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Expression of β -(1,3-1,4)-glucanase gene of *Orpinomyces* sp. GMLF18 in *Escherichia coli* EC1000 and *Lactococcus lactis* subsp. *cremoris* MG1363

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Abstract: A gene encoding β -(1,3-1,4)-glucanase (*licA*) was amplified from *Orpinomyces* sp. GMLF18 and expressed in *Escherichia coli*. The DNA sequence of *licA* showed that the gene was 707 bp and encoded a protein with a molecular mass of 26 kDa that belongs to family glycosyl hydrolase 16. The main LicA activity was observed to be cell-associated for the *licA* containing transformant *E. coli*, and the enzyme expressed in *E. coli* showed the highest activity at pH 5.0-6.0 and at temperatures of 40-50 °C. The enzyme was found to be stable at 40 °C; however, 12% of LicA activity was lost at 50 °C in 20 min. The *licA* was then introduced into the facultative anaerobic bacterium *Lactococcus lactis* subsp. *cremoris* MG1363 by a stable recombinant plasmid, pIL253. Although the enzymatic activity was lower than that in *E. coli*, the gene encoding the fungal originated lichenase was successfully expressed in *L. lactis*.

Key words: *Orpinomyces*, β -(1,3-1,4)-glucanase, *licA*, *Lactococcus lactis*, lichenan

Orpinomyces sp. GMLF18'e ait β -(1,3-1,4)-glukanaz geninin *Escherichia coli* EC1000 ve *Lactococcus lactis* subsp. *cremoris* MG1363'de ekspresyonu

Özet: β -(1,3-1,4)-Glukanaz (*licA*) kodlayan bir gen *Orpinomyces* sp. GMLF18'den izole edilmiş ve *Escherichia coli*'de klonlanmıştır. *licA*'nın DNA dizilimi, 707 bp uzunluğunda bir gen olduğunu ve 26 kDa moleküler büyüklüğünde olan glikozil hidrolaz 16 ailesine ait bir protein kodladığını göstermiştir. *licA* içeren transformant *E. coli*'de LicA enzim aktivitesinin büyük bir kısmının hücresel olduğu bulunmuş ve LicA'nın pH 5,0-6,0 ve 40-50 °C arasında en yüksek aktiviteye ulaştığı gözlenmiştir. Enzim 40 °C'de stabilitesini korurken, 50 °C'de 20 dk içerisinde aktivitesinin % 12'sini kaybetmiştir. *licA* stabil bir rekombinant plazmit olan pIL253 yardımıyla fakültatif anaerob bakteri olan *Lactococcus lactis* subsp. *cremoris* MG1363'e aktarılmıştır. Enzim aktivitesi *E. coli*'ye göre düşük olsa da anaerobik fungus kaynaklı likenaz kodlayan bir gen *L. lactis*'de başarılı bir şekilde ifade edilmiştir.

Anahtar sözcükler: *Orpinomyces*, β -(1,3-1,4)-glukanaz, *licA*, *Lactococcus lactis*, likenan

Introduction

β -Glucan is a major component of the cell walls of cereals such as oats and barley (1). 1,3-1,4- β -D-glucan 4-glucanohydrolase (β -glucanase; lichenase) (E.C. 3.2.1.73) hydrolyzes β -glucans via from b-1,4 glycosidic bonds on 3-O-substituted glucosyl residues (2). β -Glucanases have been reported from bacteria such as *Bacillus licheniformis* (3), *Clostridium thermocellum* (4), and *Streptococcus bovis* (5); fungi such as *Trichoderma reesei* (6) and *Orpinomyces* (7); and plants such as barley (8). The ruminal anaerobic fungus *Orpinomyces* sp. PC-2 was the first reported that could synthesize 1,3-1,4- β -D-glucanase (7). β -(1,3-1,4)-Glucanase coding gene *licA* of *Orpinomyces* sp. PC-2 was cloned into *E. coli* and it was suggested that the gene had a bacterial origin (7).

Cereals constitute a major source of nutrients for animals, and β -glucans occur at different levels in various cereals (9). However, β -glucans in cereals have several adverse effects, particularly for monogastric animals, and are responsible for the poor nutritive value of some cereals (10). Therefore, the enhancement of β -glucan degradation by the addition of β -glucan degrading enzymes (11) or by using recombinant lactic acid bacteria, which could express β -glucanase, has been attempted by other researchers (5,12).

L. lactis subsp. *cremoris* MG1363 is generally regarded as a safe microorganism that can survive passage through the gastrointestinal tract, and it is not a natural inhabitant of the gut (13). *L. lactis* MG1363 was also used for the delivery of biologically active molecules in the gastrointestinal tract (13). Accordingly, the aim of this study was to isolate a β -(1,3-1,4)-glucanase coding gene of an anaerobic ruminal fungus and express this gene in *L. lactis* MG1363. Thus, an anaerobic fungal isolate, *Orpinomyces* sp. GMLF18, was used to obtain a β -(1,3-1,4)-glucanase coding gene. The gene encoding β -(1,3-1,4)-glucanase was cloned into a pCT vector and transferred into *E. coli*. Following sequence confirmation, the *licA* gene was transferred into *L. lactis* subsp. *cremoris* MG1363 by the aid of pIL253. We also studied the β -(1,3-1,4)-glucanase production of fungal isolate GMLF18 and the expression level of the β -(1,3-1,4)-glucanase gene in recombinant *E. coli* EC1000 and *L. lactis* subsp. *cremoris* MG1363 strains.

