Kafkas Univ Vet Fak Derg
19 (4): 575-581, 2013
DOI: 10.9775/kvfd.2012.8400

Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

RESEARCH ARTICLE

Cloning and Expression of *Cellulosimicrobium cellulans* β-1,3-Glucanase Gene in *Lactobacillus plantarum* to Create New Silage Inoculant for Aerobic Stability [1][2]

Bahri Devrim ÖZCAN [1] Numan ÖZCAN [2] Makbule BAYLAN [3] Ali İrfan GÜZEL [4]

[1] *This study was supported by The Scientific and Technological Research Council of Turkey (TUBITAK), Project No: 108T919*
[2] *This work is carried out in the Animal Biotechnology and Genetic Laboratory, Department of Animal Science, Faculty of Agriculture, Çukurova University*

[1] Osmaniye Korkut Ata University, Faculty of Arts and Sciences, Department of Biology, TR-80000 Osmaniye - TURKEY
[2] Çukurova University, Faculty of Agriculture, Department of Animal Science, TR-01330 Adana - TURKEY
[3] Çukurova University, Faculty of Fisheries, Department of Basic Sciences, TR-01330 Adana - TURKEY
[4] Recep Tayyip Erdoğan University, Faculty of Medicine, Department of Basic Medical Sciences, TR-53100 Rize - TURKEY

Makale Kodu (Article Code): KVFD-2012-8400

Summary

In this study, the recombinant plasmid pTE353-βG was created by inserting the p353-2 cryptic plasmid region of pLP3537 into pTEG5 recombinant plasmid contains pUC18 and β-1,3-glucanase gene of *Cellulosimicrobium cellulans*. The recombinant plasmid pTE353-βG was then introduced into *Lactobacillus plantarum* by electroporation. Insert analysis of pTE353-βG digested with *Sac*I produced 1.9 kbp β-1,3-glucanase gene band on agarose gel as well as 1.9 kbp DNA encoding β-1,3-glucanase gene insert amplified on the recombinant vector via PCR indicated the integration of the gene into the plasmid. Recombinant *L. plantarum* colonies with pTE353-βG on MRS-laminarin-agar plate showed clear positive zones by Congo-red staining that revealed the expression of β-1,3-glucanase encoding gene. The β-1,3-glucanase enzyme of recombinant strain produced the same activity band with *C. cellulans* enzyme in terms of molecular weight, which showed the activity of secreted protein without any proteolytic degradation. Optimal temperature and pH values of *L. plantarum* β-1,3-glucanase have been determined 40°C and 6.0 respectively, by enzymatic analysis. These results revealed that recombinant *L. plantarum* could be considered as a silage inoculant for aerobic spoilage of silage.

Keywords: *Cellulosimicrobium cellulans, β-1,3-Glucanase, Lactobacillus plantarum, Cloning, Gene expression, Recombinant silage inoculant*

Aerobik Stabilite için Yeni Silaj İnokülantı Oluşturmak Amacıyla *Cellulosimicrobium cellulans* β-1,3-Glukanaz Geninin *Lactobacillus plantarum*'da Klonlanması ve Ekspresyonu

Özet

Bu çalışmada, rekombinant pTEG5 plazmit DNA'sına (pUC18 + *Cellulosimicrobium cellulans*'ın β-1,3-glukanaz geni) pLP3537 plazmitinin p353-2 kriptik plazmit bölgesinin aktarılması ile rekombinant pTE353-βG plazmit DNA'sı oluşturulmuştur. Rekombinant pTE353-βG plazmiti *Lactobacillus plantarum*'a elektroporasyon tekniği ile transfer edilmiştir. *Sac*I enzimi ile kesilmiş pTE353-βG'nin agaroz jelde 1.9 kbç büyüklüğünde gen bandı üretmesinin yanı sıra, β-1,3-glukanaz genini şifreleyen 1.9 kbç büyüklüğündeki DNA parçasının PCR ile rekombinant vektörden amplifiye edilmesi genin plazmite entegrasyonunu göstermektedir. pTE353-βG içeren rekombinant *L. plantarum* kolonileri MRS-laminarin-agar plağında Congo-red boyaması ile β-1,3-glukanaz geninin eksprese olduğunu gösteren açık renkli pozitif zon vermişlerdir. Rekombinant suş tarafından üretilen enzim, *C. cellulans* enzimi ile aynı moleküler ağırlıkta aktivite bandı üretmiş ve böylece enzimin herhangi bir proteolitik parçalanmaya maruz kalmadığı anlaşılmıştır. Enzimatik analizler sonucunda *L. plantarum* tarafından üretilen β-1,3-glukanaz enziminin optimum sıcaklık ve pH değerleri sırasıyla 40°C ve 6.0 olarak bulunmuştur. Bu sonuçlar, rekombinant *L. plantarum*'un silajın aerobik bozulmasını önlemek için inokülant olarak düşünülebileceğini ortaya çıkarmıştır.

Anahtar sözcükler: *Cellulosimicrobium cellulans, β-1,3-Glukanaz, Lactobacillus plantarum, Klonlama, Gen ifadesi, Rekombinant silaj inokülantı*

İletişim (Correspondence)
+90 328 8271000/2560
devrimozcan@osmaniye.edu.tr

INTRODUCTION

Endo-1,3-β-glucanases (EC 3.2.1.6 and EC 3.2.1.39) are widely distributed among bacteria and higher plants [1]. These enzymes catalyse the hydrolysis of β-1,3-glucan component found in the yeast cell wall and other β-1,3-glucans such as laminarin, curdlan and pachyman [2,3]. The bacterium *Cellulosimicrobium cellulans* (also known with the synonyms *Cellulomonas cellulans*, *Oerskovia xanthineolytica*, and *Arthrobacter luteus)* has been regarded as a major source of yeast-lytic enzymes, particularly endo-β-1,3-glucanases, proteases and mannanases [4]. Commercially available yeast-lytic glucanase preparations derived from this organism, namely Lyticase, Zymolyase, and Quantazyme, have been produced and widely used for yeast protoplast preparation and yeast DNA isolation [4-6]. Only one of these preparations (Quantazyme, Quantum Biotechnology, Canada) is produced recombinantly and protease-free [4,5].

