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HETEROLOGOUS EXPRESSION OF LICHENASE GENE FROM *Streptococcus bovis* in *Escherichia coli* STRAIN XL1 BLUE MRF′

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ABSTRACT

In this study, *Streptococcus bovis* lichenase gene was cloned into recombinant vector pBR325SE2 (pBR325 plus *Spirulina platensis* serine esterase gene) and thus the integrative vector pBR325SLic was constructed. The pBR325SLic vector was transferred into the competent *E. coli* XL1 Blue MRF′ cells. Transformed bacteria *E.coli*/pBR325SLic grew in the selective medium containing chloramphenicol (Cm) while there were no non-transformant in the same medium. 1800 bp gene product using recombinant vector as template in PCR experiment was showed on the agarose gel. Lichenase production by recombinant *E. coli* cells cultivated in liquid media reached a maximum at 36 h, with levels of 192.8 μmol mg^{-1} protein/min. Optimum pH and temperature values for recombinant enzyme activity were 7.0 and 40 °C respectively. The recombinant enzyme lost most of its activity after pre-incubation at 50 °C for 15 min. However, the enzyme maintained its stability significantly in the pH range 4.0-10.0. The molecular weight of the enzyme was estimated to be 95.6 kDa by sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

KEYWORDS:
Lichenase, *Streptococcus bovis*, *Escherichia coli*, Integrative vector, Cloning

INTRODUCTION

β-1,3-1,4-glucan is the major cell wall component of barley and other cereal endosperms. It consists of a polymer of glucose monomers joined by β-1,3- and β-1,4-glycosidic bonds in an irregular fashion [1, 2, 3]. 1,3-1,4-β-D-Glucan 4-glucanohydrolase (β-1,3-1,4-glucanase; lichenase) (EC 3.2.1.73) cleaves β-1,4 linkages adjacent to β-1,3 bonds in glucans, yielding cellobiosyltriose and cellotriosyltetraose [4, 5, 6]. Lichenases genes have been found in several microorganisms such as *B. subtilis* [7], *B. amyloliquefaciens* [8], *B. macerans* [9], *B. licheniformis* [10], *B. brevis* [11], *B. circulans* [12], *B. polymyxa* [13], *Clostridium thermocellum* [14], *Streptococcus bovis* [15], *Trichoderma reesei* [16] and *Orpinomyces* [6]. The β-(1,3-1,4)-glucanase gene from *Streptococcus bovis* was also cloned and expressed in *Lactococcus lactis* and *Enterococcus faecalis* [15].

Microbial lichenases used widely in animal feedstuff, especially for broiler chickens and piglets to improve digestibility and to reduce sanitary problems such as sticky droppings by enzymatic preparations containing bacterial β-glucanases [17, 18] and control of fungal pathogens and release of intracellular materials from microbial cells [19]. Therefore, the 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 [20].

The serine esterase family includes enzymes responsible for the hydrolysis of a wide range of carboxyl ester substrates ranging from acetylcholine to long-chain fatty-acid esters of cholesterol or triacylglycerol and the juvenile hormones of insects. A particular serine residue at the active site of these enzymes is rendered reactive by the presence of histidine and a glutamate (or aspartate) residue, the components of the catalytic triad. Serine esterases catalyse hydrolysis of carboxylate esters [21].

In the present study, we aimed to construct the integrative vector pBR325SLic by cloning the *S. bovis* lichenase gene into pBR325SE2, and thus express the gene in *E. coli* XL1 Blue MRF' cells recombinantly.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions. *E. coli* XL1 Blue MRF' and pBR325 plasmid DNA were purchased from Stratagene and Sigma-Aldrich respectively. Recombinant plasmid DNA pBR325SE2 (pBR325 plus *S. platensis* serine esterase gene) was created in our laboratory [22]. pBR325SLic (pBR325SE2 plus lichenase gene) was created in this study. β-(1,3-1,4)-glucanase gene of *Streptococcus bovis* in host *E. coli* were obtained from culture collection of Prof. Dr.

Mehmet Sait Ekinci (University of Kahramanmaraş Sütçüimam, Kahramanmaraş, Turkey). *E. coli* XL1 Blue MRF' was cultured in LB (Luria-Bertani, 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl, the pH was adjusted to 7.5 with 1 M NaOH) and LB-Agar (1.5% wt/v agar) at 37 °C. For cultivation the bacteria *E. coli*/pBR325SE2 and *E. coli*/pBR325SLic, LB and LB-agar were supplemented with 25 μg mL^{-1} chloramphenicol (Cm).

