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Cloning of β-(1,3-1,4)-Glucanase Gene in Probiotic Bacterium *Bacillus coagulans*

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Abstract

Endo-β-(1,3-1,4) glucanase (or lichenase, EC3.2.1.73) is an endoacting enzyme that hydrolyses mixed linkage β-(1,3-1,4)-glucan from the walls of starchy seed endosperm. *Bacillus coagulans* is a lactic acid producing and sporeforming probiotic bacteria. The aim of this work is to clone and express lichenase gene in *B. coagulans* to create better probiotics for poultry. The Lichenase gene was inserted into the *Escherichia coli-Bacillus* sp. shuttle vector pMK3. The construct pMK3Lic was introduced into *B. coagulans* DSM1 by electrotransformation. Insert analysis of pMKLic obtained from recombinant *B. coagulans* confirmed the lichenase gene fragment on agarose gel electrophoresis. Despite of cloning of lichenase gene in *B. coagulans*, no the lichenase enzyme activity was detected on lichenan overlay plates. Both the extracellular and intracellular lichenase enzyme activity wasn't detected from cultures of *B. coagulans* carrying pMK3Lic when analyzed by SDS-PAGE zymogram. In the study, an electrotransformation protocol for *B. coagulans* was developed and transformed strain DSM1 with plasmid pMK3, an *E. coli-Bacillus* sp. shuttle vector. A gene was also successfully transferred into *B. coagulans*. The shuttle vector and electroporation method could be used in other genetic engineering studies of *B. coagulans*.

Key words: Cloning, β-(1,3-1,4) glucanase, *Bacillus coagulans*, Probiotic

INTRODUCTION

Probiotics are commonly used as viable microbial feed supplements that affect the host animal by improving its intestinal microbial balance [1]. Few studies have been carried out on spore forming lactic acid producing bacteria (SFLAB) as probiotics. SFLAB are a group of Gram-positive bacteria, sharing characteristics common to the genera *Bacillus* (spore forming, motile) and *Lactobacillus* (micro-aerophilic, lactic acid production) [2]. *B. coagulans* is a lactic acid producing and sporeforming bacteria. It has the characteristics of resistance to high temperature and acid. It grows well, colonizes in intestine and has excellence in producing digestive enzymes and L(+) lactic acid which has got better metabolic rate compared to DL or D(-) type of lactic acid produced by most of the traditional probiotics that reduces cell metabolism during fermentation of sugars. Just like seeds, spores are capable of germination and development into living microorganisms. When they are administered to animals through feed or water can transform into active bacterial cells in existing specific environmental conditions such as body temperature, acidity, bile and other secretions of the upper digestive tract of poultry [3].

β-(1,3-1,4)-glucans are polysaccharide components of the cell walls of the higher plant family Poaceae, apparently restricted to members of the Graminiae, and particularly abundant in the endosperm cell walls of cereals with commercial value such as barley, rye, sorghum, rice, and wheat [4]. β-(1,3-1,4)-glucanases (or lichenases, EC 3.2.1.73) hydrolyse linear β-glucans and lichenan, with a strict cleavage specifity for β-(1,4) glycosidic bonds on 3-O-substituted glucosyl residues [5] Applications of β-glucanases such as lichenase in processing of animal food, control of fungal pathogens, and release of intracellular materials from microbial cells have been reviewed [6]. Recently, the utilization of enzymatic technologies to improve the quality of β-glucanases and the economy of their production has received increased interest. In animal feedstuff, especially for broiler chickens and piglets, addition of enzymatic preparations containing bacterial β-glucanases improves digestibility of barley-based diets, and reduces sanitary problems (sticky droppings) [4]. Therefore, economic value of barley, oats, rye, and even wheat can be improved by the addition of the appropriate preparation of β-glucanase and xylanase enzymes [7].

The present study is aimed to express β-(1,3-1,4)-glucanase gene in *B. coagulans* to create candidates that could be used for poultry as probiotic, which combines beneficial effects of both probiotic and lichenase enzyme.

