, a medium containing (g/L) 1 glucose, 0.2 yeast extract, 10 peptone, 0.1 $CaCl_2$, 0.1 $MgSO_4$ and 0.5 K_2HPO_4 (pH 7.0) was used [17]. The overnight culture with an optical density of 0.3 (600nm) was inoculated at 1% into 500 mL erlenmeyer flasks containing 150 mL of enzyme production medium, and incubated at 18, 24, 28, 30, 32, 40, 44, 48, 64 and 72 h in a shaking incubator at 37°C and 150 rpm. At the end of each incubation period, the cultures were centrifuged (6000 rpm, 10 minutes). The resulting supernatant was used as an enzyme source for the determination of activity.

Bacteria growth was measured spectrophotometrically at 600 nm (Beckman Coulter-UD 700).

2.5. Protease Activity Assay

Protease activity was determined by a modification of the Anson Method using casein as substrate [18]. Casein was used at 2% and dissolved in 0.1 M NaOH. The casein solution was brought to the desired volume with 0.05 M phosphate buffer (pH 7.0). 1 mL of the supernatant was mixed with substrate, and incubated at 37°C for 10 min. Then, the reaction was stopped by the addition of 2 mL of 0.4 M Trichloroacetic acid. and hold at 37°C for 20 minutes, and the precipitate formed was removed by centrifugation (6000 rpm,10 minutes). 5 mL of 0.4 M $NaCO_3$ and 1 mL of diluted Folin-Ciocalteu reagent (1:3) were added to 1 mL of supernatant. The mixture was left in a dark environment at room temperature for 20 minutes. Optical densities were then measured at 660 nm. A standard curve was formed by preparing 0-60 μg mL^{-1} tyrosine solutions. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg mL^{-1} tyrosine under the experimental conditions used.

2.6. Optimization of the Bacterial Growth Conditions for Protease

In the present study, physical and nutritional factors were optimized for the production of protease by mutant strain. For this, different carbon sources such as glycerol, fructose, maltose, sucrose, wheat starch, wheat bran, corn starch and potato starch [1 g/L] were tested by using instead of glucose in the basal medium. As organic nitrogen sources, skim milk powder, corn steep liquor, tryptone and meat extract were used instead of yeast extract and peptone in the basal medium [total 10.2 g/L]. As organic nitrogen sources, skimmed milk powder, corn steep liquor, tryptone and meat extract were used instead of yeast extract and peptone in the basal medium [total 10.2 g/L]; and inorganic nitrogen sources were KNO_3, NH_4NO_3, $NaNO_3$, NH_4Cl and $(NH_4)_2SO_4$. To investigate the effect of metal ions in the medium on bacterial growth and enzyme activity, $CaCl_2$, and $MgSO_4$ in the enzyme production medium were removed and at their total amounts [0.2 g/L] $MnSO_4$, $MgSO_4$, $CaCl_2$, $LiSO_4$, $FeSO_4$, KCl and NaCl were used. The effects of physical parameters like temperature, pH, inoculum age (days), inoculum size and agitation were investigated. For this purpose, the optimum temperature value was determined by using different temperature degrees such as 30, 37 (control), 40 and 50ºC. In determining the effect of pH, the optimum pH value was determined by using different values such as 4.0, 4.5, 5.0, 5.5, 6.0, 7.0 (control) and 8.0. The effect of the agitation was performed at 0 rpm, 50 rpm, 100 rpm, 150 rpm (control), and 200 rpm. The optimum inoculum size rate was obtained by using different inoculum sizes (0.5%, 1% (control), 2%, 3%, 4% v/v). Different inoculation ages (18 hours (control), 1, 2 and 3 days) were used to determine the optimal inoculation age.

A new modified growth medium was developed, consisting of the best factors obtained as a result of optimization studies. The protease production of the mutant bacteria in this medium was compared with the parental type produced in the basal medium. Protease activity and bacterial growth values were determined in all experimental studies.

2.7. Gel Electrophoresis (SDS-PAGE) and Zymogram Analysis

SDS-PAGE and zymogram analysis were determined to show the profile of the protease enzyme of parental strain and mutant. The molecular weights of both enzymes were also determined [19]. Acrylamide was used at the rate of 4% in the stacking gel and 10% in the separation gel. The electrophoresis was accomplished first at 80V during running through stacking gel and at 150 V in vertical mini-slab gel (Mini-ProteanTetra Cell, Bio-Rad, USA). After, the protein bands were observed by 0.5% Coomassie Brilliant Blue R-250 staining. The molecular weights of the polypeptide bands were evaluated by comparing them with standard protein markers (Cell Signalling Technology Prestained Protein Marker, #13953S). Zymogram was also performed to determine the activity of proteases in the gel [20]. For this, a 10% polyacrylamide slab gel containing SDS and 0.5% gelatin in the separation gel was used. Then, the gel was soaked in 1% Triton x-100 for 20 minutes to remove SDS, and this was done 3 times. The gel was stained with 0.5% coomassie brilliant blue R-250 in 45% (v/v) methanol and 10% (v/v) acetic acid. After destaining, protease activity was visualized.

2.8. Statistical Analysis

Standard deviation and student t-test were used for each experimental result, Excel Spreadsheets in Microsoft Excel were used. The results in the studies are the average of three repetitions, the bars represent the standard deviation.

3. RESULTS AND DISCUSSION

3.1. Selection of Best-Proteolytic Mutant by UV and EtBr

A total of 150 mutant strains were obtained as a result of UV mutation with the *Bacillus subtilis* E6-5 strain. Three mutant strains with high proteolytic activities compared to the wild type (8mm, Figure 1A) were chosen, and these mutants were named EB3 and EB7 (13 mm, Figure 1B and D, respectively) and EB4 (14 mm, Figure 1C). UV exposure conditions for EB3 were at a distance of 10 cm and an irradiation time of 10 minutes, while these conditions were 10 cm and 15 minutes for EB4 and 15 cm and 15 minutes for EB7. The lethality rate for these mutants was 94%. While the colony zone diameter of the parental strain was 4 mm, the colonies of the mutants obtained were found to be smaller in size. On the other hand, the protease zone diameters of the mutants were larger.

