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Research Article

Expression of the β-(1,3-1,4)-Glucanase Gene in *Streptococcus salivarius* subsp. *thermophilus*

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Abstract: The purpose of this study was the transformation and expression of the β-(1,3-1,4)-glucanase (lichenase) gene in *Streptococcus salivarius* subsp. *thermophilus* to create a recombinant probiotic for poultry and improve the thermostability of the lichenase enzyme. The recombinant plasmid TL1R containing the β-(1,3-1,4)-glucanase gene was introduced into *S. salivarius* subsp. *thermophilus* by electrotransformation. The expressing of the β-(1,3-1,4)-glucanase gene in *S. salivarius* subsp. *thermophilus* was confirmed on lichenan plate, SDS-PAGE, and zymogram analysis. The β-(1,3-1,4)-glucanase enzyme expressed by *S. salivarius* subsp. *thermophilus* cells seemed to increase its capacity for thermoresistance and so it maintained its activity at 70 ºC for 15 min. In contrast, the enzyme produced by *Lactococcus lactis* and *Escherichia coli* cells easily ceased activity when exposed to the same temperature. The enzyme expressed by all the recombinant bacteria resisted denaturation and somehow remained soluble after heat treatment from 37 to 100 ºC for 15 min.

Key Words: *Streptococcus salivarius* subsp. *thermophilus*, β-(1,3-1,4)-glucanase, expression, thermostability

β-(1,3-1,4)-Glukanaz Geninin *Streptococcus salivarius* subsp. *thermophilus*'da Ekspresyonu

Özet: Bu çalışmada, kanatlılara yönelik rekombinant probiyotik geliştirmek amacıyla β-(1,3-1,4)-glukanaz (likenaz) geninin *Streptococcus salivarius* subsp. *thermophilus*'a aktarılması, ekspresyonu ve likenaz enziminin sıcaklığa direncinin artırılması amaçlanmıştır. β-(1,3-1,4)-glukanaz genini taşıyan rekombinant TL1R plazmidi *S. salivarius* subsp. *thermophilus*'a elektrotransformasyon yöntemi ile aktarılmıştır. β-(1,3-1,4)-glukanaz geninin *S. salivarius* subsp. *thermophilus*'da ekspresyonu likenanlı besiyeri, SDS-PAGE ve zymogram analizleri ile tespit edilmiştir. *S. salivarius* subsp. *thermophilus*'un sentezlediği β-(1,3-1,4)-glukanaz enzimi, sıcaklığa direnci artarak 70 ºC'de 15 dakika aktivitesini korumuştur. Buna karşılık, *Lactococcus lactis* ve *Escherichia coli* tarafından sentezlenen β-(1,3-1,4)-glukanaz enzimi aynı sıcaklık uygulamasında aktivitesini kaybetmiştir. Tüm rekombinant bakterilerce sentezlenen β-(1,3-1,4)-glukanaz enzimi 37-100 ºC'lerde 15 dakika sıcaklık uygulamasından sonra denatürasyona direnerek bir şekilde suda çözünürlüğünü kaybetmemiştir.

Anahtar Sözcükler: *Streptococcus salivarius* subsp. *thermophilus*, β-(1,3-1,4)-glukanaz, ekspresyon, sıcaklığa dirençlilik

Introduction

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 (1). β-(1,3-1,4)-glucanases (or lichenases, EC 3.2.1.73) hydrolyze linear β-glucans and lichenan, with a strict cleavage specificity for β-(1,4) glycosidic bonds on 3-O-substituted glucosyl residues (2).

A number of *Bacillus* species secrete lichenases, and genes have been cloned and characterized from *Bacillus subtilis* (3), *Bacillus amyloliquefaciens* (4), *Bacillus macerans* (5), *Bacillus circulans* (6), *Bacillus polymyxa* (7), *Bacillus licheniformis* (8), and *Bacillus brevis* (9). Genes encoding β-(1,3-1,4)-glucanases have also been isolated and cloned from non-*Bacillus* species. The β-(1,3-1,4)-glucanase gene from *Streptococcus bovis* was also cloned and expressed in *Lactococcus lactis* IL2661 and *Enterococcus faecalis* JH2-SS (10).