MATERIALS and METHODS

Strains, Plasmids and Growth Media

B. coagulans DSM1 was purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). The host bacterium *E. coli* DH5α was purchased from Stratagene

(USA). pMK3 was obtained from BGSC (*Bacillus* Genetic Stock Centre, USA). pL1Hc plasmid DNA containing β-(1,3-1,4)-glucanase gene of *Streptococcus bovis* in *E. coli* were obtained from M. Sait EKİNCİ (University of Kahramanmaraş Sütçüimam, Kahramanmaraş, Turkey). *E. coli* cells were cul tured in LB (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, the pH was adjusted to 7.5 with 1 M NaOH) at 37ºC. *B. coagulans* was maintained in MediumI (7.8 g/l Peptone (meat), 7.8 g/l Peptone (caseine), 2.8 g/l Yeast Extract, 5.6 g/l NaCl, 1.0 g/l Glucose, the pH was adjusted to 7.5) at 40ºC.

DNA Manipulations

For general molecular biological techniques such as enzyme digestion, ligation, dephosphorylation and purification of insert DNA, etc, the methods described in Molecular Cloning [8] were employed.

Construction of pMK3Lic

To construct pMK3Lic plasmid, the pL1Hc plasmid, containing the lichenase gene was digested with HincII and the 1800 bp fragment was eluted from the gel. The excised lichenase gene was cloned into the vector pMK3, *E. coli-Bacillus* sp. shuttle vector, which had been digested with the SmaI restriction endonuclease and then dephosphorylated. The lichenase gene was cloned in pMK3. The ligations were transformed into *E. coli* DH5α competent cells by electroporation [9] and transformants were selected on LB, containing 15 g/l agar and 40 µg/ml X-gal plus 100 µg/ml Ampicillin (Amp). Plasmid DNA was isolated from *E. coli and B. coagulans* according to Birnboim and Doly [10].

Transformation of *Bacillus coagulans* by Electroporation

The electroporation procedure to transfer of the recombinant pMK3Lic into *B. coagulans* was used by modification of the protocol described by Vehmaanpera [11] in the present study. For electroporation, *B. coagulans* cells were grown in MediumI supplemented with 0.25 M sucrose and 50 mM K-phosphate, pH 7.5 (MediumI-SP) at 40ºC to OD600nm 0.6. Cells were recovered by centrifugation, washed three times in cold SHMG electroporation buffer (0.25 M sucrose, 1 ≅mM HEPES, 1 mM $MgCl_2$, 10% (v/v) glycerol, pH 7.0), and resuspended in the buffer in 1/100 of the original cultivation volume. For transformation, 100 µl of the cells were incubated with plasmid DNA (1 µg) in a sample cuvette (Bio-Rad) with interelectrode distance of 0.2 cm on ice for 20 minutes and pulsed by a Invitrogen ElectroporatorII apparatus set at 25µF, 200 Ω and 0.9 kV/cm. The output of the pulse generator was directed through a Pulse Controller unit (Invitrogen). Immediately after the pulse the cells were diluted 10-fold in MediumI-SP supplemented with 10% (v/v) glycerol (MediumI-SPG) at room temperature and shaken at 40ºC for 60 minutes. Transformants with pMK3Lic were selected on MediumI plates containing 10 µg/ml Kanamycine (Km) at 40ºC.

Detection of Lichenase Activity on Lichenan Plate

Lichenase positive transformants were detected using LB and MediumI plates containing 0.1% (w/v) Lichenan. After overnight growth, the petri plates were flooded with a 0.1% (w/v) Congo-Red solution. After 15 minutes incubation at room temperature, the Congo-Red solution was replaced with a 1 M NaCl solution for 15 minutes. The NaCl solution was removed and clear zones were appeared around positive colonies [12].

Detection of Lichenase Enzyme on SDS-PAGE and SDS-Lichenan-PAGE

To determine of β-(1,3-1,4)-glucanase (lichenase) enzyme secreted by original and recombinant bacteria, the cell culture or sonicated cell extract was centrifuged at 15.000 rpm for 30 minutes to remove cell or cell debris. The supernatant was then mixed with an equal volume of tricholoroacetic acid (TCA, 20% w/v). Total proteins were collected by re-centrifugation. Protein analysis was performed by using a denaturating polyacrylamide gel electrophoresis (SDS-PAGE, 12% w/v) [13].

Zymogram analysis was performed on SDS-lichenan-PAGE. Lichenan was added to the 12% SDS-PAGE gel (separating gel) to a final concentration of 0.2% (w/v). After electrophoresis, the gel was gently rocking in 50 mM sodium phosphate buffer and 20% (v/v) isopropanol solution at room temperature for an hour to remove denaturated agents. The gel was then transferred into renaturation solution (50 mM sodium phosphate buffer, 5 mM β-mercaptoethanol, 1 mM EDTA) and incubated overnight at 4°C. After renaturation of the protein, the gel was soaked 50 mM sodium phosphate buffer at 4°C for an hour. It was then covered with parafilm and incubated at 37°C for four hours. After staining of the gel with 0.2% (w/v) Congo-Red and 5 mM NaOH for an hour, it was washed with 1 M NaCl and 5 mM NaOH overnight to remove excess stain from the active bands [14].

