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Cloning of lichenase gene into *Bacillus subtilis* and partial characterization of the enzyme

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Enzymes used as feed additives increase the ability of animals to benefit from feed. From these enzymes, animals fed with lichenase enzyme can produce animal foods with high immunity and performance. Therefore, it is possible to obtain more healthy, high quality products in a shorter time. In this study, the recombinant vector pNW33N carrying the lichenase gene of *Streptococcus bovis* genome was transferred into *Bacillus subtilis* RSKK245 strain via electroporation technique. Besides, the DNA band of lichenase obtained from recombinant vector pNW33N/Lichenase after restriction endonucleases was observed on agarose gel. Enzymatic activity sites around *B. subtilis* RSKK245 colonies are shown by staining with Congo-red. The molecular weight of the enzyme was determined as 26 kDa via SDS-PAGE and zymogram analysis. This study has successfully demonstrated expression of lichenase gene in *B. subtilis* RSKK245 strain.

Keywords: Feed conversion rate, β-1,3(4)-Glucanase, Recombinant DNA

Increasing population with improved standard of living globally demands timely production of healthy and quality foods of animal origin in large quanty. Factorssuch as growth rate, care and nutrition of animals; strengthening their genetic structure; feed conversion rate; feed digestibility; and yield play an important role in achieving this goal. In order to have a high yield, it is necessary to maximize the feed conversion rate as well as protecting animal health. Feed additives are one of the important applications used to this end[1].

Enzymes used as feed additives are of fungal and bacterial origin. Various enzymes such as protease, glucanase, cellulase, pectinase, amylase, phytase and lipase are used alone or in combination in the feed industry[2]. Animals fed with glucan-containing feeds have higher levels of serum immunoglobulin and become more resistant to infectious diseases. However, externally adding beta-glucan to fish feeds is very expensive and increases the feed costs. With the advent of modern production techniques, the enzyme industry has made great progress in the last half century. In line with this progress, enzyme biotechnology has made important strides and enabled purer, cheaper, and more abundant production of industrially important enzymes[3-5].

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Lichenase are found in the cell walls of the higher plants of *Poaceae* family[6]. In addition to plants, many microorganisms such as bacteria and fungi also contain significant amounts of β-(1,3-1,4)-glucanase[7–9]. The molecular weight of *Bacillus* lichenases is typically between 25 and 30 kDa. However, their weight was reported to be 90 kDa in *Ruminococcus flavefaciens*, 40.7 kDa in *Talaromyces emersonii*, 38 kDa in *Clostridium thermocellum*, and 37 kDa in *Fibrobacter succinogenes*. Their optimum temperature values also differ among bacteria, such as 80°C in *Clostridium thermocellum*[10], 50°C in *Fibrobacter succinogenes* and *Streptococcus bovis*[11], 55°C in the bacteria with the origin of *Bacillus subtilis*, *B. amyloliquefaciens* and *B. licheniformis*[12], 45°C in *B. polymyxa*[5], and 65°C in those with the origin of *B. macerans*, *B. brevis*, and *Bacillus* sp. N137[13].

The lichenase gene which hydrolyses β-glucans at a higher level than other glucanases, plays a vital role in animal feeds preparation[14-17]. In the present study, we have made an attempt to produce the lichenase gene recombinantly, and also characterize the enzyme lichenase partially.

Material and Methods

Bacteria, plasmids and growth media

We procured *B. subtilis* RSKK245 bacterium and pNW33N/β-(1,3-1,4)-glucanase plasmid (6017 bp)

from the Animal Biotechnology and Genetic Engineering Laboratory, Department of Animal Science, Faculty of Agriculture, Çukurova University.

The bacteria were grown in Luria-Bertani (LB) broth (1% w/v tryptone, 1% w/v NaCl, 0.5%w/v yeast extract, pH 7.5) at 37°C under aerobic conditions. Then, they were stored at −20°C in LB medium containing 20% v/v glycerol. Agar 1.5% w/v and 0.1% w/v lichen were added to the solid medium for the lichenase test. The bacteria were stored in glycerol stocks (20% v/v).

Isolation, cleavage and electrotransformation of DNA

pNW33N/β-(1,3-1,4)-glucanase plasmid was isolated from the recombinant *Bacillus* sp./pNW33N/β-(1,3-1,4)-glucanase bacterium using the method reported by Hardy[18]. The isolated recombinant pNW33N/β-(1,3-1,4)-glucanase plasmid DNA was cut into linear with *Hind* III and *Bam* H1 endonuclease enzymes. Table 1 shows the components of the cleavage reaction. The cleavage reaction was viewed by electrophoresis using 0.8% agarose gel.