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Applications of β-glucanases such as lichenase in the processing of animal feed, control of fungal pathogens, and release of intracellular materials from microbial cells have been reviewed (11). 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 the digestibility of barley-based diets, and reduces sanitary problems (sticky droppings) (1). 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 (12).

Probiotics have been defined as "live microbial feed supplements that beneficially affect the host health by improving the intestinal balance" (13). Probiotics have been primarily used to establish normal intestinal flora to prevent or minimize the disturbances caused by enteric pathogens and secondarily to serve the function of antibiotic feed additives in the diets of animals. The probiotics currently on the market/under investigation contain *Lactobacilli* (*L. bulgaricus*, *L. sporogenes*, *L. plantarum,* etc.), Streptococcus (*S. salivarius* subsp. *thermophilus*, *S. faecium,* etc.), *Pediococcus* (*P. halophilus*, *P. pentasaccus*), *Bifidobacterium* spp., *Saccharomyces* (*S. cereviasiae*, *S. boulardii*), and *Bacillus* (*B. cereus*, *B. subtilis*), etc. (14). Various genes have been expressed in *S. salivarius* subsp. *thermophilus*, such as *Streptomyces* cholesterol oxidase gene (15), tyrosinase (16), rhodococcal indigo gene (17), and pediocin operon (18).

In the present study, we aimed to express the β-(1,3-1,4)-glucanase (lichenase) gene of *S. bovis* in *S. salivarius* subsp. *thermophilus* to create a recombinant probiotic for poultry, thereby combining the beneficial effects of both probiotic and lichenase enzyme. We also intended to improve the thermostability of lichenase enzyme by expression of the β-(1,3-1,4)-glucanase gene in *S. salivarius* subsp. *thermophilus* to use in pelleted poultry feeds.

Materials and Methods

Bacterial Strains, Plasmid, and Growth Media

S. salivarius subsp. *thermophilus* FI8976, *L. lactis* IL1403 strains, and TL1R plasmid DNA containing the β-(1,3-1,4)-glucanase gene of *S. bovis* in *Escherichia coli* were obtained from M. Sait EKİNCİ (Kahramanmaraş Sütçü İmam University, Kahramanmaraş, Turkey). *S. salivarius* subsp. *thermophilus* and *L. lactis* were cultured in M17 at 42 and 37 ºC, respectively. *E. coli* was cultured in LB (Luria Bertani) at 37 ºC. All recombinant techniques were performed according to Sambrook et al. (19) unless otherwise stated.

Isolation and Transformation of Plasmid DNA

The plasmid TL1R (pTRW10 vector plus the β-(1,3-1,4)-glucanase (lichenase) gene) was isolated from *E. coli* according to Birnboim and Doly (20). The recombinant plasmid DNA (TL1R) was transferred into *S. salivarius* subsp. *thermophilus* FI8976 by electrotransformation using the modified protocol for *Lactobacillus* strains (21) and *L. lactis* IL1403 (22,23).

Detection of Lichenase Activity on Lichenan Plates

Lichenase positive transformants were detected using M17 and LB 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 min incubation at room temperature, the Congo Red solution was replaced with a 1 M NaCl solution for 15 min. The NaCl solution was removed and clear zones appeared around positive colonies (24).

Thermostability Test of Lichenase Enzyme on SDS-PAGE, SDS-Lichenan-PAGE, and Non-Denaturing-Lichenan-PAGE

To determine of thermostability of β-(1,3-1,4)-glucanase (lichenase) enzyme secreted by *S. salivarius* subsp. *thermophilus*/TL1R, *L. lactis*/TL1R, and *E. coli*/TL1R, the culture supernatant was exposed to various temperatures (37, 40, 50, 60, 70, 80, 90, 100 ºC) for 15 min before centrifugation at 15,000 rpm for 15 min to remove denaturated proteins. The supernatant was then mixed with an equal volume of tricholoroacetic acid (TCA). Total proteins were collected by re-centrifugation. Protein analysis was performed using a denaturating polyacrylamide gel electrophoresis (SDS-PAGE, 12% w/v) (25).