The primary goal of making silage is to maximize the preservation of original nutrients in the forage crop for feeding at a later date [7]. Therefore, ensiled forages are the most commonly used feeds for ruminants all over the world [8]. *Lactobacillus plantarum* and other *Lactobacillus* species, *Enterococcus faecium* and *Pediococcus* species are most common silage inoculant bacteria and one or more of these bacteria to be included in silage inoculants [7,9]. One of these bacteria, *L. plantarum*, is the most important bacterium used in silage fermentation. However, aerobic spoilage by yeasts and moulds is a major cause of reduced nutritional value of silage and increases the risk of potential pathogenic microorganisms [10]. Various chemical additives with anti-fungal properties, such as propionic acid, sorbate, benzoate, acetic acid, ammonia, urea, some enzyme preparations and *Lactobacillus buchneri* as silage inoculant have been used to prevent or enhance of aerobic stability and decrease of spoilage [11]. However, usage of most of these additives has been restricted because of their other undesirable properties. For instance, propionic acid is difficult to handle because of its corrosive nature as well as at a pH of 4.8, about 50% of the acid being undissociated [7,11], sorbate, benzoate, and acetic acid being too costly [11]. Besides, *L. buchneri* as silage inoculant causes dry matter losses in silage material at early fermentation phase [12].

In the present study, we aimed to express the β-1,3-glucanase gene of *C. cellulans* in *L. plantarum* to create a recombinant silage inoculant to enhance the aerobic stability and decrease the spoilage by secreting the β-1,3-glucanase enzyme by *L. plantarum*.

MATERIAL and METHODS

Strains of Bacteria and Growth Conditions

Lactobacillus plantarum strain 5057 was cultured on MRS broth (Merck, 1.10661) and MRS agar (Merck, 1.10660) at 37ºC without shaking. For culturing of recombinant *L. plantarum*, both MRS-broth and MRS-agar supplemented with ampicillin (50 µg mL^{-1}). *Cellulosimicrobium cellulans* (*Oerskovia xanthineolytica*, ATCC 21606) was cultured in GYM Streptomyces medium (glucose (0.4% wt/v), yeast extract (0.4% wt/v), malt extract (1% wt/v), pH 7.2) at 28ºC with shaking at 250 rpm. Agar (1.2% wt/v) and $CaCO_3$ (0.2% wt/v) were added into GYM Streptomyces medium for preparation of GYM Streptomyces agar. Recombinant *Escherichia coli* carrying recombinant pTEG5 plasmid DNA was cultured in LB-broth (10 g bacto tryptone (Merck), 5 g yeast extract (Merck) and 10 g NaCl (Merck) per L, pH 7.5) at 37ºC with shaking at 250 rpm. Agar (1.5% wt/v) was added into LB medium for LB-agar. Both LB-broth and LB-agar were supplemented with ampicillin (50 µg mL^{-1}). For activity testing of recombinant *L. plantarum* on MRS-laminarin-agar plate, the plate was stained with Congo-red solution (0.1% wt/v Congo-red) for 15 min and then destained with 1 M NaCl solution for 15 min. Clear haloes on the MRS-laminarin-agar plate indicated the presence of β-1,3-glucanase activity [13].

Plasmids

Recombinant vector pTEG5 was previously created in the Animal Biotechnology and Genetic Engineering Laboratory, Department of Animal Science, Faculty of Agriculture, Çukurova University [14]. The recombinant vector pTE353-βG (pUC18 + β-1,3-glucanase gene + p353-2 cryptic plasmid) was created using *Escherichia coli*/ *Lactobacillus* shuttle vector pLP3537 (ampicillin resistant and erythromycin resistant; amp^R, er^R) [15] and pTEG5. pTE353-βG was used for *L. plantarum* transformation [16]. Recombinant *L. plantarum* strain carrying the recombinant plasmid pTE353-βG, was cultured in MRS broth and on MRS agar at 37°C supplemented with ampicillin (50 µg mL^{-1}). Recombinant plasmid pTEG5 was isolated from *E. coli* cells as described previously [17]. On the other hand, pTE353-βG was isolated from recombinant *L. plantarum* cells as described previously [18].

DNA Modification

The following modifying enzymes were purchased and used for DNA modifications; *Sac*I, *Sma*I, *Eco*RI, *Hind*III, and bacterial alkaline phosphatase, bacteriophage T4 DNA ligase as well as Pfu DNA polymerase (Fermentas, Vivantis and Promega Corporation). Restriction enzyme reactions were monitored by examining digestion by agarose gel electrophoresis using standard methods [19]. Linearised plasmid DNA and PCR products were excised from gels and purified using Genomic DNA Purification Kit (Fermentas).

Cloning Procedures

p353-2 cryptic plasmid region (2.4 kbp) was derived from pLP3537 vector by digestion with *Eco*RI and then ligated to *Eco*RI digested pTEG5 vector to construct the pTE353-βG recombinant plasmid.