RESULTS

In this study, the plasmid pMK3Lic, in which a 1800 bp HincII fragment containing the β-(1,3-1,4)-glucanase gene was inserted, was constructed as described in Materials and Methods (Fig. 1.). *E. coli* DH5α was transformed by the plasmid.

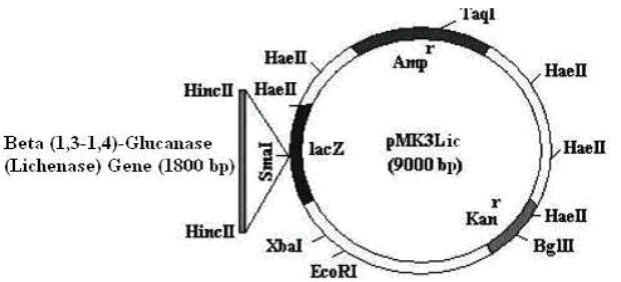

Figure 1. Structure of pMK3Lic plasmid (pMK3 (7200 bp) plus lichenase gene (1800 bp))

E. coli/pMK3lic transformants grew with non-recombinant colonies carrying pMK3 on LB/Agar medium including 40 µg/mL X-Gal and 100 µg/mL Amp. To select recombinant *E. coli* cells bearing pMK3Lic, white colonies grown on solid growth media were directly collected and the plasmid was isolated. The construct pMKLic (Fig. 1.) carrying the cloned lichenase gene was then introduced by electrotransformation into *B. coagulans*. The colonies were observed on MediumI agar medium supplemented with Km. To determine the recombinant colonies, both the non-recombinant and the colonies grew on the solid medium after transformation were reinoculated on the MediumI agar medium supplemented with Km. The original *B. coagulans* bacterium with no Km resistance gene didn't grow on the medium, whereas the recombinant *B. coagulans*/

pMK3Lic bearing Km resistance gene on the vector grew well on the medium supplemented with Km (Fig. 2.).

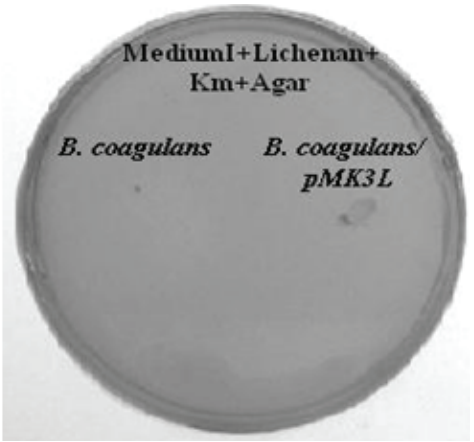

Figure 2. Screening lichenase activity of *B. coagulans*/pMK3Lic on MediumI/Lichenan/Km/Agar

Expression of lichenase enzyme of transformed bacteria was detected on Lichenan and Km overlay plates and clear zones were expected around of the recombinant colonies. The original and recombinant *B. coagulans* bacteria didn't show lichenase activity on the plates (Fig. 2.). The original pMK3, recombinant pL1Hc and the newly constructed recombinant pMK3Lic plasmids were isolated from *E. coli*. Recombinant pMK3Lic was also isolated from *B. coagulans* cells. They were subjected to insert analysis on 0.8% w/v agarose gel electrophoresis and photographed. Lichenase gene fragment (~1800 bp) from both original plasmid pL1Hc and newly constructed pMK3Lic from *E. coli* and *B. coagulans* cells were determined on the range with line on the gel (Fig. 3.).

