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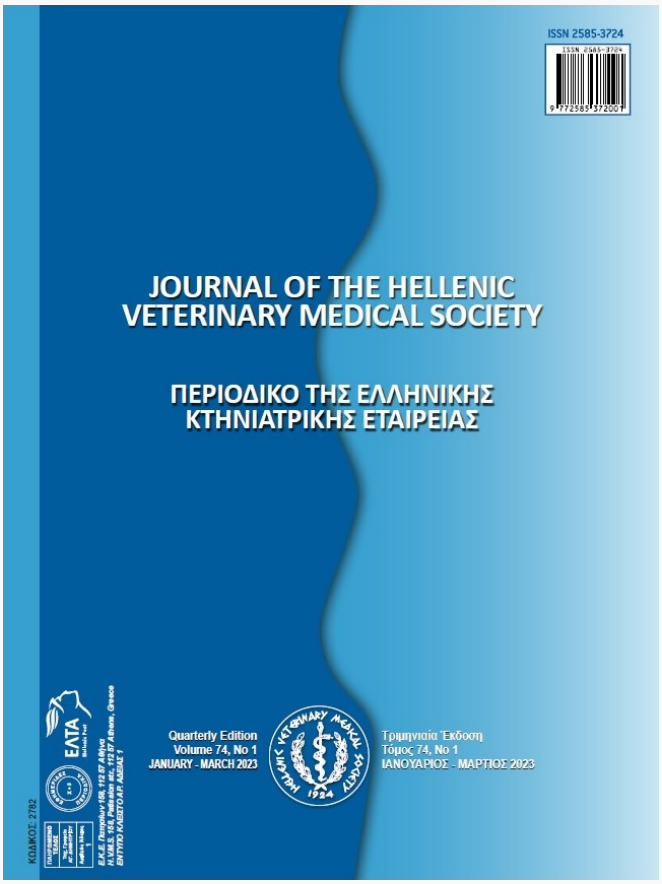

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Creation and Characterization of the β-1,4-Glucanase (CMCase) Producing Mutant Strains of *Bacillus* sp. with Ultraviolet Radiation and Ethidium Bromide

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ABSTRACT: In this study, the β-1,4-glucanase-producing *Bacillus* sp. strains were isolated from soil; then, in these strains, the undesired enzyme genes such as α-amylase, xylanase, protease were inactivated by mutation using Ethidium bromide (EtBr) and UV ray; thus, only β-1,4-glucanase producing strains were obtained. The enzyme activities (pH, temperature, thermostability, etc.) of the wild strainand its two mutant variants (UV mutant (mUV), EtBr mutant (mEB)) producing β-1,4-glucanase were partially characterized. It was found that the optimum pHs for the mUV, mEB, and the wild strain were 7.0, 6.0 and 9.0, respectively; and the optimum temperatures were 50, 60, and 50 °C, respectively. Finally, the molecular weights of the cellulase enzymes of all the three bacteria were found to be 40 kDa by SDS-PAGE and zymogram analysis.

Keywords: CM Case, β-1,4-glucanase, mutation, ethidium bromide, UV radiation, *Bacillus* sp.

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INTRODUCTION

Cellulose, the active use of which dates back a long way in the history of mankind, can be used in the industry for many different purposes. One of the main reasons for its widespread use is its ability to hydrolyze to glucose. Therefore, the hydrolysis of cellulose, which also functions as a carbon source, is also important. Cellulose, which is an organic molecule having the most biomass in nature, can be easily obtained economically, which paves the way for its effective use. In this regard, the developing scientific research methods are also important for the most effective use of this organic molecule (Bhat, 2000).

Cellulose, which is abundant in plant cell walls, is formed by binding of glucose molecules with β(1,4) glucosidase bonds. Being a hard substance with a fibrillary structure in general, it is located in the cell wall of plants and constitutes about 40% of the plant biomass (Niehaus et al., 1999). Cellulose fibrils can be found in amorphous form as well as crystalline form in nature (Kulshreshta and Dwelz, 1973). The cellulases in the hydrolytic enzyme group, which can break down the cellulose molecule into glucose, differ from each other depending on their activity to hydrolyze cellulose. In general, cellulases are categorized under 3 groups according to their activities (Lynd et al., 2002): Endoglucanase (endo-β-1,4 glucanase, CMCase, Endocellulase) (EC3.2.1.4), Exoglucanases (Cellobiohydrolases) (EC3.2.2.91), and β-Glucosidases (EC3.2.1.21). Cellulose can be hydrolyzed by bacterial enzymes in both aerobic and anaerobic conditions. In the previous studies, the most used bacteria producing cellulase enzymes in aerobic conditions were as follows: *Bacillus, Cytophaga, Herpetosiphon, Pseudomonas, Cerratia, Streptomyces, Sporocytophaga, Thermoactinomyces* and *Thermomonospora*. It was reported that Bacillus, a microorganism that has been studied for many years, has the ability to produce β-glucanase enzyme. In this regard, the most studied *Bacillus* genus bacteria were as follows: *B. licheniformis, B. amyloliquefaciens, B. subtilis* and *B. macerans* (Liming and Xueliang, 2004).