The pTE353-βG was electrotransformed into *Lactobacillus plantarum* 5057 strain using the modified method [16] as follows: For electroporation, 50 µL of the competent *L. plantarum* cells were mixed with 1 µg of plasmid DNA and transferred to a prechilled electroporation cuvette (1 mm gap). After incubation for 2-3 min, the cells were exposed to a single electrical pulse using a Gene-Pulser (Invitrogen) set at 25 µF, 200 Ω, 1300 V (13 kV cm^{-1}) and 25 mA resulting in time constant of 5 ms. After electroporation, electrotransformed *L. plantarum* cells were diluted with 500 µL MRS broth and incubated at 32°C for 4 h. Finally the cells were plated on MRS-agar containing ampicillin (50 µg mL^{-1}) and incubated at 37°C for 48 h.

PCR and Restriction Endonuclease Analysis

The sequences of the primers used for amplifying of β-1,3-glucanase gene from the recombinant vector were 5'-AGAGCTCGTGGCACTGCACTCGTTCGAGTCT-3' (forward) and 5'-AGAGCTCGACGGGCGCGGTCAGAGCGTCCAG-3' (reverse) based on the gene sequence [20]. The PCR mixture consisted of 5 µL of reaction buffer, 1 µL of 40 mM dNTP mix (200 µM each final), 1 µL each of forward and reverse primers (20 pmol each primer), 0.5 µL of Pfu DNA polymerase (2.5 U/µL), 1 µL of 50% wt/v DMSO (1% wt/v final), and 210 ng of template in a total volume of 50 µL. The following amplification program was used: Initial denaturation step at 94°C for 2 min, then 30 cycles of denaturation at 98°C for 10 s, annealing and elongation at 68°C for 5 min. A final extension step was performed as 72°C for 5 min. PCR reaction performed with recombinant plasmid analysed by agarose gel (0.8% wt/v) electrophoresis.

For restriction endonuclease analysis, recombinant plasmid pTE353-βG was isolated from *L. plantarum* transformant. To detect the β-1,3-glucanase gene, recombinant plasmid was cleaved with *Sac*I+*Eco*RI restriction endonuclease mixture and analysed by agarose gel (0.8% wt/v) electrophoresis. Furthermore, pTE353-βG was digested by *Hind*III to obtain a single linearized band.

Electrophoretic Analysis of Extracellular Proteins

To obtain the extracellular proteins of *C. cellulans* and *L. plantarum* strains from culture supernatants, the overnight grown cells were pelleted by centrifuge. The extracellular extracts (supernatants) were mixed with 1:1 volume of 20% TCA for precipitation. After the incubation at room temperature for overnight, protein pellets were obtained by centrifuge. Air-dried proteins were dissolved in 0.1 M Tris-HCL buffer (pH 8.0).

SDS-PAGE and SDS-Laminarin-PAGE (0.2% laminarin) were done as described previously [21] with slab gels (12% wt/v acrylamide). After the electrophoresis, the gel was stained for 1 h with Coomassie blue R 250 dye in methanol-acetic acit-water solution (4:1:5 by volume) and destained in the same solution without dye [22,23]. For activity staining (zymogram analysis), SDS was removed by washing the gel at room temperature in solutions containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), isopropanol 20% v/v for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. Renaturation of enzyme proteins was carried out by keeping the gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol and 1 mM EDTA at 4°C. The gel was then transferred onto a glass plate, sealed with film, and incubated at 30°C for 4 h. The gel was stained in a solution of Congo-red (0.1% Congo-red, 0.2 M NaOH), for 1 h, and destained in 1 M NaCl for 30 min. Clear bands indicated the presence of β-1,3-glucanase activity [24-26].

Enzyme Assay

β-1,3-Glucanase activity was assayed by adding 1 mL enzyme to 1 mL laminarin (2%, wt/v) in 0.1 M Na-phosphate buffer, pH 6.0 and incubating at 40°C for 30 min. The reaction was stopped by addition of 3 mL of 3,5-dinitrosalicylic acid reagent and $A_{540\ nm}$ was measured in a Pharmacia spectrophotometer [27].

Temperature and pH effects on enzyme activity were assayed at different temperatures ranging from 20 to 100°C and at pH values ranging from 4 to 11 for 30 min. Following buffers were used in the reactions: 50 mM Na-acetate (pH 4-6), 50 mM Na-phosphate (pH 6-8) and 50 mM Tris (pH 8-11) [28]. Enzyme assay carried out as described previously.

For the measurement of thermal stability, the enzyme was pre-incubated at temperatures between 20 to 100°C for 30 min at optimum pH. The enzyme activity was determined under standard enzyme assay condition. Enzyme assay carried out as described previously.

Supernatants of recombinant *L. plantarum* were taken at predetermined time intervals (0 min, 12, 24, 36, 48, 60, 72 h) of culturing period. Enzyme assay of supernatants carried out as described previously and enzyme production depending on culturing period determined.

RESULTS

Transformation of Lactobacillus plantarum

The first generation recombinant vector pTEG5 (pUC18 plus β-1,3-glucanase gene of *C. cellulans*) was created previously [14]. In present study, the 2.4 kbp *Eco*RI-*Eco*RI p353-2 cryptic plasmid region including replication origin of *L. plantarum* was derived from the pLP3537 and ligated to pTEG5 to create the second generation recombinant plasmid pTE353-βG *(Fig. 1)*.

The pTE353-βG recombinant plasmid was then electrotransformed into *L. plantarum* to express the β-1,3-glucanase gene. Recombinant *L. plantarum* colonies showed β-1,3-glucanase activity on MRS-agar plate supplemented with ampicillin (50 µg mL^{-1}) and laminarin (0.1% wt/v) with clear

zones around of the recombinant colonies *(Fig. 2)*.

While original *L. plantarum* with no ampicillin resistance gene did not grow on solid growth medium contain ampicillin, recombinant *L. plantarum*/pTE353-βG bearing *Amp* resistance gene on the vector grew well on the same medium (data not shown).