The recombinant pNW33N/β-(1,3-1,4)-glucanase DNA was transferred to the strain of *Bacillus subtilis* using the electrotransformation method and the protocol reported by Xue *et al*. [19] .

Recombinant colony selection and phenotypic test

Recombinant colonies grown on transformation plates were marked and then cultured on LB/Agar/Lichenan/Cml (34 μg/mL cml) test plates using sterile toothpicks. Then, the plates were incubated for colony growth in an incubator at 37ºC until the next day. Then, the phenotypic determinations of the recombinant bacterial colonies were performed for lichenase activity using 0.2% w/v Congo-red staining. In this treatment, 0.2% w/v Congo-red stain was placed on the bacterial plate in an amount enough to cover its surface. After an incubation of 15 min, the stain was removed, and 1M NaCl solution was added to the plate, and then the plate was left for another 15 min to remove excess stain from the solid medium. Congo-red stains the lichen medium red; therefore, the colonies forming a yellowish zone around the red-stained surface were identified as lichenase-positive bacteria.

For the comparative phenotypic test of the recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase and the non-recombinant *B. subtilis* RSKK245, the bacteria that were cultured and grown overnight were cultured on antibiotic (34 μg/mL cml) and non-antibiotic plates using toothpick and grown at 37ºC for one more night.

The plates on which the bacteria were cultured and grown overnight for the lichenase test of the recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase bacteria and non-recombinant *B. subtilis* RSKK245 bacteria were stained with 0.2% w/v Congo-red. Then, the recombinant and non-recombinant bacteria were compared in terms of lichenase activity.

SDS-PAGE and zymogram analysis

The recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase bacteria were precipitated by centrifugation to obtain extracellular proteins. The supernatant was mixed with TCA (20% w/v) at a ratio of 1:1 until homogenous and left overnight at room temperature. The total protein precipitated by centrifugation was dissolved in 1 M Tris (pH 8) solution. The polyacrylamide gel electrophoresis (12 wt/v SDS-PAGE and 0.2% lichenan SDS-Lichenan-PAGE) was performed to analyze the proteins using the method reported by Laemmli [20].

Results

The recombinant pNW33N/β-(1,3-1,4)-glucanase plasmid was isolated from *Bacillus* sp. and transferred to *B. subtilis* RSKK245 using electrotransformation. Lichenase-positive *B.subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase strains were obtained as a result of the electrotransformation. When examined in 0.8% agarose gel, pNW33N plasmid DNA with a size of 4217 bp and the bands of β-(1,3-1,4)-glucanase gene with a size of 1800 bp were observed as a result of the cleavage reaction of the plasmid DNA (Fig. 1).

Identification of recombinant colonies

The *B. subtilis* RSKK245 bacterium, which was used as a control after electrotransformation, showed an extensive colony growth on LB/Agar/Lichenan plates not containing chloramphenicol (Fig. 2A), but no colony growth was observed on plates containing chloramphenicol (Fig. 2B). It was observed that the recombinant *B.subtilis*RSKK245/pNW33N/β-

Table 1 — Components of cleavage reaction of pNW33N/β-(1,3-1,4)-glucanase plasmid DNA

Content of the Reaction Mixture	Amount (μL)
dH_2O	17
DNA	7
Reaction buffer	3
Enzyme (*Hind*III+*Bam* H1) (10 U/μL)	3
Total	30

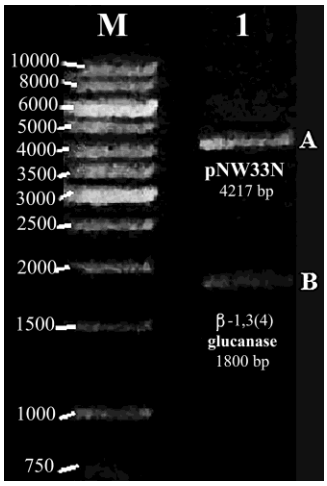

Fig. 1 — The cleavage analysis result of the recombinant pNW33N/β-1,3(4)-glucanase plasmid DNA. [Lane M: Marker and Lane 1: pNW33N/β-(1,3-1,4)-glucanase. A: pNW33N plasmid (4217 bp); and B: β-(1,3-1,4)-Glucanase gene (1800 bp)]

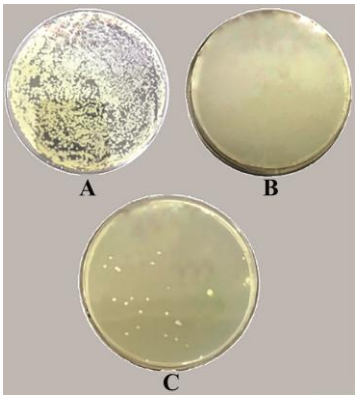

Fig. 2 — Electrotransformation results for the recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase colonies and the control group. *B. subtilis* RSKK245 colonies on (A) LB/Agar/Lichenan plate; (B) LB/Agar/Lichenan/Cml plate; and (C) Recombinant colonies carrying plasmid pNW33N/β-(1,3-1,4)-glucanase on LB/Agar/Lichenan/Cml plate

(1,3-1,4)-glucanase bacteria formed colonies on LB/Agar/Lichenan/Cml plates thanks to their chloramphenicol resistance gene (Fig. 2C).