In the animal feed industry, in order to reduce the cost of feed, some scientific studies have been carried out on the plant sources that are easily available and have the potential of being a protein source. In this context, the use of oilseeds has generally come to the fore. Soybean meal, sunflower seed meal, cottonseed meal, corn gluten flour, rapeseed and canola meal have been among the sources of vegetable protein (Hendricks and Bailey, 1989). Enzymes can be added to the feed in order to facilitate the digestion of the undigested parts due to the high cellulose content, to decrease the effect of the factors that reduce the effect of nutritional factors such as arabinoxylase, and to increase the energy values of some feed raw materials. It is important that the enzyme to be added as an additive to the feed can remain unchanged in the feed after the feed production and can continue their activities in the intestines of animals without harming them (Liang, 2000). The cost of the enzymes added as an additive to the feed is also economically affordable. In the previous studies, it was observed that the nutrient digestibility increased in the fish fed with enzyme-added feeds (Deguara et al., 1999). It is known that some bacterial species contain up to 70% dry protein (Çetin, 1983). On the other hand, β-1,4-glucanase, a cellulase enzyme, is used in the silage production based on the principle of storing the green parts of the grain with high cellulose by the lactic acid fermentation. It is also used to minimize the dry matter loss in the feed by means of accelerating the penetration of the cellulose molecule into the lactic acid tissues by partially breaking down and rapidly decreasing the silage acidity. β-1,4-glucanase is used in the textile, beverage, and food industries as well as the animal feed industry.

Ethidium bromide, a powerful chemical mutagen, interacts with DNA, causing single nucleotide separation or insertion in the gene. As a result, the frameshift mutation occurs and some changes take place in the phenotype. In general, this means that the enzyme in question cannot be obtained. That is why ethidium bromide is a preferred mutagen (Griffiths et al., 1993). Molecular weight changes as a result of the ethidium bromide mutation. This may decrease or increase the amount of the enzyme. On the other hand, the electromagnetic rays (UV rays, microwave rays, x-rays, etc.) can damage the DNA molecule and cause mutations on the genes. That is why many studies have been carried out on these rays. UV rays affect the DNA molecule in two ways. First, they act by breaking the sugar-phosphate bond in one of the DNA strands. Second, they prevent replication and transcription in DNA by ensuring the formation of thymine dimers through forming a bond between thymines. UV rays also form cytosine dimers or thymine-cytosine dimers (Kasap, 2010). The purpose of this study is to isolate from soil the bacteria producing the cellulase enzymes that can be used as feed additives in animal feed cultivation, to partially characterize the cellulase enzymes produced

by the bacteria, and finally to obtain only the cellulase producing mutants by inactivating the undesired enzyme genes by in-vitro mutagenesis.

MATERIAL AND METHOD

Bacillus sp. Strain and Its Growth Medium

The bacteria were isolated from 12 soil samples taken from the places near the tree roots in Adana. 1.60 g of each soil sample was vortexed and mixed with 5 ml of pure water. In order to ensure the death of vegetative cells and the isolation of endospore forms, each tube was pasteurized for 10 minutes at 80 °C (Hamilton et al., 1999; Lennete et al., 1985). In this way, we killed all bacteria in vegetative form. Bacteria with only endospore forms remaining were grown under aerobic conditions. Clostridium spores did not grow because anaerobic conditions did not occur. Thus, bacteria of members of the Bacillus genus, which developed only in an aerobic environment, developed in the environment. After the pasteurization, the samples were incubated for 24 hours at two different pHs (5.0 and 6.0) and two different temperatures (37 °C and 50 °C) in the LB (Luria Bertani) broth. After the incubation, to obtain a single colony, samples were taken from the broth medium which was observed to have a growth at both temperature and pH conditions; then they were diluted at 10^{-6} and inoculated into the agar medium (LB-agar) using the spread method with glass rod, and finally left for growth for 24 hours (Özcanet al., 2011).

Determining the Enzyme Activities of the Isolates in Media

The β-1,4-glucanase, xylanase, α-amylase, and protease activities were examined in the isolates.

Determining the CMCase and Xylanase Activity

The isolates were individually planted in the petri dishes with the LB-agar containing 0.1% (w/v) substrate (CMC or xylan) and incubated under the appropriate temperature conditions until the next day. On the following day, the Congo red (0.1% w/v) dye solution enough to cover the colonies grown in the plates was poured and kept for 15 minutes. At the end of the 15-minute period, the dye solution was discarded; and this time, a salt solution (1M NaCl) was added to the colonies and they were treated with this solution for 15 minutes. At the end of this period, the salt solution was removed from the plates. The plates were examined on a negatoscope, and the enzyme activity of the colonies (β-1,4-glucanase or xylanase) with a yellowish zone around in the red-dyed medium was determined as positive (Özcan, 1992).

Determining the Amylase Activity in Bacteria

The isolates were individually planted in the petri dishes with the LB-agar containing 0.5% (w/v) starch and incubated under the appropriate temperature conditions until the next day. On the following day, the iodine crystals were crushed on the lid of the plates and the bacteria were inverted and placed on the plates. After a while, the α-amylase enzyme activity of the colonies with a white transparent zone around in the blue medium dyed by the iodine vapor was determined as positive (Özcan, 1992).

Determining the Protease Activity in Bacteria

The isolates were individually planted in the milk powder medium with sterile toothpicks and incubated until the next day under the appropriate temperature conditions.On the following day, the colonies with a transparent zone around in the white background were identified as protease positive isolates (Bron et al., 1999).