DNA Modification and Cloning Procedures. The following modifying enzymes were purchased and used for DNA modifications; *Hind* III, *Bam* HI, 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 [23]. The lichenase gene was derived from the pL1Hc using *Hind* III and *Bam* HI restriction endonucleases. The lichenase gene was excised from gels and purified using the Genomic DNA Purification Kit (Fermentas). The shuttle vector pBR325SE2 was digested by *Hind* III and *Bam* HI and then dephosphorylated. The excised lichenase gene was cloned into the linearized pBR325SE2 and thus pBR322SLic was created. Recombinant plasmid pBR325SLic was transformed into the competent *E. coli* MRF' cells using the method as described previously [24].

PCR Analysis of Cloned Gene. For PCR analysis, recombinant plasmid pBR325SLic was subjected for isolation from recombinant *E. coli* cells according to [25]. The recombinant plasmid was used as a template in the PCR experiment to produce DNA fragment including gene sequence. Following primers were used for amplification of lichenase gene from the recombinant vector: Primer F: 5'-AAGTGAATTTAACTACGA-3', Primer R: 5'-GCTCTTCCATCAACATAC-3'. Amplification was performed on a Blue-Ray Biotech thermocycler and the PCR program was as follows: 95 °C for 3 min, 45 cycles of 94 °C for 40 s, 49 °C for 40 s, 72 °C for 40 s, and a final extension at 72 °C for 10 min [26]. The product (5 μL) was separated on a 0.8% agarose gel and analyzed for the yield of amplicons of expected sizes.

Enzymatic Properties of Recombinant Enzyme. β-(1,3-1,4) Glucanase (lichenase) activity was assayed by adding 1 mL enzyme (supernatant - extracellular proteins of 24 hours cultured recombinant *E. coli* cells) to 1 mL lichenan solution (2%, wt/v) in 0.1 M Na-phosphate buffer, pH 6.0 and incubating at 40°C for 30 min. After the reaction was stopped by the addition of 3 mL of 3,5-dinitrosalicylic acid reagent and incubating the sample tubes in boiling water for 5 min, 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 10 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 8-10) [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.

The pH stability of lichenase was investigated over a pH range of 4–11 in 0.05 M buffers. Buffers used were Na-acetate (pH 4–6), Na-phosphate (pH 6–8), and tris (pH 8–11). Aliquots were taken after 30 min of incubation at 40°C and immediately assayed for residual lichenase activity [29].

Electrophoretic Analysis of Extracellular Proteins. To obtain the extracellular proteins of recombinant *E. coli* from culture supernatants, the overnight cultured cells were pelleted by centrifuge. The extracellular extracts (supernatants) were mixed with a 1:1 volume of 20% TCA for precipitation. After the incubation at room temperature 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-Lichenan-PAGE (0.2% lichenan) were done as described previously [30] 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 acid–water solution (4:1:5 by volume) and destained in the same solution without dye [31, 32]. 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 lichenase activity [33, 34, 35].

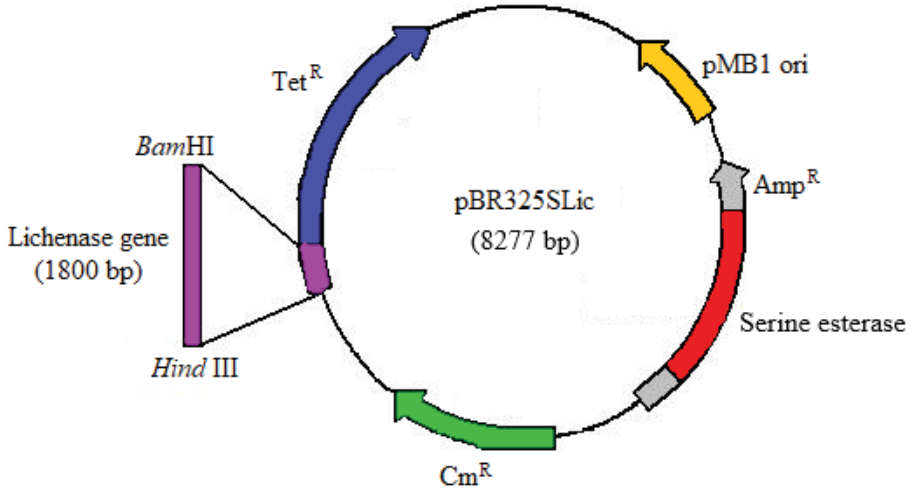


FIGURE 1
Structure of pBR325SLic (8277 bp; pBR325SE2 plus lichenase gene)