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 rocked in 50 mM sodium phosphate buffer and 20% (v/v) isopropanol solution at room temperature for 1 h to remove denaturated agents. The gel was then transferred into renaturation solution buffer (50 mM sodium phosphate, 5 mM β-mercaptoethanol, 1 mM EDTA) and incubated overnight at 4 ºC. After renaturation of the protein, the gel was soaked in 50 mM sodium phosphate buffer at 4 ºC for 1 h. It was then covered with parafilm and incubated at 37 ºC for 4 h. After staining of the gel with 0.2% (w/v) Congo Red and 5 mM NaOH for 1 h, it was washed with 1 M NaCl and 5 mM NaOH overnight to remove excess stain from the active bands (26).

Non-denaturing-lichenan-polyacrylamide gel electrophoresis was performed by using a modified protocol for SDS-Lichenan-PAGE. Denaturing agents such as SDS (sodium dodecyl sulfate) and β-mercaptoethanol were omitted from all solutions and the Tris concentration in all buffers was reduced to half. All samples were directly applied to the gel without boiling and TCA precipitation after heat treatment at various temperatures, followed by centrifugation at 15,000 rpm for 15 min (27).

Thermostability Test of Lichenase Enzyme

The culture supernatant of recombinant bacteria was exposed to various temperatures such as 37, 40, 50, 60, 70, 80, 90, and 100 ºC for 15 min and then centrifuged for 15 min to precipitate denaturated proteins. The supernatant was then dropped on a lichenan plate. After overnight incubation at 37 ºC, the petri plates were stained with Congo Red and then destained with NaCl solution (24).

Results

TL1R construct (Figure 1), carrying the β-(1,3-1,4)-glucanase (Lichenase) gene, was transferred into *S. salivarius* subsp. *thermophilus* FI8976 and *L. lactis* IL1403 cells by electrotransformation. Recombinant *S. salivarius* subsp. *thermophilus*/TL1R and *L. lactis*/TL1R colonies were observed on M17 agar medium supplemented with 10 μg mL^{-1} erytromycine. Lichenase activity of transformed bacteria on lichenan plates was detected with Congo Red staining and clear zones were than appeared around the recombinant colonies.

Culture supernatant of *S. salivarius* subsp. *thermophilus, L. lactis,* and *E. coli* carrying TL1R was heat-treated for 15 min at various temperatures (37, 40, 50, 60, 70, 80, 90, and 100 ºC) and then centrifuged at 15,000 rpm for 15 min to precipitate denaturated proteins. Total proteins remained soluble in the supernatant after heat treatment was applied to SDS-PAGE and SDS-Lichenan-PAGE to visualize total proteins and zymogram analysis, respectively. Denaturated proteins were renaturated on SDS-Lichenan-PAGE after removing denaturating agents from the gel and then

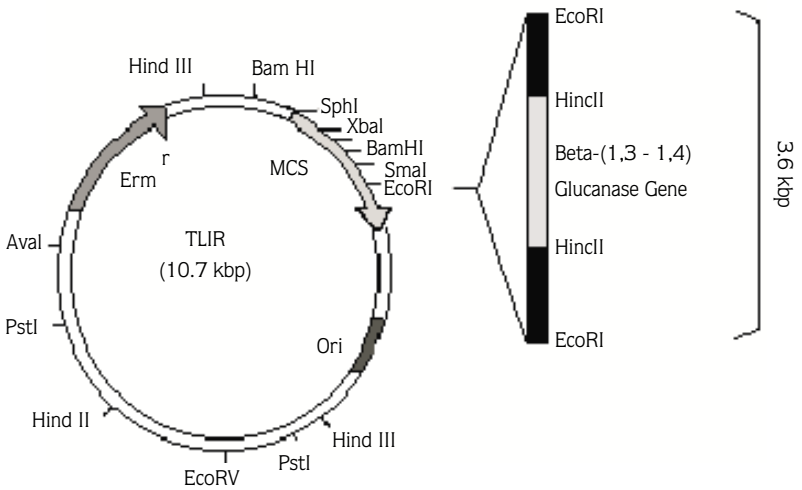

Figure 1. Structure of TL1R plasmid (pTRW10 plus β-(1,3-1,4)-glucanase (Lichenase) gene).

allowing to the enzyme to digest substrate, thereby producing clear zones on the gel for all the temperature ranges for all the recombinant bacteria tested here. The culture supernatant was also applied directly to both non-denaturing-lichenan-PAGE and lichenan assay plates for detecting remaining enzyme activity after heat treatment at various temperatures for TL1R transformed clones.