Recombinant pTE353-βG plasmid was isolated from recombinant *L. plantarum* strain. It was then subjected to restriction fragment length analysis together with PCR amplified DNA encoding the gene on agarose gel electrophoresis (0.8% wt/v). β-1,3-glucanase gene fragment (~1.9 kbp) amplified by PCR and restriction endonuclease digested recombinant plasmid confirmed the success of the cloning experiments. Recombinant plasmid digested with *Sac*I+*Eco*RI was yielded the same DNA fragment consisting of pUC18, p353-2 and β-1,3-glucanase gene *(Fig. 3)*.

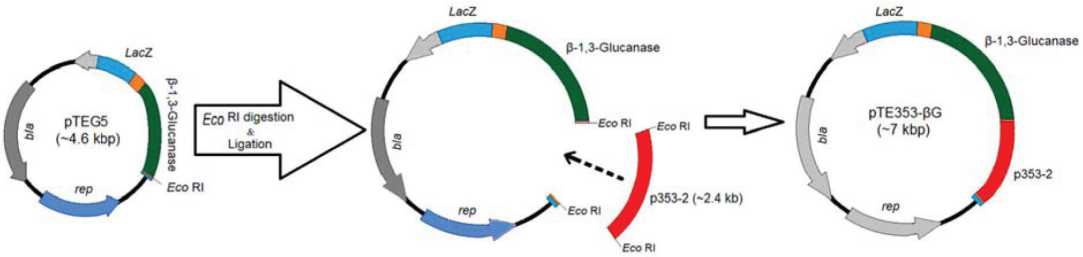

Fig 1. Construction of pTE353-βG plasmid (~7 kbp) by ligating p353-2 cryptic plasmid region including replication origin of *L. plantarum* (~2.4 kbp) into pTEG5 (~4.6 kbp)

Şekil 1. *L. plantarum* replikasyon orijinini içeren p353-2 kriptik plazmit bölgesinin (~2.4 kbç) pTEG5'e (~4.6 kbç) ligasyonu ile pTE353-βG plazmitinin (~7 kbç) oluşturulması

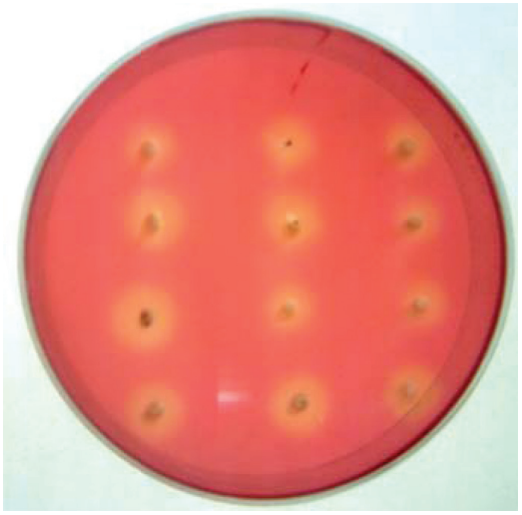

Fig 2. Recombinant *L. plantarum* colonies showing clear β-1,3-glucanase activity on MRS-agar plate with Congo red staining

Şekil 2. MRS-agar plağında Congo-red boyaması ile β-1,3-glukanaz aktivitesi gösteren rekombinant *L. plantarum* kolonileri

Culture supernatants of *C. cellulans*, *L. plantarum*/pTE353-βG, and *L. plantarum* were applied to SDS-PAGE and SDS-Laminarin-PAGE to visualize total proteins and zymogram analysis, respectively. For zymogram analysis, denaturated proteins were renaturated on SDS-Laminarin-PAGE after removing denaturating agents from the gel and then allowing to the enzyme to digest substrate, thereby producing clear zones on the gel. On zymogram analysis, only β-1,3-glucanase protein band of *C. cellulans* with 54.5 kDa in size was showed a clear zone together with extracellular protein counterpart of other recombinant *L. plantarum* strain *(Fig. 4)*. It is clearly indicated that β-1,3-glucanase gene of *C. cellulans* successfully expressed in *L. plantarum* without any significant proteolytic degradation.

Enzyme Properties

The optimum activity of the enzyme isolated from recombinant *L. plantarum* was observed at 40ºC. The mean enzyme activities were 91, 86 and 62% at 30, 50 and 60ºC

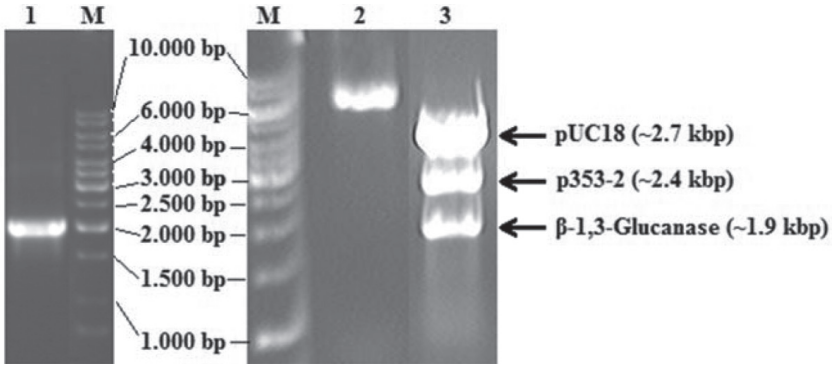

Fig 3. Restriction endonuclease and PCR analysis of pTE353-βG plasmid on agarose gel (M: 1 kbp DNA marker, 1: PCR amplified fragment of β-1,3-glucanase gene from pTE353-βG, 2: pTE353-βG/*Hind*III, 3: pTE353-βG/*Sac*I+*Eco*RI)