RESULTS AND DISCUSSION

In this study, the recombinant vector pBR325SLic (Figure 1) was created by ligating the lichenase gene into the *S. platensis*/*E. coli* integration vector pBR325SE2 [22] and transferred into the competent *E. coli* cells. *E. coli* colonies carrying the lichenase gene produced an activity zone as a result of Congo-red staining in the LB/Lichenan/Agar plate (Figure 2). PCR analysis using recombinant vector also produced a single band on agarose gel electrophoresis belonging to the lichenase gene of 1800 bp (Figure 3).



FIGURE 2
Congo-red staining of LB-lichenan-agar plate carrying recombinant and non-recombinant *E. coli* colonies (1: *E. coli* MRF′ strain as control (without any plasmid-no growth), 2: Recombinant *E. coli*/pBR325SLic colony showing clear lichenase activity)

Lichenase is one of the three distinct kinds of endoglucanases which active on lichenan and cereal glucans but not on carboxymethylcellulose and laminarin [36]. *Streptococcus bovis* JB1 lichenase gene cloned and characterized previously [15]. The 710 bps length gene is responsible for the production of 25 kDa protein. If plasmids carry sequences homologous to those of the host organism they could integrate into the chromosome [37]. Such an integrative vector had previously been developed cloning the cyanobacterium *Spirulina platensis* serine esterase gene into the plasmid pBR325 for *S. platensis* [22]. Although the *Streptococcus bovis* lichenase gene was previously cloned in *Escherichia coli* with different vectors [18], it was first cloned in this study using an integrative vector.

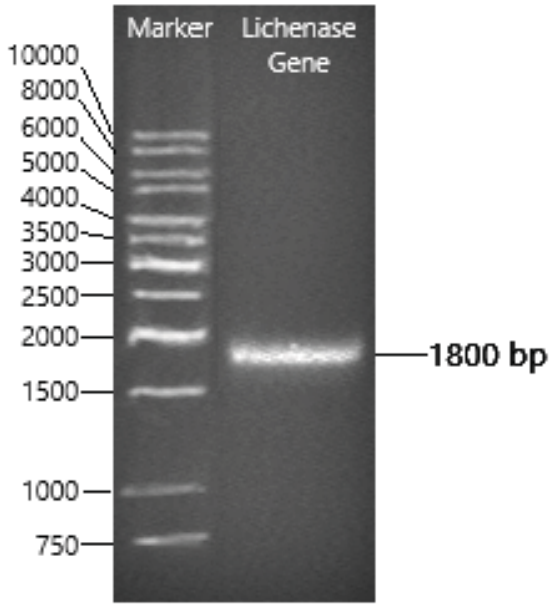


FIGURE 3
Agarose gel electrophoresis of lichenase gene amplified from recombinant vector via PCR

Temperatures between 20 and 100 ºC were used to test recombinant lichenase activity. Maximum lichenase activity was observed at 40 ºC (Figure 4A). The relative enzyme activities of the enzyme were 54, 94, 77 and 43% at 20, 30, 50, and 60 ºC respectively, after 30 min incubation. The enzyme lost most of its activity after incubation at 90 and 100 ºC. Besides,the relative enzyme activitiy between 20-60 ºC was calculated as 73.6%. Lichenase enzymes show a wide variation in pH and temperature optimum values. Teng et al [38] reported the optimum temperature of the *B. licheniformis* lichenase as 40 ºC. However, it was reported by other literature reviews that bacterial lichenases mostly show optimum activity above 40 ºC. Ekiz [39] and Kim et al [40] reported the optimum temperature of lichenase enzyme as 45 ºC, while Sun et al [41] and Furtado et al [42] 50 ºC, Afshin et al [43] and Yang et al [44]] 55 ºC, and Yang et al [45] 70 ºC. Optimum temperature of several lichenases from other studies were reported as 45 ºC [39, 40], 50 ºC [41, 42], 55 ºC [43, 44], and 70 ºC [45]. These reports indicated that optimum temperature of the recombinant lichenase is between normal values.