Materials and methods

Fungal strain and culture conditions

The *Orpinomyces* sp. GMLF18 had been isolated in our previous study (14) and was deposited in the culture collection of the Biotechnology and Gene Engineering Laboratory of Kahramanmaraş Sütçü İmam University, Kahramanmaraş, Turkey. To ensure the maintenance of the fungus, an anaerobic medium was prepared according to Orpin (15) and dispensed into Hungate tubes under strictly anaerobic conditions. The anaerobic medium contained 15% clarified rumen fluid, and wheat straw was used as a carbon source for the maintenance media. For enzyme production, the fungus was grown in a medium containing lichenan (Sigma, 0.5% w/v) as a sole energy source. To obtain relatively higher amounts of cells for DNA extraction, glucose (0.5%, w/v) was used as an energy source. All incubations for the fungus used in this study were performed at 39 °C.

Bacterial strains, culture conditions and plasmids

Escherichia coli strains were grown in Luria-Bertani (LB) broth at 37 °C in a shaking incubator (150 rpm). *Lactococcus lactis* subsp. *cremoris* MG1363 was routinely grown in M17 (Merck) medium containing 0.5% glucose as an energy source (GM17) without shaking at 30 °C. Agar plates were prepared by the addition of agar (Merck) at a concentration of 1.5% (w/v) into the broth medium. The MoFavor Cloning Kit (Favorgen Biotech Corp., Taiwan), which contains an "A" sticky end pCT vector, was used to clone the PCR product. pIL253, which is a derivative of pAMb1 (16), was used to construct a shuttle vector. The bacterial strains and plasmids used in this study are listed in Table 1.

Genomic DNA extraction and PCR analysis

The anaerobic fungus *Orpinomyces* sp. GMLF18 was grown on glucose containing anaerobic medium for 2 days and the fungal biomass was harvested by centrifugation at 1250 \times g for 10 min. Approximately 10 mg of biomass was frozen using liquid nitrogen and immediately broken down using a Mixer Mill (Retsch MM301). DNA extraction was carried out using a DNA Extraction Kit (Favorgen Biotech. Corp., Taiwan) according to the manufacturer's protocol. The DNA

Table 1. Strains and plasmids used in this study.

Strains and plasmids	Relevant features	Source or reference
Strains		
<i>Orpinomyces</i> sp. GMLF18	Lichenase coding gene (<i>licA</i>) source	14
<i>E. coli</i> EC1000	Km ^r ; RepA ⁺ , Cloning host	17
<i>L. lactis</i> MG1363	Plasmid free <i>L. lactis</i> subsp. <i>cremoris</i> MG1363 (IFR)	18
<i>E. coli</i> pCTL18	<i>E. coli</i> EC1000 carrying pCTL18	This study
<i>E. coli</i> pILL18	<i>E. coli</i> EC1000 carrying pILL18	This study
<i>L. lactis</i> pILL18	<i>L. lactis</i> MG1363 carrying pILL18	This study
Plasmids		
pCT	Amp ^r ; cloning vector; 2.7 kb	Favorgen (Taiwan)
pIL253	Ery ^r ; Gram-positive vector; 4.8 kb	16
pCTL18	Amp ^r ; derivative of pCT vector containing 707 bp of <i>licA</i>	This study
pILL18	Amp ^r , Ery ^r ; fusion of pCTL18 with pIL253 to obtain <i>E. coli</i> -Lactococcus shuttle vector	This study

samples were dissolved in 50 µL of TE (Tris EDTA, pH 8.0) buffer and then stored at -20 °C for further analysis. To screen the β-(1,3-1,4)-glucanase gene from the fungal genomic DNA, forward 5'-GAAAATATTTTCATT-3' and reverse 5'-TTGGTGCATCATAA-3' primers were designed using the previously published β-(1,3-1,4)-glucanase sequence [OSU63813]. Amplification was performed on an Eppendorf (MasterCycle, USA) thermocycler and the PCR program was as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The products (5 µL) were separated on a 1% agarose gel and analyzed for the yield of amplicons of expected sizes.

Sequencing and alignment procedures

The resultant plasmid, pCTL18, was extracted using the Plasmid DNA Extraction Kit (Favorgen Biotech Corp., Taiwan) according to the manufacturer's protocol and the β-(1,3-1,4)-glucanase gene (*licA*) insert was sequenced on both strands by Iontek (İstanbul, Turkey), a commercial company. Sequence alignment and data analysis were performed using ChromasPro V1.34 and Clone

Manager 5, respectively, whilst similarity analysis was conducted using CLUSTAL W (<http://www.ebi.ac.uk/clustalw/>; 19). Published data of various β-(1,3-1,4)-glucanases were derived from the CAZY web-server (<http://www.cazy.org/>; 20) and used for similarity comparison.