Mutagenesis

EtBr Mutagenesis

50-100 µL of the isolates produced in LB-broth overnight was planted on the agar plates using the spread method with sterile glass rods and allowed to dry for 15 minutes. At the end of this period, 3 µL of EtBr was added onto the middle of the petri dishes and allowed to dry for 5 minutes. After the drying, the plates were turned upside down and left in the incubator at the appropriate temperature until the next day. On the following day, the colonies that had a contact with EtBr were individually collected from the brink of the region where EtBr was dropped with sterile toothpicks and planted on a separate plate. The enzyme activities of these colonies were determined as described before. The mutant variants that preserved their β-1,4-glucanase activities but lost other enzyme activities were stocked for further studies.

UV Mutagenesis

The isolates grown in LB-broth overnight were transferred to the 10 ml sterile transparent tubes and exposed to UV ray at a wavelength of 260 nm for 30 minutes in a dark and icy environment. At the end of this period, the UV light-treated bacteria samples were planted in the LB-agar using the spread method with glass rod and allowed to grow in the incubators

set at 37 °C-50 °C. One day later, the colonies that grew on the medium were individually collected using sterile toothpicks and their enzyme activities were determined as described before. The variants that preserved their β-1,4-glucanase activities but lost other enzyme activities were stocked for further studies.

SDS-PAGE

SDS-PAGE analysis was carried out using 12% (w/v) gel in line with the protocol reported by Laemmli (1970). The proteins in SDS-PAGE gel were revealed by Coomassie blue staining.

Quantitative Analysis of Enzyme

The CMCase enzyme activities of the mutant strains and the wild strain were analyzed in terms of optimum temperature, temperature resistance, and optimum pH values; the production levels of the enzymes (CMCase) of these three strains were compared; and the effectiveness of this enzyme against some metal ions (EDTA, urea, $MgCl_2$, $CaCl_2$ KCl_2) was examined. In order to find the optimum temperature for the β-1,4-glucanase activity; it was reacted with the substrate in the water baths set at 30, 40, 50, 60, 70, and 80 °C. On the other hand, in order to measure the resistance of β-1,4-glucanase enzyme to temperature, the tubes containing only the enzyme were kept separately in the water baths set at 30, 40, 50, 60,70, 80, 90, 100°C for 15 minutes.In order to find the pH value at which β-1,4-glucanase works best; the experimental setup was prepared under different pH conditions such as 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0. The effects of some metal ions, that is, EDTA, urea, $MgCl_2$, $CaCl_2$, and KCl_2 on the β-1,4-glucanase were examined. To this end, the erlenmeyer flask inoculated with 100 ml of bacteria was incubated at 37°C for 48 hours. 10 ml of bacterial supernatant (obtained as a result of centrifugation at 4950 rpm) was added to the experimental setup in which there was 5 mM of EDTA, urea, $MgCl_2$, $CaCl_2$, and KCl_2 and the control tube.

Experimental Method

The bacterial strains were inoculated into 50 ml of LB medium in 500 ml erlenmeyer flask and incubated at 37°C for 24 hours, and then the bacteria were centrifuged at 4950 rpm for 10 minutes. The supernatant portions (the portions containing the enzymes) were transferred into sterile bottles and kept at +4°C until use. Reaction setup was as follows: 1 ml enzyme was mixed with 1 ml substrate and this mixture was prepared in 4 parallels; the enzyme control was prepared in 3 parallels with 1 ml enzyme and 1 ml sodium phosphate solution at pH 7.0; and the substrate (2% w/v carboxymethylcellulose) control was prepared in 3 parallels with 1 ml substrate and 1 ml sodium phosphate solution at pH 7.0. For the control purposes, the 2 ml tube containing only sodium phosphate was incubated with the others for 1 hour in a water bath set at 37°C. After the incubation, 3 ml of DNS solution was added to the tubes to stop the enzyme activity and they were incubated in boiling water for 5 minutes for the color reaction to take place. The samples left to cool were then transferred into the spectrophotometer cuvettes, and it was first reset with the control samples at OD_{540} nm and then the samples were read. The real level was computed by subtracting the arithmetic mean of the enzyme control and substrate control samples from the arithmetic mean of the enzyme substrate samples obtained (Özcan, 1998) (Miller, 1959).

RESULTS

Qualitative Enzyme Analysis of the Wild Strain of *Bacillus* sp.

The *Bacillus* sp. AZH (wild-type strain) isolate isolated at 37°C and pH 6.0 from the soil samples taken from Adana was selected for the mutagenesis studies. The β-1,4-glucanase, α-amylase, xylanase, and protease activities of this isolate were examined. For the β-1,4-glucanase and xylanase activity, the colonies were checked for forming a yellow zone around as a result of dying the plates with Congo red; for protease activity, they were checked for forming a transparent zone in the medium containing milk powder; and for the amylase activity, they were checked for a transparent zone formed by the isolate as a result of iodine staining. The data obtained are given in the Table 1 below.