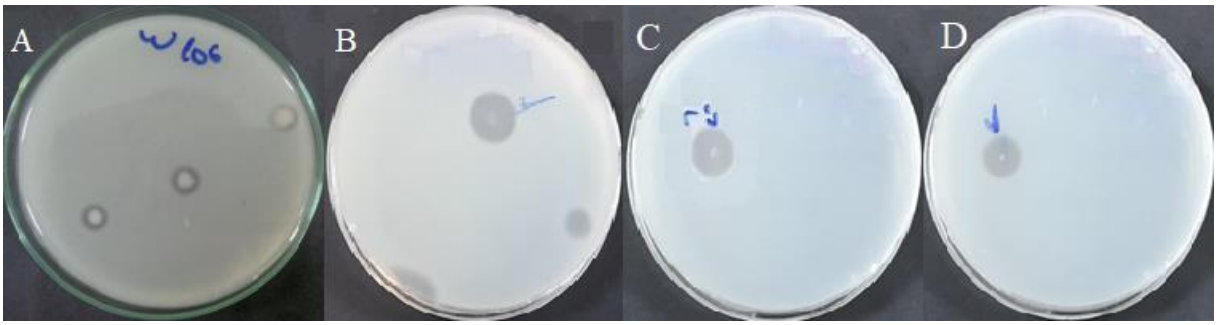

Figure 1. *Protease zone diameters of parental strain (8mm) (A) and UV mutants EB3 (13 mm) (B), EB4 (14 mm) (C) and EB7(13 mm) (D) on skim milk agar medium*

EB3, EB4, and EB7 mutant strains obtained from physical mutation were chemically mutated with EtBr. It has been determined that the concentrations of EtBr between 10-500 mg/mL used to obtain mutant strains are 100% lethal doses. Growths were detected at concentrations between 0.005-0.1 µg/mL. Mutants with

larger zone diameters than the parental strain were obtained at concentrations of EtBr between 0.015 and 0.7 µg/mL. The lethality rate at these concentrations was 80-98%. The zone diameters of the mutants obtained were between 19-25 mm (Table 1). The protease zone diameters of the parent strain (8 mm, Figure 2A) and mutans on skimmilk agar medium were compared. The mutant obtained as a result of 0.5 µg/mL EtBr treatment of UV mutant EB4 had the largest proteolytic zone diameter (25 mm, Figure 2B) and was named ATA38. Subsequent studies were continued with this strain.

Table 1. *Qualitative detection of protease productions by mutagenic effects of different EtBr concentrations on UV mutants*

EtBr concentration (µg/mL)	Mutant EB3			Mutant EB4			Mutant EB7		
	Number of colonies	Colony diameter (mm)	Zone diameter (mm)	Number of colonies	Colony diameter (mm)	Zone diameter (mm)	Number of colonies	Colony diameter (mm)	Zone diameter (mm)
0.005	23	8	15	>25	8	16	20	8	16
0.015	20	5	19	20	5	16	25	5	14
0.5	25	6	19	10	9	**25**	20	6	14
0.7	20	4	16	9	6	9	20	6	12
0.1	20	5	15	15	5	5	20	5	15

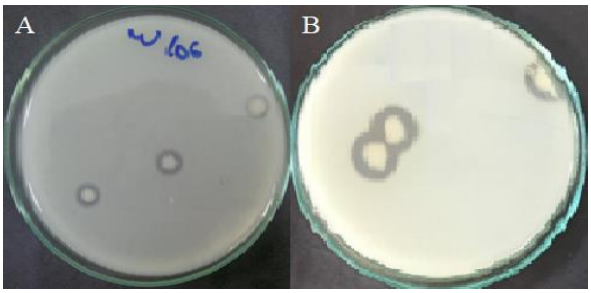

Figure 2. *Protease zone diameters of parental strain (8 mm, A) and EtBr mutant ATA38 (25 mm, B) on skimmilk agar medium*

The enzyme production capacity of the mutant obtained as a result of combined mutation experiments was investigated in a liquid medium. The maximum protease activity for the main strain was 60 IU/mL at 32 hours, while the maximum enzyme activity for mutant ATA38 was 404 IU/mL at 24 hours. The protease activity and growth of the mutant were significantly increased compared to the parental strain. It was determined that the enzyme yield in the mutant increased 6.7 times compared to the parental strain. Bacterial growth showed parallelism with enzyme production. Maximum enzyme production was determined in the stationary phase of growth (Table 2).

Table 2. *Protease activities and growth of the wild type and mutant ATA38*

Incubation time (h)	The wild type E6-5 strain		Mutant strain ATA38	
	Enzyme Activity (IU/ mL)	Bacterial growth (OD_{600})	Enzyme Activity (IU/ mL)	Bacterial growth (OD_{600})
18	45	0.23	369	1.08
24	48	0.33	404	1.09
28	55	0.32	367	1.09
32	60	0.29	292	0.81
44	53	0.23	198	0.59
48	51	0.17	135	0.53
52	47	0.14	102	0.47

Many researchers have obtained efficient mutant strains by using various chemical and physical mutagens to obtain industrially important enzymes in high yield. When cellulase producing fungi were exposed to triple (1-Methyl-3-nitro-1-nitrosoguanidine (MNNG), ethidium bromide (EtBr) and (UV)) and double

(EtBr and MNNG) mutagen agents for 30 min and 1 h, the best efficiency was obtained in the application of double mutagens [21]. In another study, they obtained the best mutant strains in their ultraviolet mutation studies with *Bacillus pumilus*, at 20 min exposure time to UV and 5 cm distance [22]. When *Bacillus licheniformis* N-2 was exposed to UV, MMS and NTG mutagens, 9 protease positive mutants were selected and it was reported that the mutant called UV-9 had 1.4 times more protease activity [23].

