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RESEARCH ARTICLE

Cloning and Expression of β-1,3-Glucanase Gene from *Cellulosimicrobium cellulans* in *Escherichia coli* DH5α [1] [2]

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Summary

In this study, β-1,3-glucanase gene of *Cellulosimicrobium cellulans* was amplified by PCR and cloned in pUC18 cloning vector to construct the recombinant plasmids pTEG5 and pTEG11. The recombinant plasmids pTEG5 and pTEG11 were transformed into competent *Escherichia coli* cells. Digestion of recombinant plasmids with *Sac*I produced 1.9 kbp β-1,3-glucanase gene band on agarose gel which indicated the gene integration. β-1,3-Glucanase gene amplification on the recombinant vectors also indicated 1.9 kbp gene insert. Recombinant enzyme was produced by *E. coli* intracellularly. Intracellular components of recombinant *E. coli* strains with pTEG5 or pTEG11 dropped on LB-laminarin-agar plate, showed clear positive zones by Congo-red staining revealing the activity of secreted protein. Based on the zymogram analysis, the intracellular produced recombinant β-1,3-glucanase enzymes exhibited the same activity bands with *C. cellulans* enzyme with respect to molecular weight.

Keywords: *β-1,3-Glucanase, Cellulosimicrobium cellulans, Cloning, Escherichia coli*

Cellulosimicrobium cellulans'dan β-1,3-Glukanaz Geninin *Escherichia coli* DH5α'da Klonlanması ve Ekspresyonu

Özet

Bu çalışmada *Cellulosimicrobium cellulans* bakterisine ait β-1,3-glukanaz geni PCR ile amplifiye edilerek pUC18 klonlama vektörüne klonlanmış, böylece pTEG5 ve pTEG11 rekombinant plazmit DNA'lar elde edilmiştir. Rekombinant plazmit DNA'lar pTEG5 ve pTEG11 kompetent *Escherichia coli* hücrelerine transfer edilmişlerdir. *Sac*I enzimi ile kesilmiş rekombinant plazmitlerin agaroz jelde elektroforezi sonucu 1.9 kbç büyüklüğündeki β-1,3-glukanaz gen bandının görünmesi, gen entegrasyonunun tamam olduğunu göstermiştir. β-1,3-glukanaz geninin rekombinant vektörlerden amplifikasyonu da 1.9 kbç büyüklüğündeki geni göstermiştir. Rekombinant enzim *E. coli* tarafından hücre içi olarak üretilmiştir. LB-laminarin-agar plağına damlatılan rekombinant *E. coli* suşlarının intraselüler içerikleri Congo-red boyaması ile pozitif zonlar üretmiş, böylece rekombinant bakterilerce üretilen proteinin aktif olduğu anlaşılmıştır. Zimogram analizinde, hücre içi üretilen rekombinant β-1,3-glukanaz enzimleri *C. cellulans* enzimi ile aynı moleküler ağırlığa ait aktivite bantları sergilemişlerdir.

Anahtar sözcükler: *β-1,3-Glukanaz, Cellulosimicrobium cellulans, Klonlama, Escherichia coli*

INTRODUCTION

The yeast cell wall consists mainly of glucan, mannoprotein, and chitin [1-3]. Among these compounds, the glucans are essential structural components, responsible for mechanical strength, shape, and elasticity of the yeast cell wall [3].

Endo-1,3-β-glucanases (EC 3.2.1.6 and EC 3.2.1.39) are widely distributed among bacteria and higher plants [4]. These enzymes catalyse the hydrolysis of β-1,3-glucan component found in the yeast cell wall and other β-1,3-

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glucans such as laminarin, curdlan and pachyman [3,5]. The bacterium *Cellulosimicrobium cellulans* (also known with the synonyms *Cellulomonas cellulans*, *Oerskovia xanthineolytica*, and *Arthrobacter luteus)* has been regarded as a major source of yeast-lytic enzymes, particularly endo-β-1,3-glucanases, proteases and mannanases [6]. Commercially available yeast-lytic glucanases preparations derived from this organism, namely Lyticase, Zymolyase, and Quantazyme, have been produced and widely used for yeast protoplast preparations and yeast DNA isolation [6-8]. Of these preparations, only Quantazyme (Quantum Biotechnology, Canada) is produced recombinantly and protease-free [6,7]. The β-1,3-glucanase gene from *C. cellulans* (ATCC 21606) was cloned and then sequenced previously [2].

In the present study, we amplified the β-1,3-glucanase gene from *C. cellulans* genome via PCR. The DNA insert carrying the gene of interest was subcloned and expressed in *Escherichia coli* strain DH5α to lead for our further studies.