In addition, 26 kDa protein bands on SDS-PAGE and visible enzyme zones on SDS-Lichenan-PAGE at all temperatures clearly confirmed that the enzyme was somehow not denaturated (or partially denaturated) and so still remained soluble in the supernatant after heat treatment at various temperature as shown on SDS-PAGE and SDS-Lichenan-PAGE (Figure 2A, B and C). On the other hand, the enzyme maintained its activity on test plates as well as non-denaturing-lichenan-PAGE (data not shown) only up to 70, 60, and 50 ºC in the supernatant of *S. salivarius* subsp. *thermophilus*/TL1R, *L. lactis*/TL1R, and *E. coli*/TL1R, respectively (Figure 3A, B, and C).

Discussion

The β-(1,3-1,4)-glucanase (Lichenase) gene from *S. bovis* was cloned and expressed in *L. lactis*, *E. coli*, and *E. faecalis* (10,28). However, this is the first report to the best of our knowledge in which the β-(1,3-1,4)-glucanase gene from *S. bovis* was expressed in *S. salivarius* subsp. *thermophilus.* Lichenase enzyme secreted from *S. salivarius* subsp. *thermophilus* was found to be active up to 70 °C on lichenan overlay plates as well as non-denaturating PAGE gels. On the other hand, the enzyme expressed by all bacteria was soluble after heat treatment at all temperature ranges up to 100 °C as shown by zymogram analysis. Zymogram analysis clearly indicated that heat-treated enzyme was still soluble (not denaturated or partially denaturated) before loading on SDS-Lichenan-PAGE. It confirmed that, after renaturation of the enzyme on the gel, enzyme activity was restored for all the heat-treated samples. Solubility of the enzyme in the culture supernatant is host-independent but the activity is clearly affected by the host by which the gene encoding the enzyme is expressed.

All these results clearly indicated that the enzyme was soluble up to 100 ºC in the culture supernatant of *S. salivarius* subsp. *thermophilus, L. lactis*, and *E. coli*, but active at 70 ºC only in the culture supernatant of *S. salivarius* subsp. *thermophilus*. Lichenase activity was generally suppressed by high temperature and low pH or other denaturating agents, but inhibition of enzyme generally was reversible. When the enzyme was preincubated with buffers, at 37 ºC, pH was then adjusted to 6.5, and enzyme activity recovered. Thus any inactivation of the cloned gene product by temperature or accumulation of high lactic acid in batch cultures is expected to be reversible. This may play an important role in the stomach and intestine environment. Similarly, high temperature may cause only temporary inactivation of the β-(1,3-1,4)-glucanase, which would become active again at a suitable temperature (28).

The constructed recombinant probitic bacterium *S. salivarius* subsp. *thermophilus* does not colonize in the

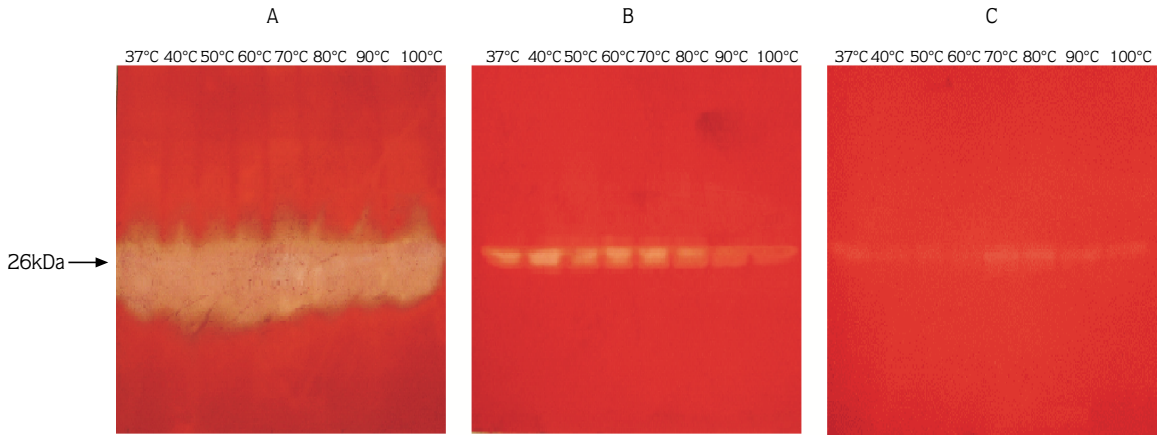

Figure 2. Thermostability of β-(1,3-1,4)-glucanase (Lichenase) in various bacterial culture supernatants at various temperatures on SDS-Lichenan-PAGE (A) *S. salivarius* subsp. *thermophilus* /TL1R, (B) *L. lactis*/TL1R, (C) *E.coli*/TL1R.