Cloning strategy and transformation

The diluted PCR product was cloned into pCT vector according to the manufacturer's protocol. The constructed plasmids were transformed into *E. coli* by CaCl₂ method to express the β-(1,3-1,4)-glucanase proteins (21). Transformed *E. coli* cells were plated on LB agar plates containing ampicillin (50 µg/mL) for the pCT based construct and erythromycin (150 µg/mL) for the pIL253 based construct. Recombinant strains were screened for β-(1,3-1,4)-glucanase activity by overlaying 0.1% lichenan and 0.4% agarose in 25 mM potassium phosphate (pH 6.5) buffer. After incubation at 37 °C for 4 h, β-(1,3-1,4)-glucanase activity was detected by congo-red staining following a 1M NaCl destaining process. β-(1,3-1,4)-Glucanase positive strains were purified and stored at -20 °C for further analysis.

In order to create a plasmid that could replicate in a gram-positive host, pIL253 was used. pCTL18 was digested with *KpnI* and ligated with *KpnI*-cut pIL253 to construct a shuttle vector expressing β -(1,3-1,4)-glucanase proteins. The constructed shuttle vector (pILL18) was transferred into *L. lactis* MG1363 via electroporation (BioRad; Pulser™) as described by Holo and Nes (22). Transformant *L. lactis* colonies were selected on 5 μ g/mL erythromycin containing GM17 agar medium. The resultant transformant colonies were also confirmed by colony PCR and congo-red staining.

β -(1,3-1,4)-glucanase production by fungal and transformed bacterial organisms

To estimate the lichenase production of *Orpinomyces* sp. GMLF18, fungus was cultivated in lichenan-containing medium for 7 days with sampling at 24-h intervals. The culture was centrifuged at 1250 \times g for 10 min and the supernatant was stored at -20 °C for further analyses. For the LicA production of bacterial strains, *E. coli* pCTL18 was grown in LB medium containing 50 μ g/mL ampicillin at 37 °C, and *L. lactis* pILL18 was grown in GM17 medium containing 5 μ g/mL erythromycin at 30 °C. β -(1,3-1,4)-Glucanase production was evaluated at 6, 12, and 24 h by sampling from the bacterial cultures. Extracellular fluid and cells were separated by centrifugation at 13,000 \times g for 5 min. The β -(1,3-1,4)-glucanase activities of the fungal isolate and bacterial strains were estimated for both the supernatant and cell extract. The cells were washed twice with 25 mM potassium-phosphate buffer (pH 6.5) and then the cells were broken down using a ball mill (Retsch) and resuspended in 25 mM potassium phosphate buffer (pH 6.5). Cellular debris was subsequently removed by centrifugation and the clarified extract was stored at -20 °C for further analyses.

Enzyme assays

The methodology used to determine β -(1,3-1,4)-glucanase activity was based on the method described by Miller (23) using 0.5% (w/v) lichenan (Sigma) as a substrate in 50 mM sodium phosphate buffer. One unit of β -(1,3-1,4)-glucanase activity was defined as 1 μ mol of reducing sugar released from the lichenan per minute. The effects of pH on activity were determined at 50 °C with the following buffers: 50 mM acetate buffer (pH 3.5 to 5.5), phosphate

buffer (pH 6.0 to 7.5), and Tris-HCl buffer (pH 8.0 to 9.0) solutions (24). The effect of temperature on β -(1,3-1,4)-glucanase activity was determined by assaying the enzyme at temperatures from 30 to 80 °C. Thermostability was performed by incubating the enzyme for 5, 10, 15, and 20 min at temperatures from 40 to 70 °C. The enzyme solution was chilled in an ice bath for 5 min and then assayed as described above. All enzyme assays were carried out in triplicate and the mean values were used.

Zymogram analysis

SDS-PAGE was performed on a 12% running gel (25) containing 0.1% lichenan. Zymogram analysis was carried out according to Liu et al. (26).

Nucleotide sequence accession number

The DNA sequence of the *licA* gene has been deposited in the GenBank database under accession number HM804955.

Results and discussion

Determination of lichenase activity in *Orpinomyces* sp. GMLF18

Lichenan degrading enzyme production by *Orpinomyces* sp. GMLF18 was investigated in lichenan containing media for 7 days and the enzyme production is shown in Figure 1. Lichenan degrading enzymes were produced in an increasing manner when lichenan was used as a sole carbon substrate. While lichenan degrading enzymes of *Orpinomyces* sp. GMLF18 were mainly secreted into the culture medium, an important fraction of activity was also

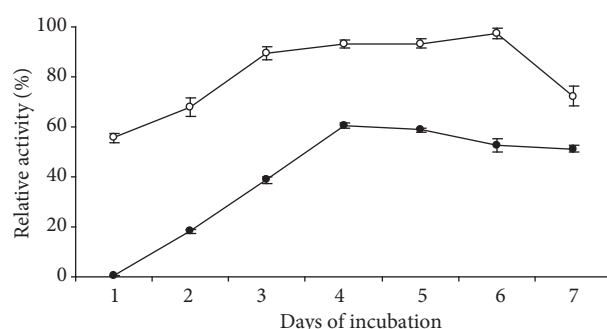


Figure 1. Relative extracellular and cell associated lichenase activity (%) of *Orpinomyces* sp. GMLF18 grown in lichenan. Symbols: (-o-) Extracellular and (-l-) cell associated enzyme activity.

accumulated in associated cells. Extracellular activity reached its maximum level on day 6 (78% of total activity), while the maximum cell associated activity was observed on day 4 (31% of total activity).