It has been reported to be used in combination with UV, NTG and EBr to increase the productivity of the parent strain *B. subtilis* M-9. Among the mutants obtained, it was determined that the BSU-5 mutant produced approximately 5.67 times more alkaline protease (81.21 ± 1.85 PU/mL) at 24 hrs of incubation than the parental strain (48 h) [24]. *Bacillus subtilis* S1-4 mutant obtained by combining UV and MNNG treatment achieved 2.5 times more protease production than the parental strain at the end of 24-hour production [25]. Acridine orange, EtBr and UV were used to increase protease production from Halotolerant Actinomycete andand increased approximately 2.2-fold from the parental strain [26]. A 1.44-fold increase in protease production from a mutant strain was reported with the UV mutation compared to the parent strain of *Bacillus pantotheneticus* [27].

When both our results and those of other researchers are compared, it is clear that the protease production pathways of bacteria are different. The frequency of spontaneous mutations also varies widely among organisms. Even within the same species, the frequency of these mutations can vary from gene to gene. This variability may occur due to the relative efficiency of error reading and repair systems in organisms.

3.2. Optimization of the Production Medium

The content of the culture medium and fermentation conditions are among the most important factors affecting enzyme production. The most notable among these are carbon and nitrogen sources, temperature, pH, inoculation age, inoculation size and agitation [28-30].

In this study, different carbon sources (sucrose, maltose, glycerol, fructose, wheat bran, wheat starch, corn starch, potato starch) at a concentration of 0.1% (w/v) were tried in the basal medium. The carbon source preference order of *Bacillus subtilis* ATA38 in terms of enzyme production was determined as Wheat Starch > Wheat Bran > Fructose > Maltose = Glucose (Control) > Glycerol > Sucrose > Potato Starch > Corn Starch, respectively (Figure 3). The best carbon source was wheat starch. Enzyme production in the medium with wheat starch (525 IU/mL) increased 1.3 times compared to the control medium with glucose (404 IU/mL). on the other hand, the mutant strain showed an increase of 8.75 times compared to the parental strain (60 IU/mL) in this medium. It was determined that there is no parallelism between growth and enzyme production.

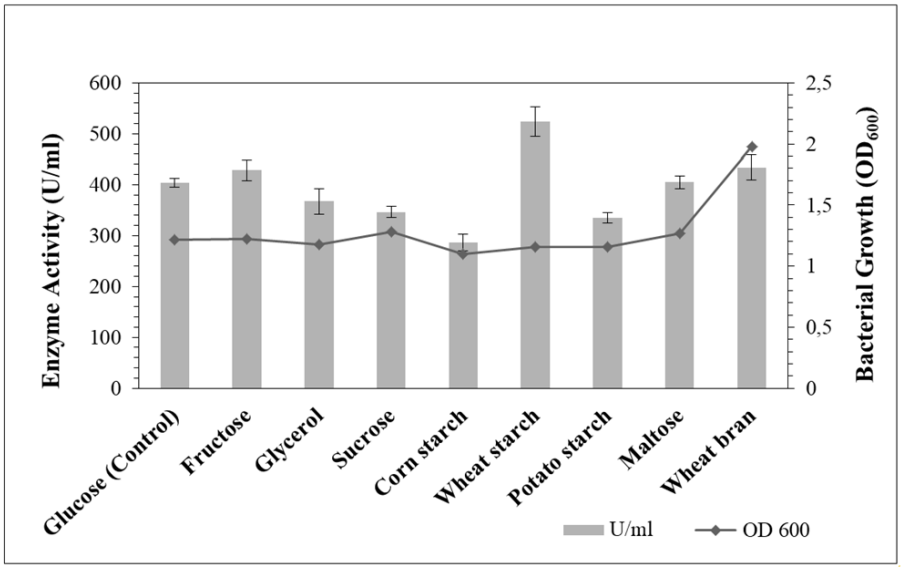

Figure 3. *Effect of carbon sources on protease production by ATA38 mutant*

Nitrogen sources are also a parameter that is effective in enzyme production. In the study, it was determined that inorganic nitrogen sources were not effective on enzyme production, whereas organic nitrogen sources had a high effect. The various organic nitrogen sources impact on growth and protease production from mutant ATA38 strain was shown in Figure 4. In terms of mutant enzyme production, the order of preference for organic nitrogen source is Meat Extract > Yeast Extract > Tryptone > Control (peptone and yeast extract > Corn step liqueur > Peptone > Skim milk, respectively. Enzyme production in the medium with meat extract (850 IU/mL**)** as the most nitrogen source increased by 2.1 times compared to the control medium with Peptone and Yeast extract (404 IU/mL). On the other hand, the mutant strain (850 IU/mL) showed a 14.1-fold increase in yield compared to the parental strain (60 IU/mL) in this medium. It was determined that there is no parallelism between bacterial growth and enzyme production.