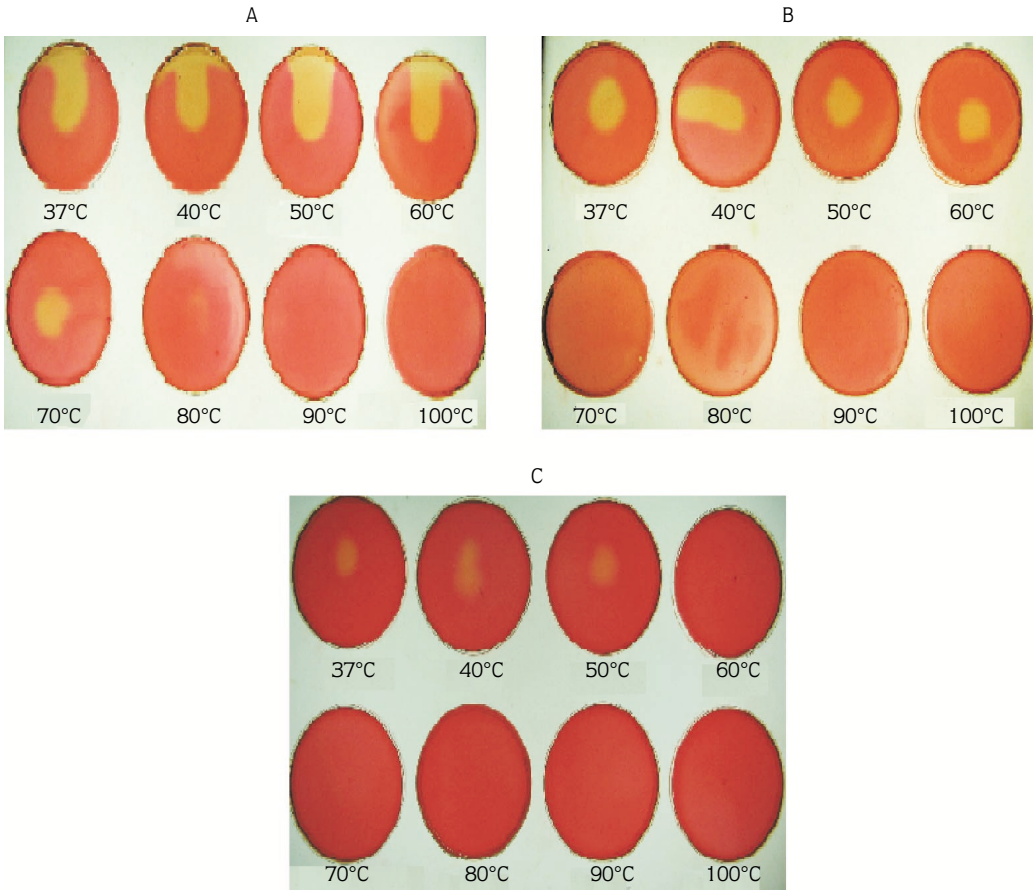

Figure 3. Thermostability of β-(1,3-1,4)-glucanase (Lichenase) in various bacterial culture supernatants at various temperatures on lichenan plates (A) *S. salivarius* subsp. *thermophilus* /TL1R, (B) *L. lactis*/TL1R, (C) *E. coli*/TL1R.

poultry intestine, but when it is added to a barley-based pelleted diet it may be viable. Because the bacterium is moderately thermophilic and because of the thermostability of β-(1,3-1,4)-glucanase, the enzyme can be renatured at the optimal conditions after the pelleting temperature (70-90 °C). The recombinant bacteria can produce β-(1,3-1,4)-glucanase and digest β-glucans in the poultry gastrointestinal system. Therefore, *S. salivarius* subsp. *thermophilus* producing β-(1,3-1,4)-glucanase may be a good candidate as a recombinant probiotic, combining the beneficial effects of both probiotic and moderately heat-resistant lichenase enzyme for poultry.

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