Table 1. The activity statuses of some enzymes of *Bacillus* sp. AZH

Isolate	Enzyme Activities			
	β-1,4-glucanase (CMCase)	Xylanase	α-Amylase	protease
Bacillus sp. AZH	+	-	+	+

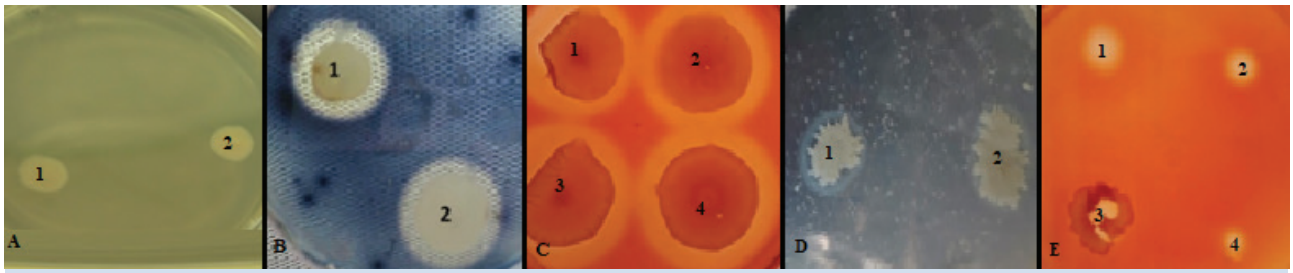


Figure 1. The Enzyme Activities of *Bacillus* sp. AZH Isolate. A) *Bacillus* sp.AZH strain B) The transparent zone formed by the α-amylase activity of the *Bacillus* sp. AZH Isolate in the LB-Starch-Agar medium. C) The activity zones formed by the β-1,4-glucanase (CMCase) activity of the *Bacillus* sp. AZH Isolate in the LB-CMC-Agar medium. D) The transparent zone formed by the protease activity of the *Bacillus* sp. AZH Isolate in the LB-Milk Powder-Agar medium. E) The *Bacillus* sp. AZH colonies that did not produce the xylanase activity zones in the LB-Xylan-Agar medium.

As can be seen in the Table 1, *Bacillus* sp AZH isolate was found to be positive in terms of β-1,4-glucanase, α-amylase, and protease activities, but negative in terms of xylanase activity (Figure 1).

The Mutagenesis Studies Carried out on *Bacillus* sp. AZH Isolate

As a result of the mutagenesis studies, *Bacillus* sp. AZH-mUVand *Bacillus* sp. AZH-mEB mutant variants were obtained and their β-1,4-glucanase, α-amylase, xylanase, and protease activities were examined. The results are given in the Table 2 and the Figure 2.

The Table 2 and Figure 3 give the β-1,4-glucanase, α-amylase, and protease activity statues of the *Bacillus* sp. AZH-mUV mutant variant exposed to UV light at a wavelength of 260 nm for 30 minutes in a dark and icy environment.

Table 2. The activity statuses of some enzymes of the *Bacillus* sp. AZH mutant variants created after the mutagenesis applications

Mutant Variants	Enzyme Activities			
	β-1,4-glucanase (CMCase)	Xylanase	α-Amylase	protease
Bacillus sp. AZH AZH-mEB	+	-	-	+
Bacillus sp. AZH-mUV	+	-	-	+

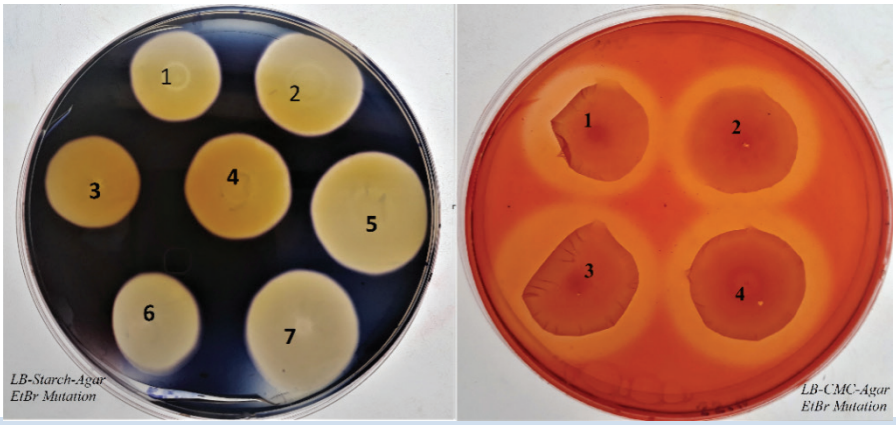


Figure 2. α-amylase (negative) and β-1,4-glucanase (positive) activity zones formed by the *Bacillus*sp.AZH-mEB mutant variant in the LB-Starch-Agar and LB-CMC-Agar media

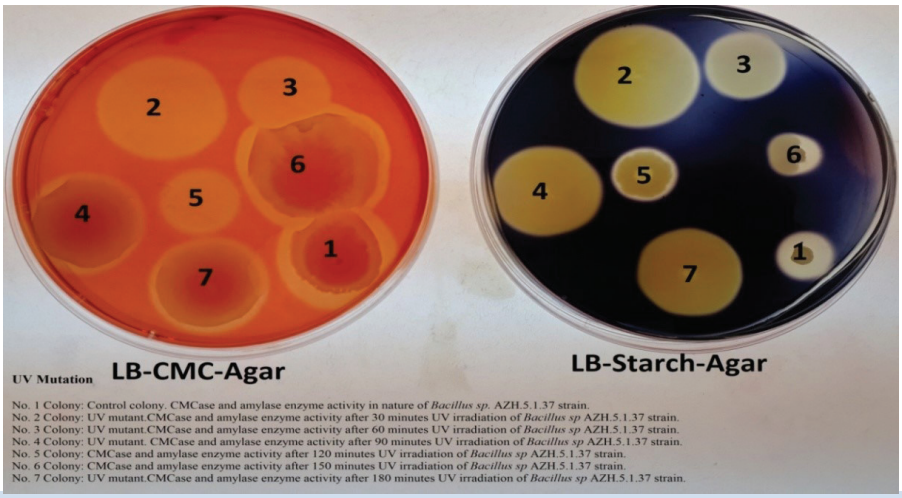


Figure 3. A-amylase (negative) and β-1,4-glucanase (positive) activity zones formed by the *Bacillus* sp.AZH-mEB mutant variant in the LB-Starch-Agar and LB-CMC-Agar media