Phenotypic test of the recombinant colonies

The recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase bacteria and the non-recombinant *B. subtilis* RSKK245 bacteria were compared on plates containing lichen with and without antibiotics for a phenotypic test. Both the recombinant and non-recombinant bacteria were observed to grow and form colonies in plates not containing antibiotics. However, while the recombinant bacteria formed colonies in the medium containing antibiotics, it was observed that the non-recombinant *B. subtilis* RSKK245 bacterium without

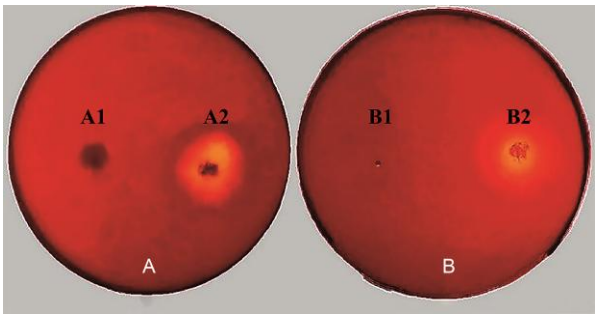

Fig. 3 — Comparative lichenase activity test for the recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase and the non-recombinant *B. subtilis* RSKK245 bacteria. (A) LB/Agar/Lichenan (A_1: Non-recombinant *B. subtilis* RSKK245 bacterium and A_2: Recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase bacterium); and (B) LB/Agar/Lichenan/Cml (B_1: Non-recombinant *B. subtilis* RSKK245 bacterium (No growth) and B_2: Recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-Glucanase bacterium)

plasmids could not grow. As for the Congo-red staining of the colonies cultivated on selective media, it was observed that the recombinant colonies on both plates produced an activity zone [Fig. 3 A(ii) and B(ii)], but the non-recombinant bacterial colonies without plasmids could not produce an activity zone [Fig. 3 A(i) and B(i)]. These results show that the β-(1,3-1,4)-glucanase gene produces the relevant enzyme by being expressed in *B. subtilis* RSKK245 bacterium.

SDS-PAGE and zymogram analysis

The recombinant *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase bacteria were compared with the non-recombinant *B. subtilis* RSKK245 bacteria in SDS-PAGE. It was observed that, after the transfer of the non-recombinant *B. subtilis* RSKK245 strain into the pNW33N/β-(1,3-1,4)-glucanase plasmid by electrotransformation, a new band with a molecular weight of 26 kDa was formed (Fig. 4A). As a result of staining the gel with Congo-red after zymogram analysis, β-(1,3-1,4)-glucanase gene showed an enzyme activity (Fig. 4B).

These results proved that when the recombinant pNW33N/β-(1,3-1,4)-glucanase plasmid was transferred to *B. subtilis* RSKK245 strain by electrotransformation, the β-(1,3-1,4)-glucanase gene was produced by being expressed in this bacterium.

Discussion

Recombinant enzyme production is not a widely used in Turkey, although it is less costly and more sustainable. In this study, biotechnological methods and genetic engineering techniques were applied to

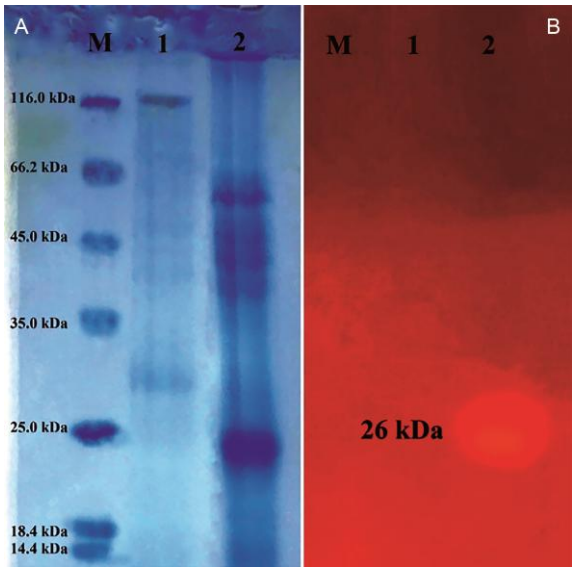

Fig. 4 — Comparison of proteins of the recombinant and non-recombinant bacteria by SDS-PAGE and zymogram analysis. (A) SDS-PAGE analysis; and (B) Zymogram analysis [Lane M: Marker, Lane 1: *B. subtilis* RSKK245 bacterium, and Lane 2: *B. subtilis* RSKK245/pNW33N/β-(1,3-1,4)-glucanase bacterium)]

transfer the β-(1,3-1,4)-glucanase gene, which is responsible for the production of lichenase enzyme and can be used as an alternative antimicrobial agent in the future, to the *B. subtilis* RSKK245 strain using recombinant pNW33N vector and to express it. By this means, a new recombinant bacterium that produces this enzyme was developed.