Cloning and sequencing of *licA*

The β -(1,3-1,4)-glucanase coding region was amplified from the genomic DNA of *Orpinomyces* sp. GMLF18 using *LicA* primers. Approximately 800 bp amplification product was obtained from the PCR and this amplicon was cloned into pCT vector and transferred into *E. coli* EC1000. After transformation, 38 lichenase positive colonies were counted over the 3000 transformed colonies. Two of these colonies were randomly selected and stored for further studies. The nucleotide sequence of *licA* was derived from pCTL18. *licA* consisted of 707 bp and contained a complete open reading frame (ORF) encoding polypeptide of 235 amino acids with a molecular mass of 26,710 Da. Poly (A) tail was not found at its 5' end. GC content of ORF of *licA* was found to be 35.5%. The bias in the codon

usage was also investigated in the *licA*, and 51.91% and 8.94% of codons involved T and G, respectively, in the wobble position. The codons have A and C in the third position at a proportion of 19.15% and 20%, respectively. It was observed that 19 codons were not utilized in *licA*. The predicted amino acid sequence of *LicA* from *Orpinomyces* sp. GMLF18 was found to be considerably similar to that of *Orpinomyces* sp. PC2 *LicA* with a 97% homology. *LicA* differed from other β -(1,3-1,4)-glucanases from *Anaeromyces* sp. W98, *Piromyces communis*, and *Neocallimastix patriciarum* at rates of 19%, 20%, and 23%, respectively. A high degree of identity was also observed for β -(1,3-1,4)-glucanases from *Streptococcus bovis* (59%), *Bacillus licheniformis* (47%), and *Clostridium thermocellum* (53%). The multiple alignment of the amino acid sequences of *LicA* with other published β -(1,3-1,4)-glucanases derived from the Carbohydrate-Active Enzymes (CAZY) Database (<http://www.cazy.org>; 18) suggested that *LicA* belongs to the family glycosyl hydrolase 16 (Figure 2).

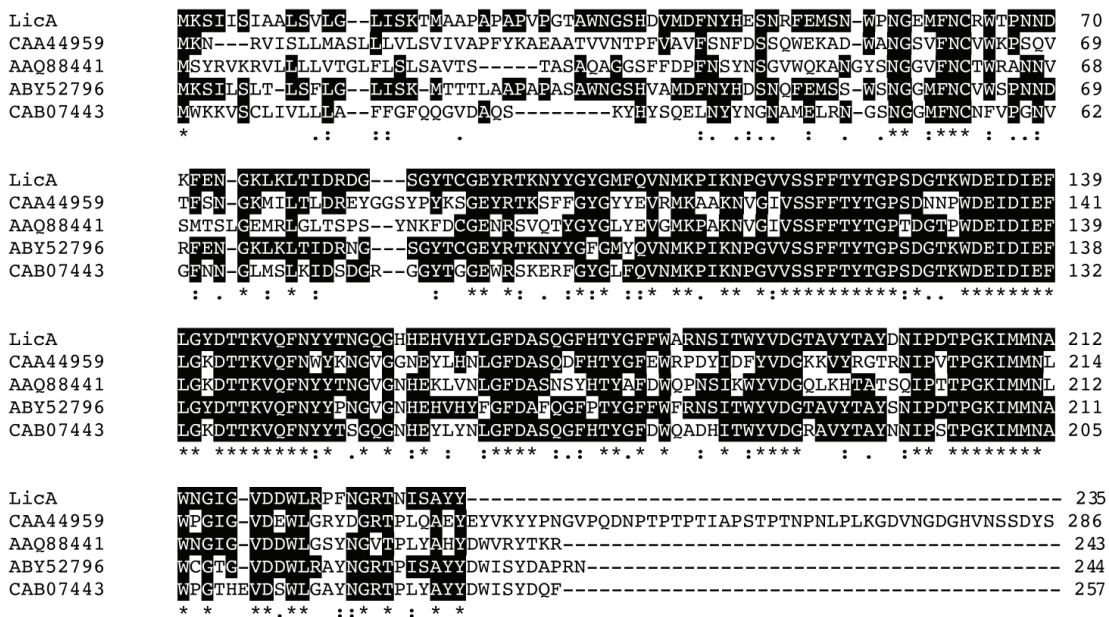


Figure 2. Multiple amino acid alignment of β -(1,3-1,4)-glucanase (*LicA*) isolated from *Orpinomyces* sp. GMLF18 and 4 other β -(1,3-1,4)-glucanases from other glycosyl hydrolases family 16. Residue positions that are identical in all 5 sequences are indicated with asterisks. The sources of the β -(1,3-1,4)-glucanase sequences: *C. thermocellum* (GenBank Accession No. CAA44959), *B. licheniformis* (AAQ88441), *P. communis* (ABY52796), *S. bovis* (CAB07443).