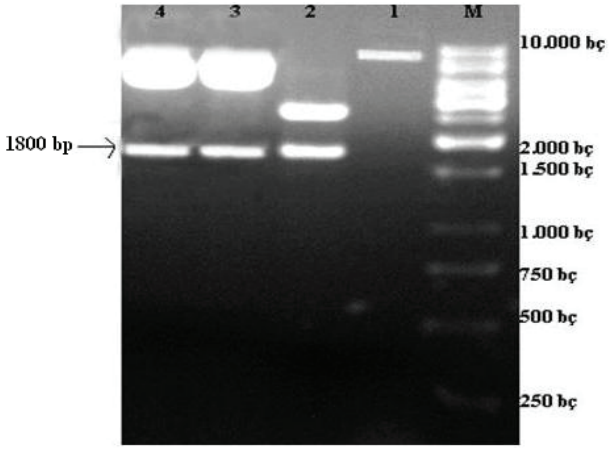

Figure 3. Insert analysis of pMK3Lic plasmid on agarose jel M: 1kb DNA Markers, 1: pMK3/SmaI, 2: *E. coli*/pL1Hc/HincII, 3: *E. coli*/pMK3Lic/SmaI, 4: *B. coagulans*/pMK3Lic/SmaI

Lichenase activity and total proteins of various bacteria (*E. coli*/pL1Hc, *E. coli* DH5α, *E. coli*/pMK3Lic, *B. coagulans*/pMK3Lic) were compared on SDS-PAGE and SDS-Lichenan-PAGE.

The similar results were observed from intracellular and extracellular proteins of these bacteria. Visible enzyme zones (26 kDa) on SDS-Lichenan-PAGE were only screened from *E. coli*/pL1Hc and *E. coli*/pMK3Lic by zymogram analysis. Lichenase activity wasn't observed in both extracellular and intracellular culture of *B. coagulans*/pMK3Lik cells (Fig. 4A. and B.).

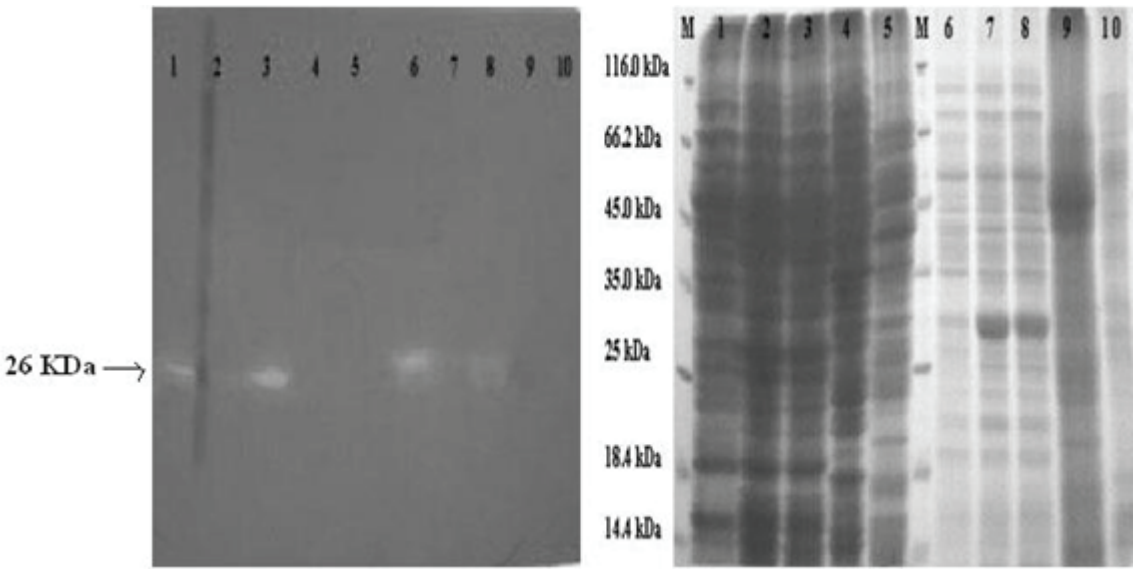

Figure 4. Comparison of lichenase activity (A) and total proteins (B) obtained from intracellular proteins (1-5) and extracellular proteins (6-10) of various bacteria (*E. coli*/pL1Hc, *E. coli* DH5α, *E. coli*/pMK3Lic, *B. coagulans*, *B. coagulans*/pMK3Lic, respectively) on SDS-PAGE

DISCUSSION

The β-(1,3-1,4)-glucanase (Lichenase) gene from *S. bovis* was cloned and expressed in *E. coli, Lactococcus lactis* and *Enterococcus faecalis* (15,16). In the study, the cloning and expression of β-(1,3-1,4)-glucanase gene in *B. coagulans* was studied. *B. coagulans* has been found to be a source of many commercially valuable products, such as lactic acid [17, 18], thermostable enzymes [19, 20] and coagulin, an antimicrobial peptide [21, 22]. It was also demonstrated that administering *B. coagulans* cells as a dietary additive to piglets or chickens had a growth promoting and prophylactic effect [23-26]. These reports suggest the use of *B. coagulans* as a probiotic in animal feed, as an alternative to antibiotics.