SDS-PAGE and Zimogram Analysis Results of the *Bacillus* sp. AZH Isolate and Its Mutant Variants

According to the data obtained from the SDS-PAGE analysis, *Bacillus* sp. AZH and its mutant variants (*Bacillus* sp. AZH-mEB, *Bacillus* sp. AZH-mUV) were compared and it was found that the total band profiles were compatible with each other. On the other hand, the activity bands responsible for the β-1,4-glucanase enzyme of all the three bacteria were compared and the molecular weight of the β-1,4-glucanase enzyme for all the three bacteria was found to be about 40 kDa (Figure 4).

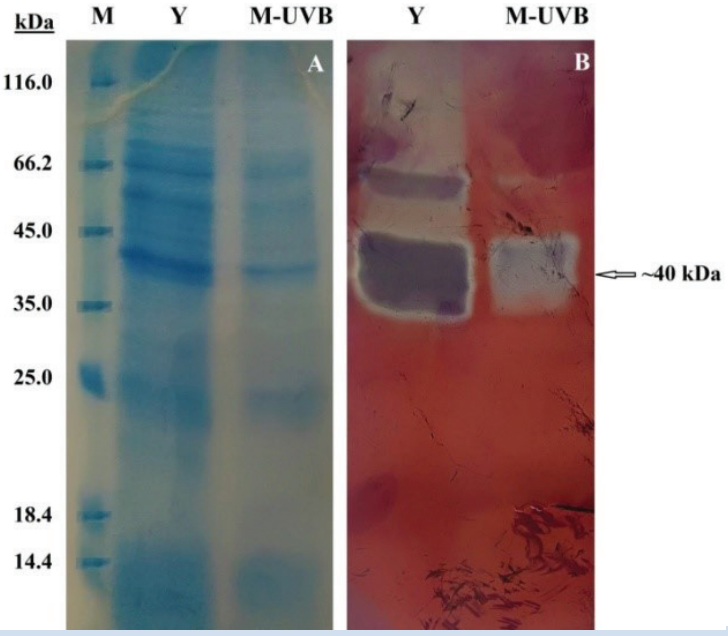
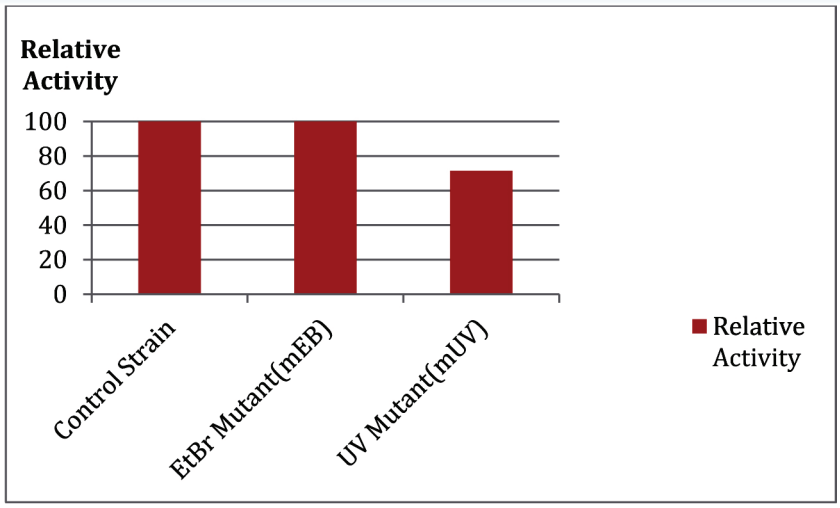


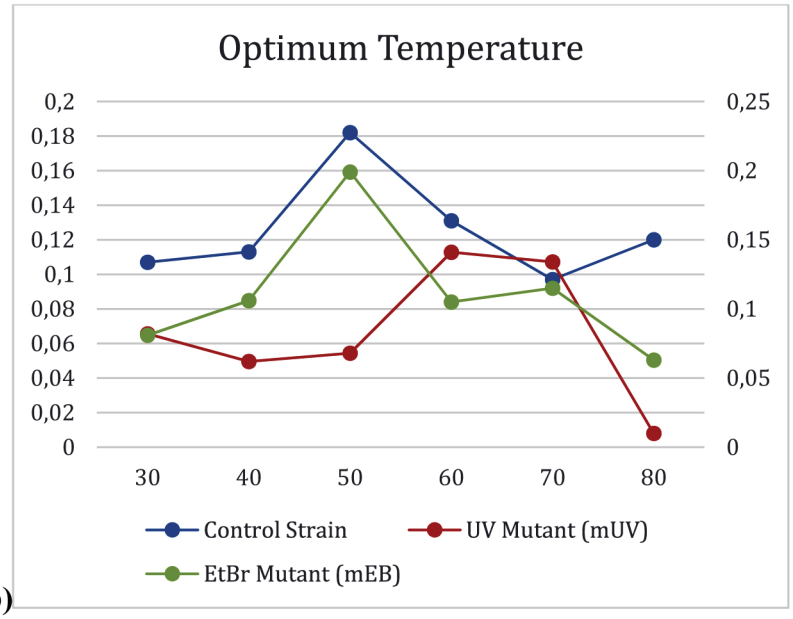
Figure 4. (A) Comparison of the total proteins of the wild and mutant variants in SDS-PAGE, (B) Comparison of their β-1,4-glucanase enzyme activities in SDS-CMC-PAGE (M: Marker, Y: *Bacillus* sp. AZH, M-UVB : *Bacillus* sp. AZH-mUV)