Şekil 3. pTE353-βG plazmitinin agaroz jelde restriksiyon endonükleaz ve PCR analizleri (M: 1 kbç DNA markır, 1: pTE353-βG'dan PCR ile amplifiye edilmiş β-1,3-glukanaz gen fragmenti, 2: pTE353-βG/*Hind*III, 3: pTE353-βG/*Sac*I+*Eco*RI)

respectively, whereas only 23% activity was retained after incubation at 70ºC for 30 min *(Fig. 5A)*. The enzyme also showed a significant relative activity (80.6%) between pH 5.0 and 7.0 with an optimum pH of 6.0 *(Fig. 5B)*. For thermal stability estimation, the retaining activity was determined at optimum pH and temperature *(Fig. 5C)*. The retained original enzyme activity obtained from 20 to 60ºC was 94% for 15 min. The enzyme was stable for 15 min between at 20 and 40ºC, while at 50, 60, 70 and 80ºC, 9, 22, 71 and 87% of the original activities were lost, respectively.

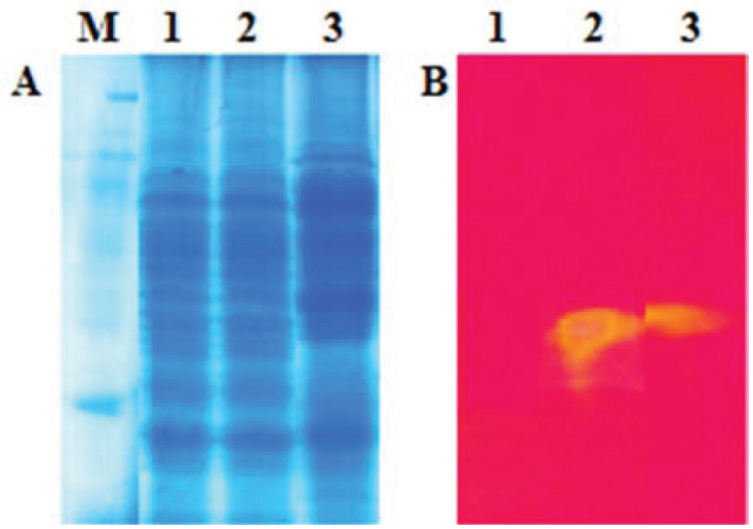

Fig 4. SDS-PAGE (A) and SDS-Laminarin-PAGE (B) analysis of recombinant and non-recombinant bacterial proteins (M: Marker, 1: *L. plantarum*, 2: *L. plantarum*/pTE353-βG, 3: *C. cellulans*)

Şekil 4. Rekombinant ve rekombinant olmayan bakteriyel proteinlerin SDS-PAGE (A) ve SDS-Laminarin-PAGE (B) analizleri (M: Markır, 1: *L. plantarum*, 2: *L. plantarum*/pTE353-βG, 3: *C. cellulans*)