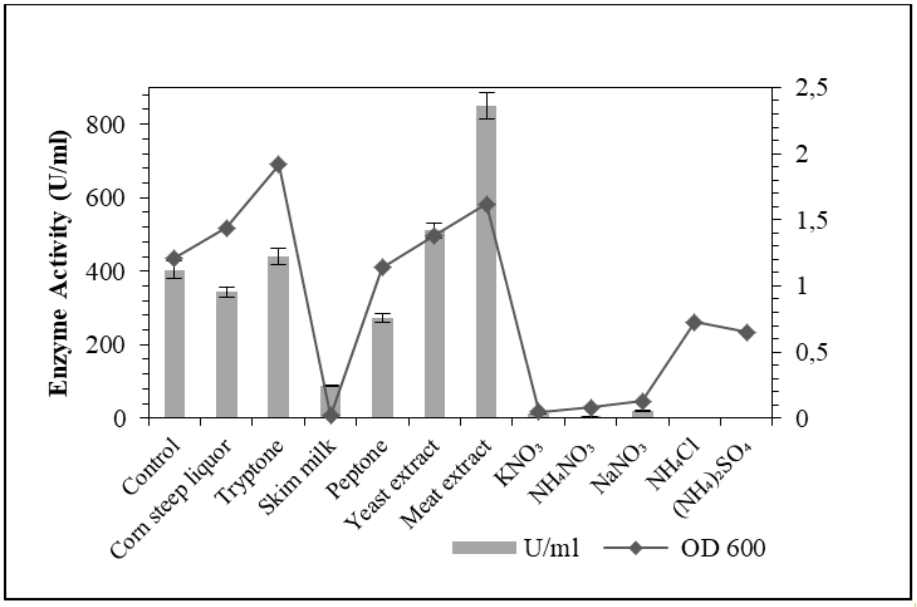

Figure 4. *Effect of organic and inorganic nitrogen sources on protease production by ATA38*

Metal ions in the medium are among the factors affecting enzyme production, as they have an inducing role. For this, different metal ions were tested. The best metal ion for mutant ATA38 was KCl. It was observed that the mutant preferred the metal source in enzyme production as KCl > Control ($MgSO_4$+$CaCl_2$) > NaCl, respectively (Figure 5). It has been determined that $MnSO_4$, $CaCl_2$, $MgSO_4$, $LiSO_4$, and $FeSO_4$ have an inhibitory effect. When $MgSO_4$ and $CaCl_2$ in the control medium were tested separately, it was determined that they were not effective in protease production alone. The mutant strain (460 IU/mL) showed a 7.6-fold increase in yield compared to the parental strain (60 IU/mL) in the presence of KCl.

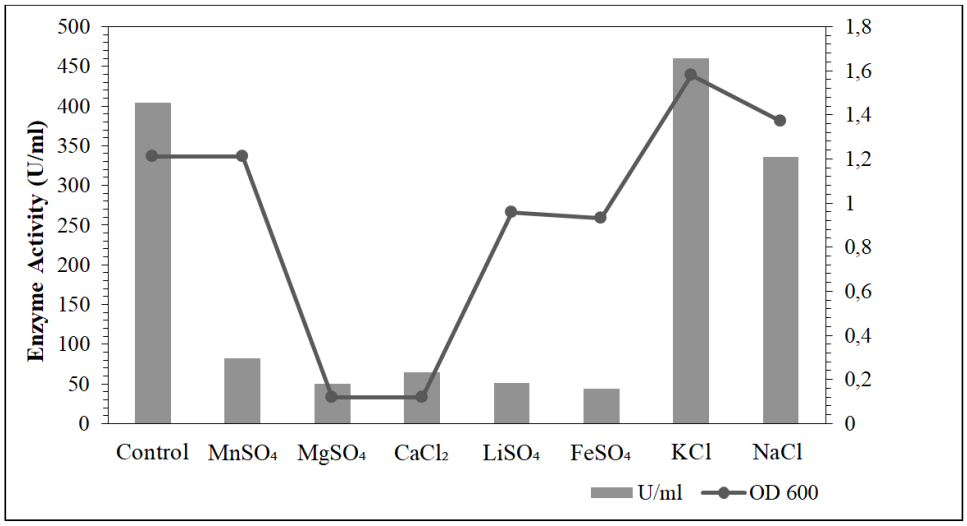

Figure 5. *Effect of different metal ions on protease production by ATA38*

Various researchers have used different carbons sources, nitrogen sources and metal ions for the production of high-level enzymes from mutants obtained by using physical mutagens and different chemical mutagens, and they have reached different results as a result of these researches.

UV, EMS, and Ethidium Bromide were applied to *Bacillus cereus* bacteria by random mutation method. In the nutritional optimization study, the best carbon and nitrogen sources were found to be 1% fructose and 1% peptone, respectively [31]. For UV-mutated *Bacillus subtilis* G-4, glucose (1%) and peptone (1%) were determined as the best sources [32]. It has been reported that casamino acid and yeast extract are the most suitable sources [33]. It has been stated that ammonium salts, which are inorganic nitrogen sources, inhibit the growth and protease production of *B. licheniformis* N-2 [34].

In order to determine the optimal physical factors in protease production of mutant ATA38, pH, temperature, graft size, graft age and agitation values were investigated. In present study, maximum production was obtained at 37°C, which is the control temperature, and it was observed that the high temperature of 50°C dramatically reduced enzyme production and the growth decreased with the increase in temperature and the mutant had mesophilic character, while the maximum synthesis of protease was found at pH 6.0. Enzyme production was obtained in an acidic medium compared to a basic medium (Figure 6). At pH 6.0 (622 IU/mL), where the maximum enzyme production was achieved, it increased 1.5 times compared to the control medium with pH 7.0 (404 IU/mL). The mutant strain (622 IU/mL) showed a 10.3-fold increase in yield compared to the wild type (60 IU/mL) at pH 6.0.