The β-1,4-glucanase Enzyme Analysis Results for the *Bacillus* sp. AZH and Its Mutant Variants

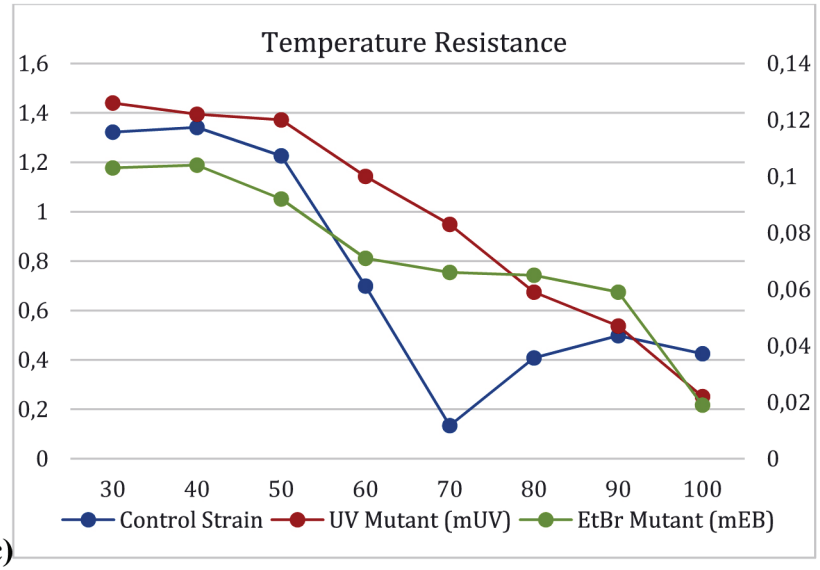
Relative activities, optimum temperature values, resistance to the temperature at different temperature values of the enzyme, optimum pH values, and effect of metal ions (EDTA, urea, $MgCl_2$, $CaCl_2$, and KCl_2) on the CMCase activities of Bacillus sp. AZH, Bacillus sp. AZH-mEB ve Bacillus sp. AZH-mUV mutant varieties were investigated. The results are given in Figure5a, Figure5b, Figure5c, Figure5d and Figure5e respectively.


Relative Activity
100
80
60
40
20
0
Control Strain
EtBr Mutant(mEB)
UV Mutant(mUV)
Relative Activity

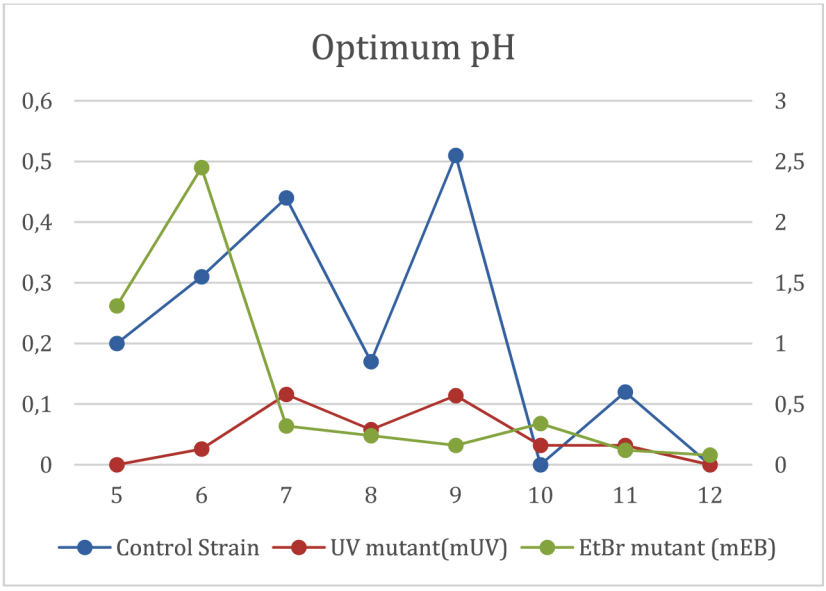
a)


Optimum Temperature
0,2
0,18
0,16
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
0,25
0,2
0,15
0,1
0,05
0
30
40
50
60
70
80
Control Strain
UV Mutant (mUV)
EtBr Mutant (mEB)