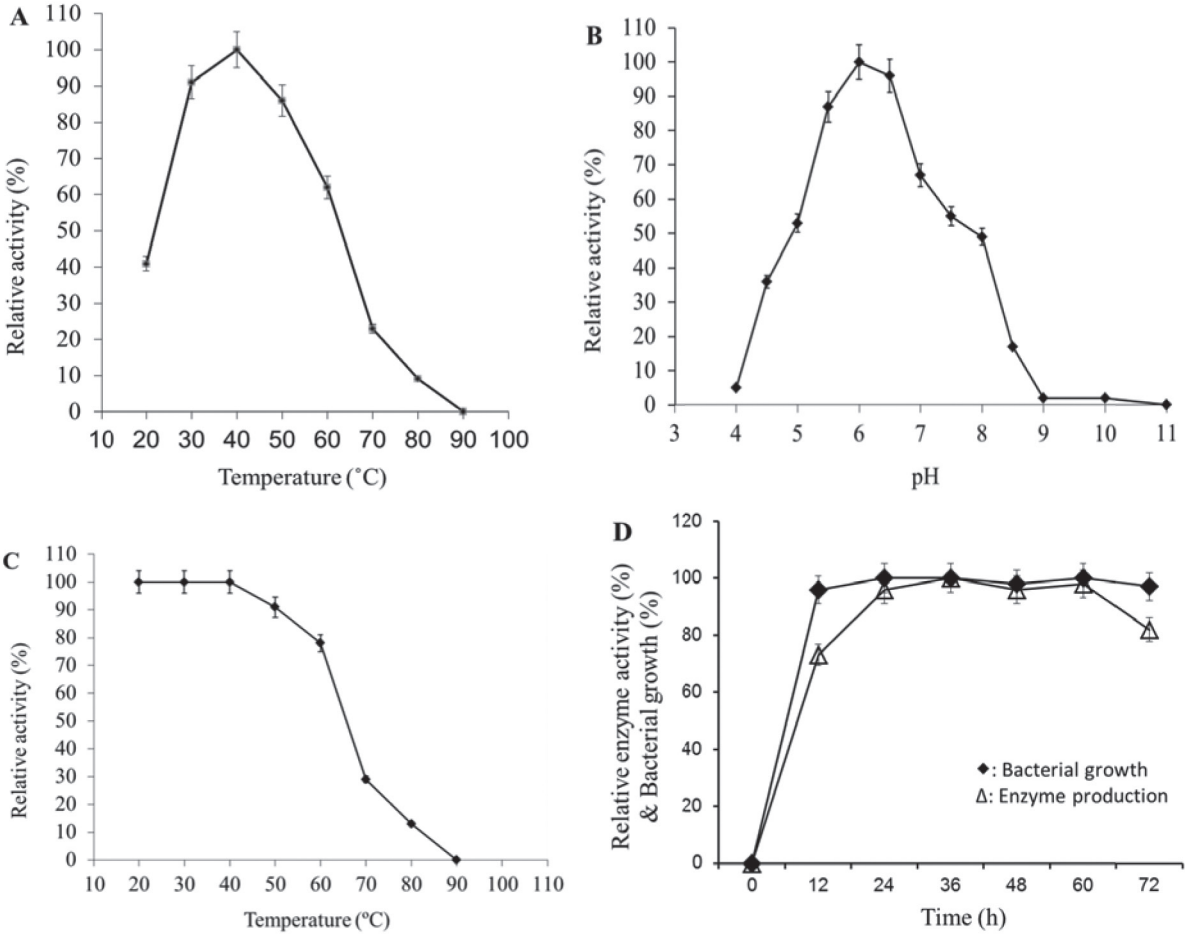

Fig 5. Enzyme properties of recombinant β-1,3-glucanase (A: Effect of temperature, B: Effect of pH, C: Thermal stability, D: Enzyme production depending on bacterial growth)

Şekil 5. Rekombinant β-1,3-glukanazın enzimatik özellikleri (A: Sıcaklığın etkisi, B: pH'nın etkisi, C: Termal stabilite, D: Bakteri gelişimine bağlı enzim üretimi)

The production of β-1,3-glucanase by the recombinant *L. plantarum* was reached maximum activity at 36 h of incubation period *(Fig. 5D)*. After 60 h of incubation period, the enzyme activity started to decrease.

DISCUSSION

With this study, β-1,3-glucanase gene of *C. cellulans* was cloned and expressed in *L. plantarum*. The enzyme secreted from *L. plantarum* was found to be active with clear zones on MRS-agar plate containing laminarin. On the other hand, zymogram analyses clearly indicated that activity bands with same size surrounded with clear zones confirming the expression of *C. cellulans* β-1,3-glucanase gene in *L. plantarum* successfully without any significant proteolytic degradation.

β-1,3-Glucanase gene of *C. cellulans* (ATCC 21606) was well studied and enzymatic properties were revealed [29]. Furthermore, the nucleotide sequence of the gene was determined [20]. In our previous study, the DNA fragment of gene was generated by PCR from *C. cellulans* genome, ligated into *Sma*I digested pUC18 and then first generation recombinant vector pTEG5 was created [14]. In the present study, p353-2 cryptic plasmid region (2.4 kbp) including replication origin of *L. plantarum* was derived from *Escherichia coli*/*Lactobacillus* shuttle vector pLP3537 then ligated into *Eco*RI digested pTEG5 and second generation recombinant vector pTE353-βG was created. The pTE353-βG recombinant vector was then successfully transferred into *L. plantarum* 5057.

Silage can spoil rapidly if exposed to air during storage or feed out stage of silage. Although a common misconception is that molds are responsible for spoilage of silage, yeasts are the primary microorganisms that cause aerobic spoilage and heating [7]. Some chemical additives and inoculant microorganisms were used to increase the aerobic stability and to prevent the spoilage of silage. Molecular studies on silage inoculants have been increased and then some genes were cloned in lactic acid bacteria for improvement for DM loses.

Aerobic deterioration of silage is a complex process which depends on many factors and usually it is initiated by aerobic yeasts [30]. Various chemical additives with antifungal properties, such as propionic acid, sorbate, benzoate, acetic acid, ammonia, urea, some enzyme preparations and *Lactobacillus buchneri* as silage inoculant have been used to prevent or enhance of aerobic stability and decrease of spoilage [11]. However usages of most of these additives have been restricted because of their other undesirable properties.

Previously, several genes from other microorganisms were cloned and expressed in *L. plantarum* such as α-amylase [31-34], cellulase, xylanase and endoglucanase [8,35], endo-1,4-β-glucanase [36], levanase [37], β-galactosidase [38] and chitinase [39]. Besides, herbal β-1,3-glucanase genes from soybean [40], jujube fruit [41] and rice [42] were cloned previously. On the other hand, the *C. cellulans* β-1,3-glucanase gene was cloned and expressed in *Bacillus subtilis* and *E. coli* [1-3,20,43]. But this is the first report to the best of our knowledge in which the β-1,3-glucanase gene from *C. cellulans* was cloned and expressed in *L. plantarum* to create new silage inoculant for better aerobic stability.

In our study, the optimum temperature of the recombinant enzyme was found with a wide range between 30-60°C. Although the gene has been cloned in several bacteria including *E. coli* and *B. subtilis*, temperature properties have not been investigated. On the other hand, temperature optimums of the β-1,3-glucanases from strain DSM 10297 and TK-1 were reported as 40 and 60°C, respectively [4]. Our finding is in agreement with optimum temperatures of the enzymes from the strains DSM 10297 and TK-1. On the other hand, optimum pH of our recombinant enzyme was found as 6.0 with a significant relative activity (80.6%) between pH 5.0 and 7.0. The present study indicates that, this enzyme prefers slightly acidic pH for optimal activity. Similar pH conditions for activity of native β-1,3-glucanase from *C. cellulans* have been reported previously [29]. pH values of various silages observed in other studies were reported as 4.3-4.5 (alfalfa silage with 30-35% DM), 4.7-5.0 (alfalfa silage with 45-55% DM), 4.3-4.7 (grass silage with 25-35% DM), 3.7-4.2 (corn silage with 35-40% DM), and 4.0-4.5 (high moisture corn silage with 70-73% DM) [7]. Our enzyme has been protected almost 40 and 55% of its activity in 4.0 and 5.0 pH conditions, respectively. With our results, the recombinant enzyme could be considered as a solution partially for aerobic spoilage at feed out stage. Besides, considering the development of *L. plantarum* at 30°C, this temperature value is quite appropriate for activation of the recombinant enzyme. Our enzyme has been suffered the loss of activation over 60°C for 30 min. So, under the unsuitable silage conditions with 60°C, our enzyme began to denature.