Enzyme characterization

The effect of pH and temperature on lichenase activity was investigated for both *E. coli* pCTL18 and *Orpinomyces* sp. GMLF18 (Figure 3). The activity of lichenase was determined from pH 3.5 to 9.0 by using 3 different buffer systems with lichenan as the substrate. The maximum activity of LicA from *E. coli* pCTL18 was observed at pH 5.0-6.0 and temperatures of 40-50 °C. Similar pH and temperature profiles were observed in lichenan degrading enzymes from *Orpinomyces* sp. GMLF18. LicA lost 9% of activity at pH 4.0, but *Orpinomyces* sp. GMLF18 lichenase recorded 39% of activity at the same pH value. No activity was observed at pH 9.0 regardless of enzyme source. However, LicA from transformed *E. coli* was more active than *Orpinomyces* sp. GMLF18 lichenase at 20 °C (52% and 29% of activity, respectively). The thermal stability of LicA was tested by incubating the enzyme at different temperatures for different preincubation times. LicA lost all of its activity in the first 5 min at 60 and 70 °C, and 88% of LicA activity was left at 50 °C in 20 min. LicA preserved its activity after 20 min preincubation at 40 °C.

Transformation of *Lactococcus lactis* subsp. *cremoris* MG1363

For the expression of the *licA* gene in *L. lactis*, a second construct was made by fusing the plasmid pCTL18 with the vector pIL253. The constructed pILL18 carrying the *licA* gene was introduced by electroporation into *L. lactis*. A congo-red plate assay was applied to confirm the β -(1,3-1,4)-glucanase

enzyme activities of the transformants. β -(1,3-1,4)-glucanase activity was readily observed by clear zone formation around the transformant colonies, indicating lichenan degradation. *L. lactis* MG1363 was also checked for β -(1,3-1,4)-glucanase activity by the same method and no activity was observed around the colonies (Figure 4). The expression of plasmids from the transformant colonies was tested by PCR and restriction analysis, and the expected results were obtained from the constructed plasmid. To prevent plasmid loss in further studies, erythromycin was added to the media at a concentration of 5 μ g/mL when the transformants were cultivated.

Expression of β -(1,3-1,4)-glucanase in *E. coli* and *L. lactis*

The expression and distribution of the cloned enzyme was determined in the extracellular and intracellular fraction of the cultures (Table 2). An important amount of total lichenase activity in *E. coli* harboring the plasmid pCTL18 was found in the intracellular fraction (>95%). Enzyme production started at the early growth stages (45.18 U/mL at 6 h) of the *E. coli* pCTL18 culture and it remained stable for 24 h. Other carbon sources such as carboxymethyl cellulose, avicel, and xylan were also used as substrate; however, no detectable activity could be observed with these carbon sources.

Intracellular and supernatant β -(1,3-1,4)-glucanase activities were found very similar at 6 h but after that major activity was detected in the extracellular fraction of *L. lactis* pILL18 culture. The

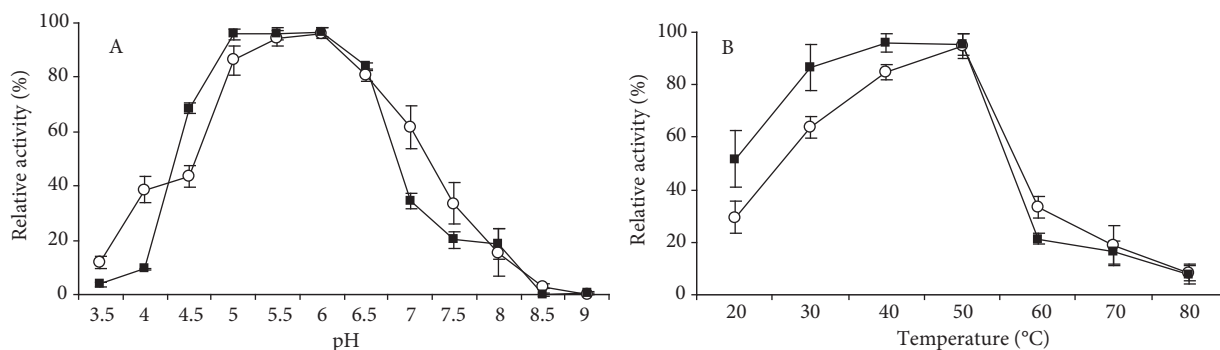


Figure 3. Influence of pH and temperature on the activity of lichenan degrading enzymes of *Orpinomyces* sp. GMLF18. (A) Effect of the pH determined at 50 °C; (B) effect of the temperature determined at pH 6.5. Symbols: (-o-) *Orpinomyces* sp. GMLF18 lichenase enzymes; (-n-) LicA from *E. coli* pCTL18.