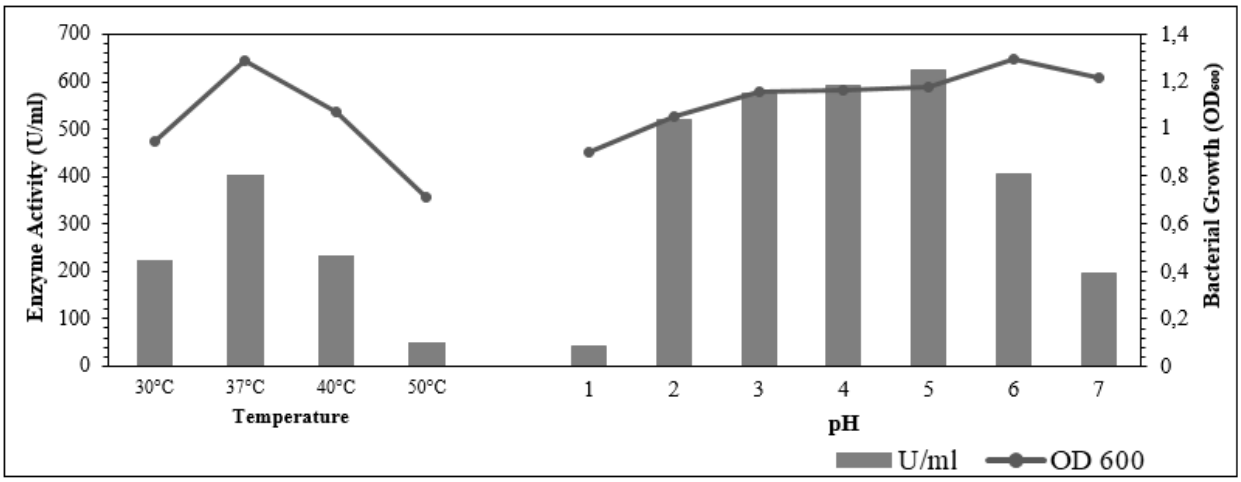

Figure 6. *Effect of temperature and pH on protease production on protease production by mutant strain ATA38*

Optimal values for protease production of the mutant strain obtained as a result of the UV mutation of the parent strain *Bacillus subtilis* G-4 were reported as pH 9.0 and 37.5 ºC [32]. Optimum pH and optimal temperature were found pH 8.5 and 37ºC, respectively, for protease production by *Bacillus subtilis* [35]. The optimum temperature of a mutant of *Bacillus licheniformis* N-2 was found to be 60ºC [23]. While the maximum protease activity of the parent strain *Bacillus subtilis* M-92 was as 70.18 ± 1.25 PU/mL at optimal pH 9.0 and 37ºC, the protease activity of the mutant strain was reported as 75.75 ± 2.84 PU/mL at optimal pH 9.0 and 45ºC [24].

Aeration leads to a homogeneous distribution of substrate, nutrients, and oxygen into the nutrient medium, especially when using the submerged technique. Thus, it can increase the metabolic activities of microorganisms resulting in the production of primary metabolites such as enzymes.

In this study, the highest productivity of protease was recorded at 200 rpm agitation rate (Figure 7). With this rpm (487 IU/mL), a 1.2-fold increase in efficiency was determined compared to the basal medium (control; 150 rpm, 404 IU/mL). It was observed that bacterial growth increased under high aeration conditions. Inoculum size is of great importance in microbial enzyme processes as it affects the morphology of bacteria as well as their progression during growth phase [36]. For this reason, it is important to choose the appropriate inoculum size according to the type of the microorganisms in order to obtain high yields from the desired products [37]. Optimum inoculum size is important, because using a low inoculum size will produce insufficient biomass, while using a high graft size will produce too much biomass and in both cases the desired product will be insufficient [38]. The optimal inoculum size was found to be 1% v/v. It was determined that growth and enzyme production were parallel. It was observed that the enzyme production and growth gradually decreased with the increase in the size of the inoculum.

Since inoculum age plays an important role in the fermentation rate, optimization of inoculum age should always be considered an important fermentation parameter. Figure 7 shows the effect of inoculum age (18 hours (control), 1, 2, and 3 days) on bacterial growth and protease production. The best protease production was achieved when 1% v/v of 18 h culture was inoculated into the fermentation medium. It has been observed that the young culture is more effective than the old culture.

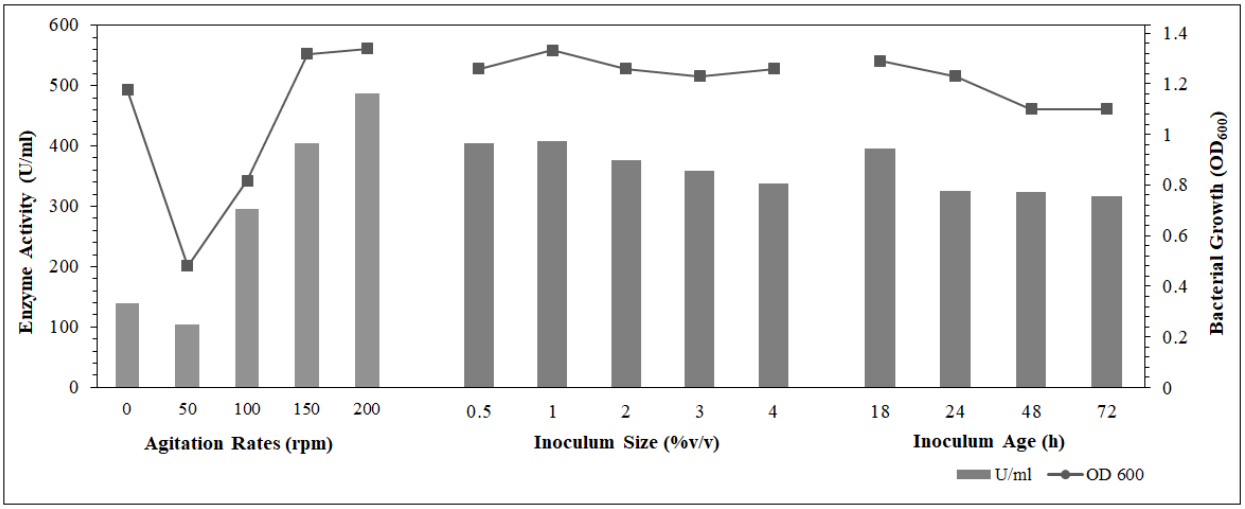

Figure 7. *Effects of agitation rate, inoculum size and inoculum age on on protease production by ATA38 mutant*