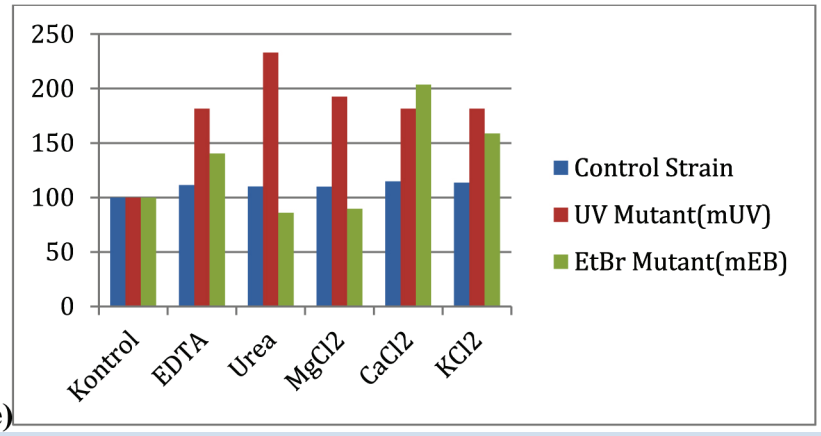
b)


Temperature Resistance
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
30
40
50
60
70
80
90
100
Control Strain
UV Mutant (mUV)
EtBr Mutant (mEB)

c)



d)



e)

Figure 5. a) β-1,4-glucanase relative activity values, b) Optimum temperature graph of the β-1,4-glucanase enzyme, c) Temperature resistance graph of β-1,4-glucanase enzyme at 30, 40, 50, 60, 70, 80, 90, and 100 °C, d) Optimum pH graph of β-1,4-glucanase enzyme, e) Effect of metal ions (EDTA, urea, $MgCl_2$, $CaCl_2$, and KCl_2) on β-1,4-glucanase for the *Bacillus* sp. AZH (Control Strain) and its mutant variants, that is, *Bacillus* sp. AZH-mEB (EtBr Mutant) and *Bacillus* sp. AZH-mUV (UV Mutant)

DISCUSSION

It is important to improve the use of bacterial cultures that are becoming more common. In general, applications are carried out using the bacteria providing lactic acid production (*Lactobacillus, Pedicoccus and Enterococcus*) (Tengerdy et al., 1991). It is possible to classify the biological silage additives under two categories, that is, lactic acid bacteria (LAB) and enzymes. The most prominent role of enzymes is that they can hydrolyze the non-fermented polysaccharides. Since the LAB additives have a proteolytic activity, the increase in the amount of lactic acid produced affects the silage quality positively and increases the feed-usability. Furthermore, the presence of these biological additives in silage reduces the dry matter loss. Cellulases, one of the enzymes applicable in silage, provide the hydrolysis of structural polysaccharides and can increase the amount of carbohydrates that can be used in fermentation. It was observed that the controlled use of these two biological factors both increased the quality of silage fermentation and improved the consumability of silage, that is, its taste (Woolford, 1999). On the other hand, it was also observed that the bacterial inoculants not only had positive effects on silage, but also promoted the increase of microbial flora in the ruminants of silage-fed animals. It was reported that the bacterial inoculants provided an increase in live weight gain and milk yield in trace amount (Weinberg and Muck, 1996). Stokes (1992) observed that the enzymes hydrolyzing the cell wall of the plants reduced the NDF (Non-Protein Nitrogenous Compounds) content to 15% in the silages ob-

tained from a mixture of alfalfa and poaceae. It was also reported that, in practice, it increased the daily dry matter consumption in dairy cows by 10% in the total daily ration. The effect of this on the daily milk yield was found to be 650 g more per animal. Bolsen and Heidker (1985) and Chen et al. (1994) asserted that the lactic acid bacteria (LAB) inoculants could be used as a silage additive in the form of a mixture with enzymes. They also reported that the use of LAB together with the cell wall-degrading enzymes such as cellulase, hemicellulase, and pectinase and the enzymes capable of hydrolyzing starch, such as amylase positively affected the silage fermentation by producing additional substrate product, lowered the cell wall content, improved the digestion of dry matter and organic matter, increased the hydrolysis of the carbohydrates insoluble in acid solvents (ADF) and the carbohydrates insoluble in neutral solvents (NDF), but did not affect the aerobic resistance.

In the present study, β-1,4-glucanase (CMCase) producing bacteria were isolated from the soil samples collected from various parts of Adana province, and the β-1,4-glucanase enzyme was partially characterized by partial purification. The isolates were wild-type, aerobic and spore-forming. Due to this reason, the isolates were identified as members of the Bacillus genus or various similar aerobic endospore-forming bacteria because they germinated from spore forms under aerobic conditions (Remize, 2017). However, to determine the genus and species of the isolates accurately, they need to be confirmed with the API 50 CHB test, which includes 50 different carbon sources (Wind et al, 1994) and 16S rDNA sequencing. Mawadza et al. (2000) characterized the cellulase enzyme produced from two *Bacillus* sp. strains. They found that the molecular weight of the two enzymes was 40 kDa and they showed their optimum activity at a temperature range of 45-70 °C and a pH range of 5.0-6.5. Hakamada et al. (2002) isolated the alkaline endoglucanase enzyme from *Bacillus circulan* and found that the optimum temperature and pH values were 55°C and 8.5, respectively; and the molecular weight of the enzyme was 43 kDa. Singh et al. (2004) isolated the alkaline cellulase enzyme from *B. sphaericus* JS1 strain. They found the molecular weight of the enzyme as 42 kDa and reported that the optimum temperature and pH values were within the range of 60-70 °C and 8.0-10.0, respectively. In the present study, according to the data obtained from the SDS-PAGE analysis, the activity bands responsible for the β-1,4-glucanase enzyme of *Bacillus* sp. AZH and its mutant variants (*Bacillus* sp. AZH-mEB and *Bacillus* sp. AZH-mUV) were compared and it was found that the molecular weight of the enzyme in all the bacteria was about 40 kDa (Figure 4). It was observed that the optimum temperature was 50 °C for the *Bacillus* sp. AZH and the *Bacillus* sp. AZH-mEB, and 60 °C for the *Bacillus* sp. AZH-mUV (Figure 5-b). On the other hand, the optimum pH was found to be 9.0 for *Bacillus* sp. AZH, 6.0 for *Bacillus* sp. AZH-mEB, and 7.0 for *Bacillus* sp. AZH-mUV (Figure 5-d).