The mixed linked 1,3-1,4-β-D-glucans form a major part of cell walls of cereals, such as oat and barley, and account for up to 70% of the cell wall in barley endosperm [27]. β-glucans consist of glucose units joined by β-1,4 and β-1,3 linkages and include lichenin or barley β-glucan. After ingestion, β-glucans become soluble, resulting in increased digesta viscosity [28]. Increased intestinal viscosity reduces the passage rate of digesta [29]. With a reduction in digesta passage rate, the overall feed consumption would be less and could contribute to a decrease in live performance. As the viscosity increases, the rate of nutrient absorption decreases, which, in turn, could reduce nutrient assimilation rate due to reduced enzyme:substrate reactions in the intestine [30]. High viscosity digesta also lead to increased incidence of sticky droppings, increasing the occurrence of dirty eggs [31]. The use of enzyme preparations containing β-glucanases in the formulation of broiler chicks feedstuff in order to improve its digestibility and reduce ''sticky dropping'' problems is important for poultry industry [28].

To express lichenase gene in the bacterium, a vector called pMK3Lic-bearing β-(1,3-1,4)-glucanase gene was constructed and then electrotransferred into *B. coagulans*. SmaI digestion of the recombinant plasmid from *B. coagulans* yielded 1800 bp fragment of DNA carrying the gene and 7200 bp fragment of pMK3 vector on agarose gel electrophoresis. Lichenase gene was cloned in *B. coagulans*, however lichenase enzyme activity wasn't detected on lichenan plates. SDS-PAGE zymogram analysis was performed to determine that lichenase enzyme may be secreted intracellularly. However, both the extracellular and intracellular lichenase enzyme activity wasn't detected from cultures of *B. coagulans* carrying pMK3Lic.

The value of expression systems based on strong and tightly regulated promoters is well recognized in modern biotechnology. In the study *lacZ* promoter or own promoter of the lichenase gene was used for expression of lichenase gene in *B. coagulans*. The promoters may be not strong enough for expressing the gene.

B. subtilis and related *Bacillus* species have been used for decades for the bulk production of industrial enzymes and native secretory enzymes such as amylases and proteases [32, 33]. Despite its potential application to heterologous protein production, a major drawback of *B. subtilis* as the host is that product levels expressed by it are generally too low for practical use. In attempting to improve the productivity of *B. subtilis,* efforts have been focused mainly on two aspects: development of protease-deficient strains and elucidation of efficient regulatory elements at both the levels of transcription and protein secretion [34]. Therefore, new expression systems for *Bacillus* strains have been developed to express heterologous proteins at high levels [35-37]. Numerous proteases secreted by these expression hosts are often responsible for the degradation of most heterologous and even homologous secreted gene products [38, 39]. Lichenase secreted from recombinant *B. coagulans* may be not enough amounts to degrade of lichenan or inactivated by proteases of the host.

Although several Gram-positive bacteria naturally develop competence for DNA uptake and can be readily transformable with both plasmid and linear DNA, *B. coagulans* is recalcitrant to genetic manipulation [40, 41]. To fully utilize the potential of this bacterium as a biocatalyst, it is important to develop a reproducible genetic system that can be used to genetically and metabolically engineer this bacterium. Rhee et al. [42] have developed an electrotransformation protocol for *B. coagulans* and using this method also identified and constructed plasmids that can be used for gene transfer into *B. coagulans*. They have transformed strain P4-102B with plasmid pMSR10, a new *Bacillus/E. coli* shuttle vector containing a *B. coagulans* plasmid replication system and plasmid pNW33N, a *Geobacillus stearothermophilus*/ *E. coli* shuttle vector.

In the study, we have developed an electrotransformation protocol for and transformed strain DSM1 with plasmid pMK3, an *E. coli-Bacillus* sp. shuttle vector. We demonstrated that pMK3 vector could be used for gene transferring into strain DSM1 in other studies. In conclusion, the lichenase gene was not expressed in *B. coagulans*. However, this was the first report to our knowledge that a gene was successfully transferred and lichenase gene was attempted to clone in *B. coagulans*.

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