Some researchers have also investigated the size, age of the inoculum, and also agitation. The optimal inoculum size was found at 3% for UV-mutated *Bacillus subtilis* G-4 and 2.0% for *Bacillus licheniformis* N-2 [32, 39]. On the other hand, 10% inoculum size was reported to be optimum for *Bacillus subtilis* PCSIR-5 [28].

From the *B. subtilis* apr-IBL04 mutant obtained with ethidium bromide, the maximum protease activity (631.09 U/mL) at 3% inoculation, pH 8,72 h and 45°C was increased 7-fold compared to the parental strain (82.32 U/mL) [40]. Inoculum size 1.0 cell/mL (7.0 x 10^3) and 24 hr aged inoculum were optimized for the production of protease by *Bacillus subtilis* [41].

The growth medium was optimized to get better yields from the ATA38 strain obtained by the random mutation technique. For this, a modified medium was obtained by combining the nutritional and physical conditions in which the highest protease activities were obtained. It was determined that the enzyme yield increased by 2.7 times in the modified medium (1096 IU/mL, OD600 2.13) compared to the basal medium (404 IU/mL, OD600 1.09) (Figure 8). The mutant strain showed an 18.2-fold increase in yield compared to the wild type (60 IU/mL) in the newly created modified medium. In this medium was also an increase in bacterial growth.

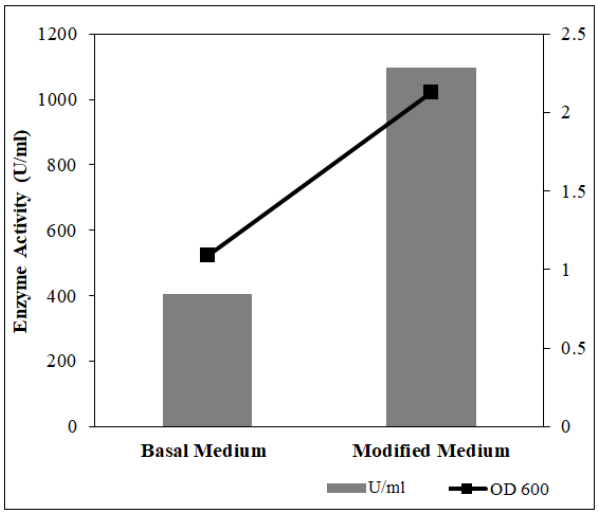

Figure 8. *Protease activity and bacterial growth in modified and basal medium*

3.3. Protease Profiles of Parental Strain and Mutant ATA38

In this study, SDS-PAGE and zymogram analyzes of the protease obtained from the parental strain and mutant ATA38 were performed, and the enzymes of both strains were compared. Both enzymes showed bands in the same place, but a thicker band was obtained in the mutant strain. The molecular weights of the enzymes obtained from both strains were estimated to be about 51 kDa (Figure 9). It is known that the molecular weight of metalloproteinases originating from *Bacillus* is generally in the range of 40-60 kDa [42]. It is observed in some exceptional cases. For example, the molecular weight of *Bacillus subtilis* (natto) protease and *B. subtilis* protease has been reported to be 90 kDa and 15 kDa, respectively [43, 44]. The neutral protease of *Bacillus subtilis* USTCMS 1011 strain has a molecular weight of 65.4 kDa [45].

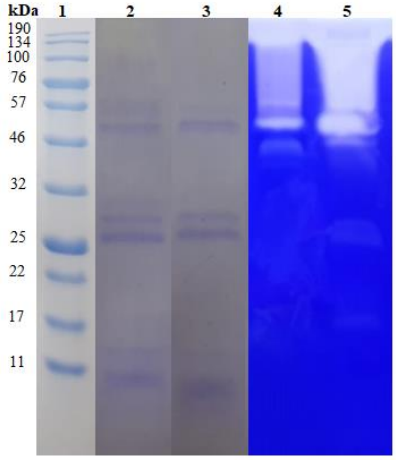

Figure 9. *SDS-polyacrylamide gel electrophoresis and zymogram analysis of the parental strain and mutant ATA38. Lane 1. Protein markers (between 11 and 190 kDa), Lane 2. The parental enzyme extract, Lane 3. The mutant ATA38 enzyme extract, Lane 4. The parental enzyme extract, Lane 5. The mutant ATA38 enzyme extract*

In conclusion, protease enzyme is an important industrial enzyme that is used in different branches of industry and its usage rate is increasing day by day. Efficient strains can be obtained by isolating the strains with high enzyme yield from nature, modifying the nutrient medium, and mutation techniques. The protease enzyme obtained from *Bacillus subtilis* ATA38, a very productive and new mutant obtained as a result of this study, might have the potential to be industrially usable.

CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

REFERENCES

[1] Shankar, S., Rao, M., and Seeta, L.R., "Purification and characterization of an alkaline protease by a new strain of *Beauveria* sp.", Process Biochemistry, 46(2): 579–585, (2011).

[2] Das, G., and Prasad, M.P., "Isolation, purification & mass production of protease enzyme from *Bacillus subtilis*", International Research Journal of Microbiology, 1(2): 26–31, (2010).