In conclusion, the β-1,3-glucanase gene of *C. cellulans* cloned in *L. plantarum*. Recombinant *L. plantarum* expressing β-1,3-glucanase encoding gene could be considered as a silage inoculant for aerobic spoilage of silage.

REFERENCES

1. Tanabe Y, Pang Z, Oda M: Cloning and sequencing of endo-1,3-β-glucanase from *Cellulosimicrobium cellulans*. *J Biol Macromol*, 8 (3): 60-63, 2008.

2. Salazar O, Molitor J, Lienqueo ME, Asenjo JA: Overproduction, purification, and characterization of β-1,3-glucanase type II in *Escherichia coli*. *Protein Express Purif*, 23 (2): 219-225, 2001.

3. Ferrer P, Halkier T, Hedegaard L, Savva D, Diers I, Asenjo JA: Nucleotide sequence of a β-1,3-glucanase isoenzyme II_A gene of *Oerskovia xanthineolytica* LL G109 *(Cellulomonas cellulans)* and initial characterization of the recombinant enzyme expressed in *Bacillus subtilis*. *J Bacteriol*, 178 (15): 4751-4757, 1996.

4. Ferrer P: Revisiting the *Cellulosimicrobium cellulans* yeast-lytic β-1,3-

glucanases toolbox: A review. *Microb Cell Fact*, 5 (10): 1-8, 2006.

5. Salazar O, Asenjo JA: Enzymatic lysis of microbial cells. *Biotechnol Lett*, 29 (7): 985-994, 2007.

6. Kitamura K, Yamamoto Y: Purification and properties of an enzyme, zymolyase, which lyses viable yeast cells. *Arch Biochem Biophys*, 153 (1): 403-406, 1972.

7. Kung L: Silage fermentation and additives. **In,** Directfed Microbial Enzyme and Forage Additive Compendium. pp. 1-18, Miller Publishing Co. Minnetonka, 2001.

8. Bates EEM, Gilbert HJ, Hazlewood GP, Huckle J, Laurie JI, Mann SP: Expression of a *Clostridium thermocellum* endoglucanase gene in *Lactobacillus plantarum*. *App Environ Microbiol*, 55 (8): 2095-2097, 1989.

9. Zahiroddini H, Baah J, McAllister TA: Effects of microbial inoculants on the fermentation, nutrient retention, and aerobic stability of barley silage. *Asian Australas J Anim Sci*, 19 (10): 1429-1436, 2006.

10. Driehuis F, Oude Elferink SJWH, Van Wikselaar PG: Fermentation characteristics and aerobic stability of grass silage inoculated with *Lactobacillus buchneri*, with or without homofermentative lactic acid bacteria. *Grass Forage Sci*, 56, 330-343, 2001.

11. Kung L: Aerobic stability of silages. *Proceedings of the Silage for Dairy Farms.* University of Delaware, Department of Animal and Food Sciences, Harrisburg, PA, 2005.

12. Holzer M, Mayrhuber E, Dannerr H, Braun R: The role of *Lactobacillus buchneri* in forage preservation. *Trends Biotechnol*, 21, 282-287, 2003.

13. Teather RM, Wood PJ: Use of Congo red polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol*, 43 (4): 777-780, 1982.

14. Özcan BD, Özcan N, Baylan M, Güzel Aİ: Cloning and expression of β-1,3-glucanase gene from *Cellulosimicrobium cellulans* in *Escherichia coli* DH5α. *Kafkas Univ Vet Fak Derg*, 19 (3): 523-528, 2013, DOI: 10.9775/kvfd.2012.8225.

15. Posno M, Heuvelmans PTHM, van Giezen MJF, Lokman BC, Leer RL, Pouwels PH: Complementation of the instability of *Lactobacillus* strains to utilize d-xylose with d-xylose catabolism-encoding genes of *Lactobacillus pentosus*. *Appl Environ Microbiol*, 57 (9): 2764-2766, 1991.

16. Alegre MT, Rodriguez MC, Mesas JM: Transformation of *Lactobacillus plantarum* by electroporation with *in vitro* modified plasmid DNA. *FEMS Microbiol Lett*, 241, 73-77, 2004.

17. Birnboim HC, Doly J: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*, 7 (6): 1513-1523, 1979.

18. Hardy KG: *Bacillus* Cloning Methods, **In,** Glover DM (Ed): DNA Cloning: A Practical Approach, Vol: II. 1-36, IRL Press Inc., Oxford, United Kingdom, 1985.

19. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, 1989.

20. Shen S-H, Chretien P, Bastien L, Slilaty SN: Primary sequence of the glucanase gene from *Oerskovia xanthineolytica*. *J Biol Chem*, 266 (2): 1058-1063, 1991.

21. Laemmli UK: Cleauage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature*, 227 (5259): 680-685, 1970.

22. Saul DJ, Williams LC, Grayling RA, Chamley LW, Love DR, Bergquist PL: *celB*, a gene coding for a bifunctional cellulase from the extreme thermophile '*Caldocellum saccharolyticum*. *Appl Environ Microbiol*, 56 (10): 3117-3124, 1990.

23. Ozcan N, Cunningham C, Haris WJ: Cloning of a cellulase gene from the rumen anaerobe *Fibrobacter succinogenes* SD35 and partial characterization of the gene product. *Lett Appl Microbiol*, 22 (1): 85-89, 1996.