The thermal stability of the enzyme was determined by measuring its residual activity after preincubating at various temperatures. Enzyme activity remained relatively stable at 50 ºC and below after 30 min pre-incubation, but decreased rapidly beyond this temperature (Figure 4A). The stability of the enzyme at different pH levels was determined by preincubating the enzymes with the appropriate buffers. We found that the enzyme exhibited a significant amount of activity within the broader range of pH 4.0–10.0 (Figure 4B). Thermostability of enzymes is highly important in industrial applications, particularly in processes that require heat treatment. Recombinant lichenase may not show the expected thermal stability in heat treated industrial processes. This problem could be overcome by transferring the lichenase gene to a thermophilic bacterium, thereby increasing the thermostability of the enzyme. Aşan and Özcan [18] reported that the thermo-stability of the enzyme increased by cloning the *Streptococcus bovis* lichenase gene in a thermophilic bacterium *Streptococcus salivarius* subsp *thermophilus* FI8976. But this may not be enough for industrial production. This problem could be solved by transferring the gene into a non-pathogen thermophilic *Bacillus* strain suitable for industrial production. Such a genetic modification can increase the thermostability of enzymes, and a significant increase in enzyme production amounts depending on the copy numbers of the plasmid DNAs in the host microorganism.

Recombinant lichenase exhibited its maximum activity at pH 7.0 (Figure 4B). The average relative enzyme activity between pH 5.0 and 9.0 was 86%. Nevertheless, the average relative enzyme activity at pH 4.0–10.0 was 74%. The average enzyme activity at acidic (4.0-6.0) and alkaline (8.0-11.0) pH values were calculated as 65 and 61%, respectively. It has been reported that many bacterial lichenases show optimum activity around neutral pH values [44]. Although the recombinant lichenase enzyme showed its optimum activity at pH 7.0, it exhibited a wide pH activity range. Similar data were observed for the pH stability of the enzyme. The recombinant lichenase showed remarkable residual activity at pH 4.0-10.0. A similar result was reported by Yang et al [44] for the thermophilic fungus *M. cinnamomea* lichenase enzyme

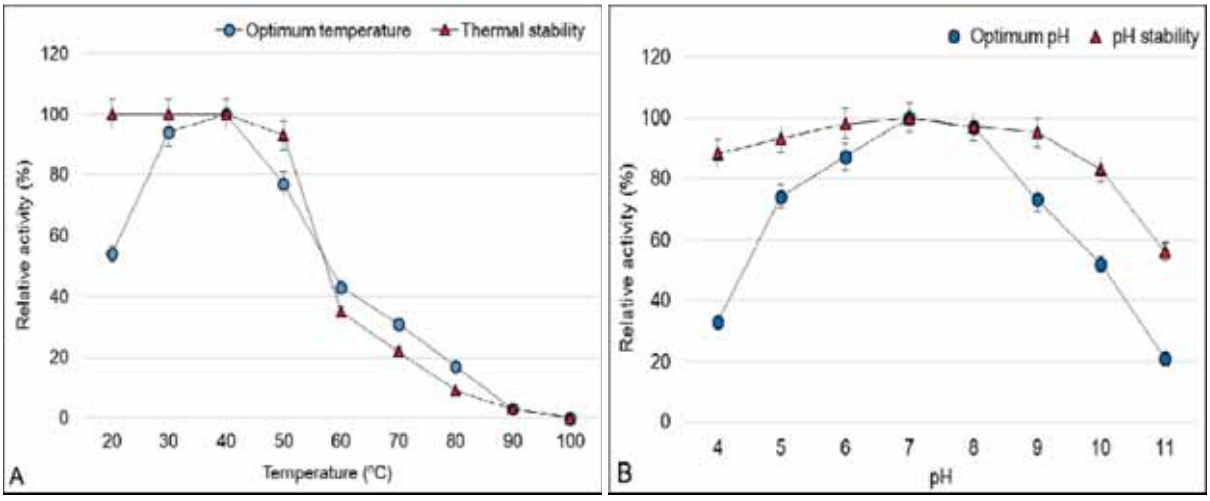


FIGURE 4
Enzymatic properties of recombinant lichenase. A) Effect of temperature on enzyme activity and stability, B) Effect of pH on enzyme activity and stability

with high activity values between 4.0-10.0 pH values. The high relative activity and stability of the lichenase enzyme at alkaline pH values make this enzyme a suitable candidate for use in areas such as detergent, paper and fruit juice industries where alkaline conditions are applied.

CONCLUSION

In the present study, *Streptococcus bovis* lichenase gene was cloned into *E. coli* XL1 Blue MRF′ via recombinant vector pBR325SE2. Research findings suggest that recombinant lichenase coud be used in industrial applications with current properties. However, the enzyme need to develop for thermal stability by cloning the gene into a thermostable bacterial strain.

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