[3] Hammami, A., Hamdi, M., Abdelhedi, O., Jridi, M., Nasri, M., and Bayoudh, A., "Surfactant- and oxidant-stable alkaline proteases from *Bacillus invictae*: characterization and potential applications in chitin extraction and as a detergent additive", International Journal of Biological Macromolecules, 96: 272–81, (2017).

[4] Gupta, R., Beg, Q.K., Khan, S., and Chauhan, B., "An overview on fermentation, downstream processing and properties of microbial alkaline proteases", Applied Microbiology and Biotechnology, 60: 381–395, (2002).

[5] Pant, G., Prakash, A., Pavani, J.V.P., Bera, S., B., Deviram, G.V.N.S., Kumar, A., Panchpuri, M., and Prasuna, R.G., "Production, optimization and partial purification of protease from *Bacillus subtilis*", Journal of Taibah University for Science, 9: 50–55, (2015).

[6] Azin, D.A., and Noroozi, R.F., "Effect of chemicals on the improved gluconate productivity by an *Aspergillus niger* strain", Applied Biochemistry and Biotechnology, 61: 393–397, (2001).

[7] Iftikhar, T., Niaz, M., Abbas, S.Q., Zia, M.A., Ashraf, I., Lee, V., and Haq, I., "Mutation induced enhanced biosynthesis of lipases by *Rhizopus oligosporus* var. *Microsporus*", Pakistan Journal of Botany, 42: 1235–1249, (2010).

[8] Zia, M.A, Rahman, K., Sheikh, M.A., and Khan, I.A., "Chemically treated strain improvement of *Aspergillus niger* for enhanced production of glucose oxidase", International Journal of Agriculture and Biology, 12: 153–154, (2010).

[9] Ribeiro, O., Magalhaes, F., Aguiar, T.Q., Wiebe, M., Penttila, M., and Domingues, L., "Random and direct mutagenesis to enhanceprotein secretion in *Ashbya gossypii*", Bioengineered, 4: 322–331, (2013).

[10] Basavaraju, S., Kathera, C., and Jasti, P.K., "Induction of Alkaline Protease Production by *Bacillus* mutants through U.V. irradiation", International Journal of Pharmaceutical Sciences Review and Research, 26(1): 78–83, (2014).

[11] Rani, M.R., Prasad, N.N., and Sambasivarao, K.R.S., "Optimization of cultural conditions for the production of alkaline protease from a mutant *Aspergillus Flavus* AS2", Asian Journal of Experimental Biological Sciences, 3: 565–576, (2012).

[12] Khedr, M.A., Ewais, E.A., and Khalil, K.M.A., "Overproduction of thermophilic α-amylase productivity and Amy E gene sequence of novel Egyptian strain *Bacillus licheniformis* MK9 and two induced mutants", Current Science International, 6(2): 364–376, (2017).

[13] Suribabu, K., Govardhan, Y.L., and Hemalatha, K.P.J., "Strain Improvement of *Brevibacillus borostelensis* R1 for Optimization of α-Amylase Production by Mutagens", Journal of Microbial and Biochemical Technology, 6(3): 123–12, (2014).

[14] Khalil, A.B., Ghandi, H., Anfoka, H., and Bdour, S., "Isolation of plasmids present in thermophilic strains from hot springs in Jordan", World Journal of Microbiology and Biotechnology, 19: 239–241, (2003).

[15] Demirkan, E., Kut, D., Sevgi, T., Dogan, M., and Baygin, E., "Investigation of effects of protease enzyme produced by *Bacillus subtilis* 168 E6-5 and commercial enzyme on physical properties of woolen fabric", Journal of the Textile Institute, 111: 26–35, (2020).

[16] Zong, H., Zhan, Y., Li, X., Peng, L., Feng, F., and Li, D., "A new mutation breeding method for *Streptomyces albulus* by anatmospheric and room temperature plasma", African Journal of Microbiology Research, 6(13): 3154–3158, (2012).

[17] Quadar, S.A., Shireen, E., Iqbal, S., and Anwar, A., "Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3", Indian Journal of Biotechnology, 8: 286–290, (2009).

[18] Keay, L., and Wildi, B.S., "Proteinases of the genus *Bacillus.* I. Neutral proteinases", Biotechnology and Bioengineering, 12: 179–212, (1970).

[19] Laemmli, U.K., "Cleavage of structural proteins during theassembly of the head of bacteriophage T4", Nature, 227: 680-685, (1970).

[20] Vijayaraghavan, P., Lazarus, S., and Vincent, S.G.P., "De-hairing protease production by an isolated *Bacillus cereus* strain AT under solid-state fermentation using cow dung: biosynthesis and properties", Saudi Journal of Biological Sciences, 21: 27–34, (2014).

[21] Chand, P., Aruna, A., Maqsood, A.M., and Rao, L.V., "Novel mutation method for increased cellulase production", Journal of Applied Microbiology, 98: 318–323, (2005).

[22] Solaiman, E.A.M., Hegazy, W.K., and Maysa, E., "Moharam Induction of overproducing alkaline protease *Bacillus* mutants through UV irradiation", The Arab Journal of Biothechnology, 8: 49-60, (2005).

[23] Nadeem, M., Qazi, J. I., and Baig, S., "Enhanced production of alkaline protease by a mutant of *Bacillus licheniformis* N-2 for dehairing", Brazilian Archives of Biology and Technology, 53: 1015–1025, (2010).

[24] Javed, S., Meraj, M., Bukhari, S. A., Irfan, R., and Mahmood, S., "Hyper-production of Alkaline Protease by Mutagenic Treatment of *Bacillus subtilis* M-9 using Agroindustrial Wastes in Submerged Fermentation", Journal of Microbial and Biochemical Technology, 5(3): 74–80, (2013).