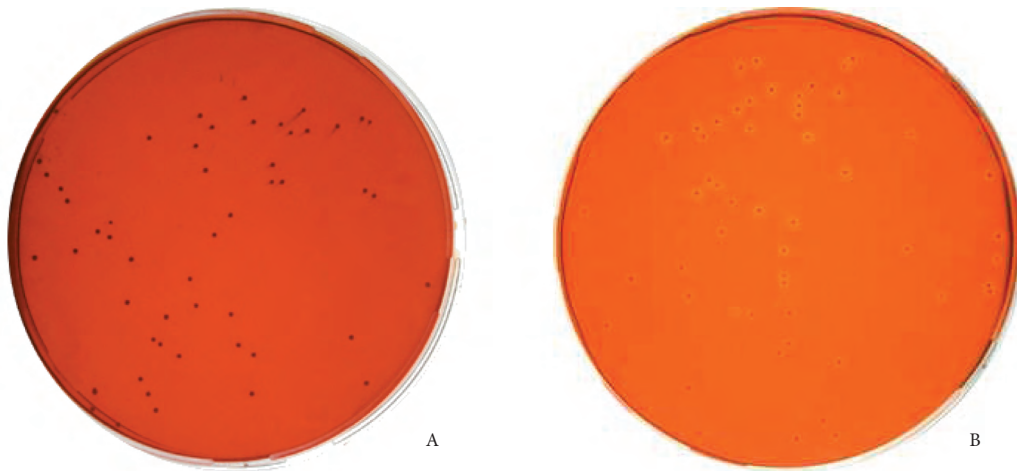


Figure 4. Screening of lichenase activity. Although lichenase activity was not detected in *L. lactis* subsp. cremoris MG1363 (A), lichenase was secreted from *L. lactis* pILL18, which reflects the expression of *licA* (B).

Table 2. β -(1,3-1,4)-glucanase activity (U/ml) produced by *E. coli* pCTL18 and *L. lactis* pILL18 carrying recombinant plasmids containing the b-(1,3-1,4)-glucanase gene.

Time (h)	<i>E. coli</i> pCTL18		<i>L. lactis</i> pILL18	
	Extracellular	Cell associated	Extracellular	Cell associated
6	ND*	45.18 \pm 2.00	1.87 \pm 0.30	1.99 \pm 0.13
12	2.08 \pm 0.29	46.47 \pm 1.85	9.98 \pm 0.54	1.02 \pm 0.09
24	2.04 \pm 0.12	52.87 \pm 1.31	5.44 \pm 0.38	0.63 \pm 0.05

* Not detected

maximum activity levels for β -(1,3-1,4)-glucanase in the supernatant fraction was determined to be 9.98 U/mL at 12 h. More than 50% of the total β -(1,3-1,4)-glucanase activity was maintained after 24 h in the culture medium.

It could be seen in the zymogram analysis of the culture supernatant from *Orpinomyces* sp. GMLF18 that there were lichenan degrading enzymes with a molecular mass higher than 50 kDa and only one active band was visualized with an approximate mass of 26 kDa (Figure 5). *E. coli* and *L. lactis* have only one band for lichenase activity and this band was equivalent to the ~26 kDa band of *Orpinomyces* sp. GMLF18, which is consistent with the deduced molecular mass of *licA*.

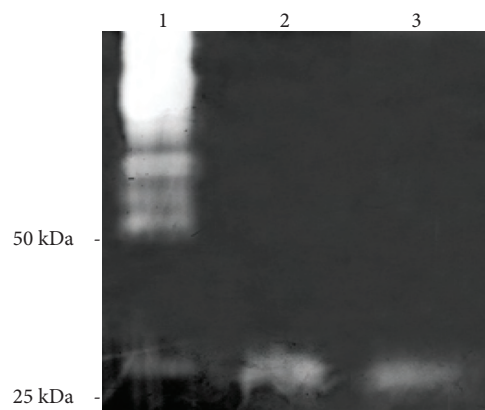


Figure 5. SDS-PAGE zymogram of lichenase activity. Lane 1, culture supernatant of *Orpinomyces* sp. GMLF18 (day 7); Lane 2, intracellular fraction of *E. coli* pCTL18 (12 h); Lane 3, culture supernatant of *L. lactis* pILL18 (12 h).

Rumen is well adapted for the fermentation of plant cell walls (27) and rumen fungi are an important group of rumen microbial ecosystems that can extensively colonize and degrade plant cell walls (28). Rumen fungi are also capable of fermenting cereal grains (29) and *Orpinomyces joyonii* was found to be more successful in the digestion of cereal grains than *N. patriciarum* and *P. communis* (30). *N. patriciarum* and *P. communis* exhibited their lichenase activities mostly on vegetative fractions (31), whereas *Orpinomyces* sp. GMLF18 secreted its lichenase enzymes mainly into the medium. Therefore, the high extracellular activity detected in this study could explain the success of *O. joyonii* in cereal digestion (30).

The production of β -(1,3-1,4)-glucanase is well known in ruminal bacteria (5,32), but *Orpinomyces* sp. PC-2 was the first reported for rumen fungi (7). The LicA isolated in this study was almost identical to the LicA from *Orpinomyces* sp. PC-2 except for only one difference: GMLF18 had valine at the position of 162 whereas PC-2 had isoleucine at the same position. High homology (77%-81%) between rumen fungal β -(1,3-1,4)-glucanases support the hypothesis of Eberhardt et al. (33) that a lateral transfer of genes could be occurring between anaerobic fungi. LicA from GMLF18 had a high degree of similarity (47%-59%) with bacterial β -(1,3-1,4)-glucanases and LicA was grouped with bacterial β -(1,3-1,4)-glucanases under the family of glycosyl hydrolases 16.

The pH and temperature effects on the activity of recombinant LicA have a similar profile to *Orpinomyces* sp. GMLF18 lichenase enzymes. Recombinant LicA was more active at pH 4.5-5.5, but GMLF18 lichenase activity was more active at pH 6.5-8.0. Our findings about pH and temperature optima were in agreement with previous reports. The optimum pH and temperature of rumen fungal enzymes have been found to be around pH 5.5-6.5 and 45-55 °C, respectively (34,35). The optimum pH and temperature values for LicA from *Orpinomyces* sp. PC-2 were reported to be pH 5.8-6.2 and 45 °C, respectively (7), which are similar to the optima values of LicA from GMLF18. A variant of the *O. joyonii* β -glucanase enzyme also had similar optimal activity conditions, while the thermal stability of the enzyme was increased by random mutagenesis and screening (36).