The mutagenesis studies carried out using the chemical or physical mutagens have been widely applied both to increase the enzyme activity and to inhibit some undesired enzyme activities. Özcan (1996) inactivated the positive α-amylase activity of the *Bacillus subtilis* ORBA (1) strain by treating it with ethidium bromide (EtBr) which caused a frameshift. Bilgilisoy (2003) treated the *Bacillus* sp. RSKK244 and RSKK246 strains with EMS (ethyl methyl sulfonate) and EtBr to change their enzyme production and obtained novel mutant strains. As a result of the treatment with EMS, the enzyme activities of 244M1, 244M2, and 246M3 strains were as follows: lichenase$^-$, xylanase$^-$, β-1,4-glucanase$^-$, α-amylase$^+$; lichenase$^-$, xylanase$^-$, β-1,4-glucanase$^-$, α-amylase$^+$; and lichenase$^+$, xylanase$^+$, β-1,4-glucanase$^-$, α-amylase$^+$, respectively. Mohamed et al. (2011) exposed the isolates obtained from the *Streptomyces pseudogriseolus* strain isolated from soil to the UV radiation at different times and obtained 139 mutant strains. Park et al. (2017) isolated the cellulase-producing bacteria from salted clam and aimed to increase their enzyme production by treating them with physical and chemical agents. In the mutagenesis (UV radiation and chemical mutagens) they carried out, they obtained the P11 mutant strain as a result of the EMS application. They compared the wild and mutant strains; and as a result, they found that the endoglucanase production increased in the mutant strain. In the present study, it was attempted to obtain an enzyme that can adapt to the pH and temperature conditions of silage. To this end, the *Bacillus* sp. AZH isolate (Figure 1) having a β-1,4-glucanase enzyme activity developing at 37°C and pH 6.0 was exposed to a chemical mutagen (EtBr) and UV radiation and its enzymes activities such as amylase, protease, xylanase were terminated; and it was attempted to create only the mutant strains producing β-1,4-glucanase enzyme (Figures 3 and 4). It was also aimed to increase the β-1,4-glucanase enzyme production in these mutant variants; and as a

result, only the *Bacillus* sp. AZH-mEB and *Bacillus* sp. AZH-mUV mutant strains, the amylase activities of which were terminated, were obtained.

Based on these data, according to the optimum temperature analysis of the β-1,4-glucanase enzyme of the three strains, it can be thought that the enzyme is moderately thermophilic. When the data obtained from the mutation studies for the optimum pH were examined, it was observed that it shifted slightly from alkaline to acidic. Although $MgCl_2$ and Urea increased the activity in the β-1,4-glucanase enzyme of the wild strain under normal conditions (Urea 10.1%, $MgCl_2$ 10%); they doubled the activity in the β-1,4-glucanase enzyme of the *Bacillus* sp. AZH-mUV mutant strain (Urea 123%, $MgCl_2$ 92.6%), and reduced the activity in the β-1,4-glucanase enzyme of *Bacillus* sp. AZH-mEB mutant strain (Urea 14%; $MgCl_2$ 10.0%). We are of the opinion that this stems from the mutagenesis method used (Figure 5-e).

In the study, after an incubation at 37 ℃ for half an hour, the β-1,4-glucanase enzyme production levels of the *Bacillus* sp. AZH and its mutant variants (*Bacillus* sp. AZH-mEB and *Bacillus* sp. AZH-mUV) were compared and their relative activities were found to be 100%, 100%, and 71.5%, respectively (Figure 5-a). The resistance to temperature was found to be 40℃ for the *Bacillus* sp. AZH, 40℃ for the *Bacillus* sp. AZH-mEB, and 30℃ for the *Bacillus* sp. AZH-mUV (Figure 5-c).

CONCLUSION

The silage fermentation taking place at low thermal values is an important indicator that affects the quality of silage. The temperature increase in the silage (rising to 35-40℃) may indicate that the fermentation is not going well. And this causes an energy and dry matter loss in the silage. pH, another indicator affecting the quality, is expected to be within the range of 3.7-4.2 in a quality silage (Kung and Shaver, 2001). If the β-1,4-glucanase enzyme of the *Bacillus* sp. AZH-mEB and *Bacillus* sp. AZH-mUV mutant variants, which are intended to be used as additives in silage, are used in line with the abovementioned optimum temperature or pH values; against the possibility of temperature or pH increase that may occur in silage fermentation, it can be an enzyme that will show activity at these values and prevent the dry matter loss by breaking down cellulose. However, it is predicted that the β-1,4-glucanase enzyme of the *Bacillus* sp. AZH and its mutant variants (*Bacillus* sp. AZH-mEB and *Bacillus* sp. AZH-mUV) will have some difficulties in adapting to the silage conditions, and thus will have an indirect effect on silage.

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CONFLICT OF INTEREST

None declared.

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