Lichenase enzyme is used effectively in the food and beverage industry and the medical/pharmaceutical industry, as well as being used to increase the digestibility of feeds in animal production[21,22]. In the future, the substrate specificity of the recombinant enzyme can be investigated and its possible uses and potentials can be revealed more accurately[23,24]. In other words, it turned out to be a very popular material in molecular biology studies. This is also evident from the number of studies on the identification, isolation, and cloning of the genes responsible for the production of lichenase enzymes of different organisms. For example, isolated the *bgaA* gene encoding the endo-β-(1,3-1,4)-glucanase enzyme from *Bacillus* sp. N137 and cloned it in *E. coli* with its own promoter [25] . Expressed *licA*, the first lichenase (β-(1,3-1,4)-D-glucanase EC 3.2. 1.73) gene consisting of 971-bp cDNA from *Orpinomyces* sp. PC-2 and isolated from fungi, in *E. coli*[6]. In other studies, lichenase genes were expressed by transferring them from *B. subtilis* MA139 to *Pichia pastoris* X-33 strain[26] from *B. subtilis* to *E. coli* JM101[27]; from *B. circulans* to *B. subtilis* RM125 and *B. megaterium* ATCC14945 strains[28]; from *Streptococcus bovis* bacterium to *Spirulina platensis* M2 and S*pirulina platensis* N-39 strains[29]; and from *Orpinomyces* sp. GMLF18 to *E. coli*[30]. Likewise, Echinci *et al*.[31] Isolated the lichenase gene from *S. bovis* JB1 genome, transferred it to *S. bovis*, *Lactococcus lactis* IL2661, and *Enterococcus faecalis* JH2-SS, and expressed[31]. Characterization of parental and mutant strains with the genotype of interest could help to map conserved regions within *B. subtilis* strains. Thus, by purifying isozymes from the relevant genotype within *B. subtilis* strains, comprehensive biochemical analyses can be performed to determine which isozymes will maximize the feed conversion rate of animals.

In the present study, as a result of SDS-PAGE and zymogram analysis, the molecular weight of the β-(1,3-1,4)-glucanase enzyme was found to be 26 kDa. Many β-(1,3-1,4)-glucanase genes were reported to have a similar molecular weight. β-(1,3-1,4)-Glucanase genes were reported to have a molecular weight of 24 kDa in both *E. coli* and *B. macerans* bacteria[32], 25 kDa in *L. lactis* IL2661 and *E. faecalis* JH2-SS bacteria[31], 25 kDa in *E. coli* BL21 bacteria[33], 26 kDa in *B. subtilis* RSKK246 bacteria[34], and 27 kDa[6], 28 kDa[35], 29 kDa in *E. coli* bacteria[36] and 55 kDa in *B. subtilis* A8-8[37]. The molecular weight found in the present study for the lichenase genes is similar to those reported in the above mentioned studies. The molecular weight of the β-(1,3-1,4)-glucanase gene of *Rhizomucor miehei* CAU432 as 35.4 kDa[38], while the molecular weight of the β-(1,3-1,4)-glucanase gene of *Ruminococcus flavefaciens* bacteria as 90 kDa[12]. The differences between species and within species in terms of molecular weights are mostly due to mutations in the evolutionary process, and this is considered normal. Many other enzyme studies reported similar results in this regard.

Multiple molecular enzyme forms that catalyze the conversion of the same substrate are called isozymes[39]. Isozymes have been categorized under three main categories based on their mutations. According to this categorization, isozymes may originate from (i) the multiple gene loci encoding different polypeptide chains; (ii) the multiple alleles at a single locus; and (iii) the secondary modifications that occur as a result of covalent modifications, conformational isomers, and the formations of

monomer, dimer, trimer, etc., of a basic subunit with more than one polypeptide chain.

Conclusion

In this study, the lichenase gene was produced recombinantly, and also, the lichease enzyme was characterized partially. It suggests that this enzyme can be used as a commercial feed additive to maximize the feed conversion rate of animals.

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Conflict of Interest

Authors declare no competing interests.

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