Zymogram analysis showed that the cloned *licA* gene product of *E. coli* pCTL18 was visualized as a single band with an expected molecular mass of 26 kDa. Several lichenase degrading enzymes were detected from the culture supernatant of *Orpinomyces* sp. GMLF18. The minor band (approx. 26 kDa) of the isolate GMLF18, which was equivalent to the cloned gene product, seems to be secreted free from a multienzyme complex because of the absence of dockerin domains in the *licA*. The other bands of molecular masses higher than 50 kDa indicated cellulases that could degrade lichenan. Several cellulase genes such as *celD* (37) from *N. patriciarum*; *celA* and *celC* (38) from *Orpinomyces* sp. PC-2; and *celB29* and *celB2* (39) from *O. joyonii* were reported to exhibit lichenase activity.

Previously, several genes of rumen fungi were expressed in lactic acid bacteria such as xylanase in *Lactobacillus reuteri* (12) and cellulase in *L. lactis* (40). In this study, the rumen fungal β -(1,3-1,4)-glucanase *licA* gene was expressed and secreted β -(1,3-1,4)-glucanase into the culture medium of *L. lactis* with the aid of the pIL253 vector. pIL253 is derived from the broad host range plasmid pAMb1, and the copy number of pIL253 in *L. lactis* has been estimated at 45-85 copies per cell (16). Rumen bacterial β -(1,3-1,4)-glucanases were previously cloned and expressed in lactic acid bacteria (5,12) and in this study we have demonstrated that a rumen fungal β -(1,3-1,4)-glucanase gene can be expressed successfully in the lactic acid bacterium *L. lactis* subsp. *cremoris* MG1363, although the enzymatic activity was lower than that in *E. coli*. Similarly, expression of the β -(1,3-1,4)-glucanase gene of *S. bovis* was also found to be much lower in *Enterococcus faecalis* and *L. lactis* than that of *S. bovis*, and it was suggested that this situation could arise from several differences, such as promoter recognition or translation, between the bacterial strains (5).

The *Orpinomyces* sp. GMLF18 *licA* gene was functionally expressed in *L. lactis* MG1363 and the resulting enzymes were exported to the medium. The ability to produce and secrete rumen fungal β -(1,3-1,4)-glucanase represents a significant potential for poultry as a probiotic. However, further experiments are required to evaluate the colonization and β -(1,3-1,4)-glucanase secretion of transformed *L. lactis* cells in the gastrointestinal tract of broilers.

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References

- Demirbas A. β -Glucan and mineral nutrient contents of cereals grown in Turkey. *Food Chem* 90: 773-777, 2005.
- Planas A. Bacterial 1,3-1,4- β -glucanases: structure, function and protein engineering. *Biochim Biophys Acta* 1543: 361-382, 2000.
- Lloberas J, Perez-Pons JA, Querol E. Molecular cloning, expression and nucleotide sequence of the endo- β -1,3-1,4-D-glucanase gene from *Bacillus licheniformis*. *Eur J Biochem* 97: 337-343, 1991.
- Schimming S, Schwarz WH, Staudenbauer WL. Structure of the *Clostridium thermocellum* gene *licB* and the encoded β -1,3-1,4-glucanase: a catalytic region homologous to *Bacillus* lichenases joined to the reiterated domain of clostridial cellulases. *Eur J Biochem* 204: 13-19, 1992.
- Ekinci MS, McCrae SI, Flint HJ. Isolation and overexpression of a gene encoding an extracellular β -(1,3-1,4)-glucanase from *Streptococcus bovis* JB1. *Appl Environ Microbiol* 63: 3752-3756, 1997.
- Saravanan R, Pavani Devi V, Shanmugam A et al. Isolation and partial purification of extracellular enzyme (1,3)- β -D glucanase from *Trichoderma reesei* (3929). *Biotech* 6: 440-443, 2007.
- Chen H, Li XL, Ljungdahl LG. Sequencing of a 1,3-1,4- β -D-Glucanase (Lichenase) from the anaerobic fungus *Orpinomyces* strain PC-2: Properties of the enzyme expressed in *Escherichia coli* and evidence that the gene has a bacterial origin. *J Bacteriol* 179: 6028-6034, 1997.
- Fric F, Huttova J. Glucanase, glucan synthase and chitinase activity in barley genotypes susceptible or resistant to *Erysiphe graminis* f.sp. *hordei*. *Biologia Plantarum* 35: 95-101, 1993.
- Genc H, Ozdemir M, Demirbas A. Analysis of mixed-linked (1-3),(1-4)- β -D-glucans in cereal grains from Turkey. *Food Chem* 73: 221-224, 2001.
- Annisson G, Choct M. Anti-nutritive activities of cereal non-starch polysaccharides in broiler diets and strategies minimizing their effects. *World Poultry Sci J* 47: 232-242, 1991.
- Wang ZR, Qiao SY, Lu WQ et al. Effects of enzyme supplementation on performance, nutrient digestibility, gastrointestinal morphology, and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. *Poult Sci* 84: 875-881, 2005.
- Liu JR, Yu B, Liu FH et al. Expression of rumen microbial fibrolytic enzyme genes in probiotic *Lactobacillus reuteri*. *Appl Environ Microbiol* 71: 6769-6775, 2005.
- Wegmann U, O'connell-Motherway M, Zomer A et al. Complete genome sequence of the prototype lactic acid bacterium *Lactococcus lactis* subsp. *cremoris* MG1363. *J Bacteriol* 189: 3256-3270, 2007.
- Çömlekcioglu U, Akyol İ, Kar B et al. Anaerobik rumen funguslarının izolasyonu, tanımlanması ve kültür koleksiyonunun oluşturulması. *Hayvansal Üretim* 49: 29-35, 2008.
- Orpin CG. Studies on the rumen flagellate *Sphaeromonas communis*. *J Gen Microbiol* 94: 270-280, 1976.
- Simon D, Chopin A. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. *Biochimie* 20: 559-566, 1988.
- Leenhouts K, Buist G, Bolhuis A et al. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol Gen Genet* 253: 217-224, 1996.
- Gasson MJ. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast induced curing. *J Bacteriol* 154: 1-9, 1983.
- Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673-4680, 1994.
- Cantarel BL, Coutinho PM, Rancurel C et al. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res* 37 (Database issue): D233-D238, 2009.
- Mandel M, Higa A. Calcium-dependent bacteriophage DNA infection. *J Mol Biol* 53: 159-162, 1970.