24. Lee SP, Morikawa M, Takagi M, Imanaka T: Cloning of the *aapT* gene and characterization of its product, α-amylase-pullulanase (AapT), from thermophilic and alkaliphilic *Bacillus* sp. strain XAL601. *Appl Environ Microbiol*, 60 (10): 3764-3773, 1994.

25. Ikeda T, Yamazaki H, Yamashita K, Shinke R: The tetracycline inducible expression of alpha-amylase in *Bacillus subtilis*. *J Ferment Bioengineer*, 74, 58-60, 1992.

26. Coral G, Arikan B, Unaldi MN, Guvenmez H: Some properties of crude carboxymethyl cellulase of *Aspergillus niger* Z10 wild-type strain. *Turk J Biol*, 26, 209-213, 2002.

27. Miller GL: Use of dinitrosalicylic acid for determination of reducing sugar. *Anal Chem*, 31 (3): 424-426, 1959.

28. Özcan N: Cloning and sequencing of a cellulase gene from *Fibrobacter succinogenes* SD35. *PhD Thesis,* Department of Molecular and Cell Biology, University of Aberdeen, Scotland, U.K., 1992.

29. Scott JH, Schekman R: Lyticase: Endoglucanase and protease activities that act together in yeast cell lysis. *J Bacteriol*, 142 (2): 414-423, 1980.

30. Ozduven ML, Kursun Onal Z, Koc F: The effects of bacterial inoculants and/or enzymes on the fermentation, aerobic stability and *in vitro* dry and organic matter digestibility characteristics of triticale silages. *Kafkas Univ Vet Fak Derg*, 16 (5): 751-756, 2010.

31. Fitzsimons A, Hols P, Jore J, Leer RJ, O'Connell M, Delcour J: Development of an amylolytic *Lactobacillus plantarum*, silage strain expressing the *Lactobacillus amylovorus* alpha-amylase gene. *Appl Environ Microbiol,* 60, 3529-3535, 1994.

32. Hols P, Ferain T, Garmyn D, Bernard N, Delcour J: Use of homologous expression-secretion signals and vector-free stable chromosomal integration in engineering of *Lactobacillus plantarum* for alpha-amylase and levanase expression. *Appl Environ Microbiol*, 60 (5): 1401-1413, 1994.

33. Jones S, Warner PJ: Cloning and expression of alpha-amylase from *Bacillus amyloliquefaciens* in a stable plasmid vector in *Lactobacillus plantarum*. *Lett Appl Microbiol*, 11 (4): 214-219, 1990.

34. Scheirlinck T, Mahillon J, Joos H, Dhaese P, Michiels F: Integration and expression of alpha-amylase and endoglucanase genes in the *Lactobacillus plantarum* chromosome. *Appl Environ Microbiol*, 55 (9): 2130-2137, 1989.

35. Scheirlinck T, Meutter JD, Arnaut G, Joss H, Claeyssens M, Michiels F: Cloning and expression of cellulase and xylanase genes in *Lactobacillus plantarum*. *Appl Microbiol Biotechnol*, 33 (5): 534-541, 1990.

36. Rossi F, Rudella A, Marzotto M, Dellaglio F: Vector-free cloning of a bacterial endo-1,4-β-glucanase in *Lactobacillus plantarum* and its effect on the acidifying activity in silage: Use of recombinant cellulolytic *Lactobacillus plantarum* as silage inoculant. *Antonie van Leeuwenhoek*, 80 (2): 139-147, 2001.

37. Wanker E, Leer RJ, Pouwels PH, Schwab H: Expression of *Bacillus subtilis* levanase gene in *Lactobacillus plantarum* and *Lactobacillus casei*. *Appl Microbiol Biotechnol*, 43 (2): 297-303, 1995.

38. Halbmayr E, Mathiesen G, Nguyen TH, Maischberger T, Peterbauer CK, Eijsink VG, Haltrich D: High-level expression of recombinant beta-galactosidases in *Lactobacillus plantarum* and *Lactobacillus sakei* using a Sakacin P-based expression system. *J Agric Food Chem*, 56 (12): 4710-4719, 2008.

39. Brurberg MB, Haandrikman AJ, Leenhouts KJ, Venema G, Nes IF: Expression of a chitinase gene from *Serratia marcescens* in *Lactococcus lactis* and *Lactobacillus plantarum*. *Appl Microbiol Biotechnol*, 42 (1): 108-115, 1994.

40. Cheong YH, Kim CY, Chun HJ, Moon BC, Park HC, Kim JK, Lee S, Han C, Lee SY, Cho MJ: Molecular cloning of a soybean class III beta-1,3-glucanase gene that is regulated both developmentally and in response to pathogen infection. *Plant Sci*, 154 (1): 71-81, 2000.

41. Tian SP, Yao EJ, Deng X, Xu XB, Qin GZ, Chan ZL: Characterization and expression of β-1,3-glucanase genes in jujube fruit induced by the microbial biocontrol agent *Cryptococcus laurentii*. *Phytopathology*, 97 (3): 260-268, 2007.

42. Yamaguchi T, Nakayama K, Hayashi T, Tanaka Y, Koike S: Molecular cloning and characterization of a novel β-1,3-glucanase gene from rice. *Biosci Biotechnol Biochem*, 66 (6): 1403-1406, 2002.

43. Ferrer P, Hedegaard L, Halkier T, Diers I, Savva D, Asenjo JA: Molecular cloning of a lytic beta-1,3-glucanase gene from *Oerskovia xanthineolytica* LLG109. A beta-1,3-glucanase able to selectively permeabilize the yeast cell wall. *Ann NY Acad Sci,* 782, 555-565, 1996.