[25] Wang, X.C., Zhao, H.Y., Liu, G., Cheng, X.J., and Feng, H., "Improving production of extracellular proteases by random mutagenesis and biochemical characterization of a serine protease in *Bacillus subtilis* S1-4", Genetics and Molecular Research, 15(2): gmr.15027831.

[26] Payal, M., Charu, S., and Pradeep, B., "Strain Improvement of Halotolerant Actinomycete for Protease Production by Sequential Mutagenesis", International Journal of Chemical Sciences, 15(1): 109, (2017).

[27] Shikha, S.A., and Darmwal, N.S., "Improved production of alkaline protease from a mutant of alkalophilic *Bacillus pantotheneticus* using molasses as a substrate", Bioresource Technology, 98: 881–885, (2007).

[28] Sajedi, R.H., Naderi-Manesh, H., Khajeh, K., Ahmadvand, R., Ranjbar, B., Asoodeh, A., and Moradian, F., "A Ca-independent amylase that is active and stable at low pH from the *Bacillus* sp. KR-8104", Enzyme and Microbial Technology, 36: 666–671, (2005).

[29] Couto, S.R., and Sanromán, M.A., "Application of solid-state fermentation to food industry- A review", Journal of Food Engineering, 76: 291–302, (2006).

[30] Souza, P.M.D., "Application of microbial a-amylase in industry-a review", Brazilian Journal of Microbiology, 41: 850–861, (2010).

[31] Raju, E.V.N., and Divakar, G., "*Bacillus cereus* GD 55 Strain Improvement by Physical and Chemical Mutagenesis for Enhanced Production of Fibrinolytic Protease", International Journal of Pharmaceutical Sciences and Research, 4: 81–93, (2013).

[32] Gulsher, M., Nadeem, M., Syed, Q., Irfan, M., and Baig S., "Protease Production from UV Mutated *Bacillus subtilis*", Bangladesh Journal of Scientific and Industrial Research, 47(1): 69–76, (2012).

[33] Prakasham, R.S., Rao, C.S., and Sarma, P.N., "Green gram husk - An inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation", Bioresource Technology, 97: 1449–1454, (2006).

[34] Nadeem, M., Iqbal J.Q., Shahjahan B., and Qurat-ul-Ain S., "Effect of medium composition on Commercially Important Alkaline Protease Production by *Bacillus licheniformis* N-2", Food Technology and Biotechnology, 46: 385–394, (2008).

[35] Mukhtar, H., and Haq, I., "Production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent in leather processing", Pakistan Journal of Botany, 40(4): 1673–1679, (2008).

[36] Darah, I., Han, L.Z., Nuraqilah, Y., Isnaeni., and Lim Sheh, H., "*Bacillus licheniformis* BT5.9 isolated from Changar hot spring, Malang, Indonesia, as a potential producer of thermostable α-amylase", Tropical Life Sciences Research, 24(1): 71–84, (2013).

[37] El-Enshasy, H.A., Farid M.A., and El-Sayed E.A., "Influence of inoculums type and cultivation conditions on natamycin production by *Streptomyces natalensis*", Journal of Basic Microbiology: An International Journal on Biochemistry, Physiology, Genetics, Morphology, and Ecology of Microorganisms, 40(5-6): 33–342, (2020)

[38] Sangeetha, P.T., Ramesh, M.N., and Prapulla S.G., "Production of fructosyl transferase by *Aspergillus* oryzae CFR202 in solid-stse fermentation using agricultural by-product", Applied Microbiology and Biotechnology, 65(5): 530–537, (2004).

[39] Nadeem, M., Qazi, J.I., Baig, S.J., and Syed, Q.A., "Studies on commercially important alkaline protease from *Bacillus licheniformis* N-2 isolated from decaying organic soil", Turkish Journal of Biochemistry, 32: 171–177.

[40] Shafique, T., Shafique, J., Zahid, S., Kazi, M., Alnemer, O., and Ahmad, A., "Screening, selection and development of *Bacillus subtilis* apr-IBL04 for hyper production of macromolecule alkaline protease", Saudi Journal of Biological Sciences, 28: 1494–1501, (2021).

[41] El-Safey, E.M. and Abdul-Raouf, U.M., "Production, purification and characterization of protease enzyme from *Bacillus subtilis*." International Conferences for Development and the Environment in the Arab World, Assiut, Egypt, March 23–25, 14, (2004).

[42] Vijayaraghavan, P., Vijayan, A., Arun, A., Jenisha, J.K. and Vincent, S.G.P., "Cow dung: a potential biomass substrate for the production of detergent-stable dehairing protease by alkaliphilic *Bacillus subtilis* strain VV", SpringerPlus, 1: 76, (2012).

[43] Kato, T., Yamagata, Y., Arai, T. and Ichishima, E., "Purification of a new extracellular 90 kDa serine proteinase with isoelectric point of 3.9 from *Bacillus subtilis* (natto) and elucidation of its distinct mode of action", Bioscience, Biotechnology, and Biochemistry, 56(7): 1166–1168, (1992).

[44] Hussain, F., Kamal, S., Rehman, S., Azeem, M., Bibi, I., Ahmed, T., and Iqbal, H.M.N., "Alkaline protease production using response surface methodology, characterization and industrial exploitation of alkaline protease of *Bacillus subtilis*", Catalysis Letters, 147: 1204–1213, (2017).

[45] Devanadera, M.K.P., Haw, S.V.O., Arzaga, M.J.J., Buenaflor, L.J., Gagarin, T.J.E., Vargas, A.G., Mercado, S.M., and Santiago, L.A., "Optimization, production, partial purification and characterization of neutral and alkaline proteases produced by *Bacillus subtilis*", Journal of Microbiology, Biotechnology and Food Sciences, 6(2): 83–838, (2016).