22. Holo N, Nes F. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. *Appl Environ Microbiol* 55: 3119-3123, 1989.
23. Miller GL. Use of dinitrosalicylic reagent for the determination of reducing sugars. *Annal Chem* 31: 426-428, 1959.
24. Aygan A, Arıkan B. Production and characterization of multifunctional endoxylanase by *Bacillus* sp. X13. *Turk J Biol* 33: 231-237, 2009.
25. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685, 1970.
26. Liu JR, Yu B, Zhao X et al. Coexpression of rumen microbial β -glucanase and xylanase genes in *Lactobacillus reuteri*. *Appl Microbiol Biotechnol* 77: 117-124, 2007.
27. Weimer PJ. Cellulose degradation by ruminal microorganisms. *Crit Rev Biotechnol* 12: 189-223, 1992.
28. Borneman WS, Hartley RD, Morrison WH et al. Feruloyl and *p*-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation. *Appl Microbiol Biotechnol* 33: 345-351, 1990.
29. Yanke LJ, Dong Y, McAllister TA et al. Comparison of amylolytic and proteolytic activities of ruminal fungi grown on cereal grains. *Can J Microbiol* 39: 817-820, 1993.
30. McAllister TA, Dong Y, Yanke LJ et al. Cereal grain digestion by selected strains of ruminal fungi. *Can J Microbiol* 39: 367-376, 1993.
31. Williams AG, Orpin CG. Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi on a range of carbohydrate substrates. *Can J Microbiol* 33: 418-426, 1987.
32. Teather RM, Erfle JD. DNA sequence of *Fibrobacter succinogenes* mixed-linkage β -glucanase (1,3-1,4- β -D-glucan 4-glucanohydrolase) gene. *J Bacteriol* 172: 3837-3841, 1990.
33. Eberhardt RY, Gilbert HJ, Hazlewood GP. Primary sequence and enzymic properties of two modular endoglucanases, Cel5A and Cel45A, from the anaerobic fungus *Piromyces equi*. *Microbiology* 2000; 146, 1999-2008.
34. Lowe SE, Theodorou MK, Trinci APJ. Cellulases and xylanases of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose and xylan. *Appl Environ Microbiol* 53: 1216-1223, 1987.
35. Akyol I, Comlekcioglu U, Kar B et al. Cloning of a xylanase gene *xyn2A* from rumen fungus *Neocallimastix* sp. GMLF2 in *Escherichia coli* and its partial characterization. *Biologia* 64: 664-670, 2009.
36. Kim SA, Cheng KJ, Liu JH. A variant of *Orpinomyces joyonii* 1,3-1,4- β -glucanase with increased thermal stability obtained by random mutagenesis and screening. *Biosci Biotechnol Biochem* 66: 171-174, 2002.
37. Xue GP, Gobius KS, Orpin CG. A novel polysaccharide hydrolase cDNA (*celD*) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities. *J Gen Microbiol* 138: 2397-2403, 1992.
38. Li XL, Chen H, Ljungdahl LG. Two cellulases, CelA and CelC, from the polycentric anaerobic fungus *Orpinomyces* strain PC-2 contain N-terminal docking domains for a cellulase-hemicellulase complex. *Appl Environ Microbiol* 63: 4721-4728, 1997.
39. Qiu X, Selinger B, Yanke LJ et al. Isolation and analysis of two cellulase cDNAs from *Orpinomyces joyonii*. *Gene* 245: 119-126, 2000.
40. Ozkose E, Akyol I, Kar B et al. Expression of fungal cellulase gene in *Lactococcus lactis* strains to construct novel recombinant silage inoculants. *Folia Microbiol* 54: 335-342, 2009.