MATERIAL and METHODS

Strains of Bacteria and Growth Conditions

Cellulosimicrobium cellulans (*Oerskovia xanthineolytica*, ATCC 21606) was cultured in GYM Streptomyces medium (glucose (0.4% wt/v), yeast extract (0.4% wt/v), malt extract (1% wt/v), pH 7.2) at 28°C. Agar (1.2% wt/v) and $CaCO_3$ (0.2% wt/v) were added into GYM Streptomyces medium for preparation of GYM Streptomyces agar. *Escherichia coli* strain DH5α was cultured in LB-broth (10 g bacto tryptone (Merck), 5 g yeast extract (Merck) and 10 g NaCl (Merck) per L, pH 7.5) at 37°C. Agar (1.5% wt/v) was added into LB medium for LB-agar. Both LB-broth and LB-agar were supplemented with ampicillin (50 μg mL^{-1}) for culturing of recombinant *E. coli* strains. For activity testing on LB-laminarin-agar plates, intracellular proteins of cultured recombinant *E. coli* strains obtained by sonication (50-60 kHz, Bandelin Electronic Sonopuls UWV 2070, Germany) and dropped onto the plate and air-dried for 15-20 min. After drying, the plate was incubated at 30°C for 4-5 h. The plate was stained with Congo-red solution (0.1% wt/v Congo-red) for 15 min and then destained with 1 M NaCl solution for 15 min. Clear bands at the dropped area indicated the presence of β-1,3-glucanase activity [9].

Plasmids

pUC18 (ampicillin resistant; amp®) was used to create recombinant vectors pTEG5 and pTEG11, harbouring β-1,3-glucanase gene. The recombinant plasmids pTEG5 and pTEG11 were used for *E. coli* transformation. Recombinant *E. coli* strains, carrying pTEG5 or pTEG11, were grown on LB-agar at 37°C supplemented with ampicillin (50 μg mL^{-1}) as a selective agent. Recombinant plasmids were isolated from *E. coli* cells containing pTEG5 or pTEG11 as described previously [10].

DNA Modification

The following modifying enzymes were purchased and used for DNA modifications; *Sac*I, *Sma*I, *Eco*RI 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 [11]. Linearized plasmid DNA and PCR product were excised from gels and purified using Genomic DNA Purification Kit (Fermentas).

PCR and Cloning Procedures

The sequences of the primers used for amplifying of β-1,3-glucanase gene were 5'-AGAGCTCGTGGCACTGCAC TCGTTCGAGTCT-3' (forward) and 5'-AGAGCTCGACGGGC GCGGTCAGAGCGTCCAG-3' (reverse) based on the gene sequence [2]. The PCR mixture consisted of 5 μL of reaction buffer, 1 μL of 40 mM dNTP mix (200 μM each final), 1 μL each of forward and reverse primers (20 pmol each primer), 0.5 μL of Pfu DNA polymerase (2.5 U/μL), 1 μL of 50% wt/v DMSO (1% wt/v final), and 650 ng of template in a total volume of 50 μL. The following amplification program was used: Initial denaturation step at 94°C for 2 min, then 30 cycles of denaturation at 98°C for 10 s, annealing and elongation at 68°C for 5 min. A final extension step was performed as 72°C for 5 min. The blunt ended PCR product was ligated into *Sma*I digested pUC18 plasmid DNA to create pTEG5 and pTEG11 using standard methods [11]. The ligation mixes contained approximately 360 ng of PCR product and 630 ng of linearized plasmid.

Recombinant plasmids pTEG5 and pTEG11 were transformed into *E. coli* DH5α strain using the method as described previously [12].

Electrophoretic Analysis of Extracellular and Intracellular Proteins

To obtain the extracellular proteins of *C. cellulans*, *E. coli* DH5α and recombinant *E. coli* strains from growth medias, the cells were pelleted by centrifuge. The extracellular extracts (supernatants) were mixed with 1:1 volume of 20% wt/v TCA for precipitation. After the incubation at room temperature for overnight, protein pellets were obtained by centrifuge. Air-dried protein pellets were dissolved in 0.1 M Tris-HCL buffer (pH 8.0). To obtain the intracellular proteins of recombinant *E. coli* strain from LB-broth, the cells were pelleted by centrifuge and then the pellets were dissolved in equal volume of water. After the sonication process (50-60 kHz) the samples were centrifuged. The protein pellets were obtained from the supernatant using 20% wt/v TCA as described above.

SDS-PAGE and SDS-Laminarin-PAGE (0.2% laminarin) were done as described previously [13] 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 acit–water solution (4:1:5 by volume) and destained in the same solution without dye [14,15]. 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. Gel was then transferred onto a glass plate, sealed with a film, and incubated at 30°C for 4 h. Gel was stained in a solution of Congo-red (0.1% wt/v Congo-red, 0.2 M NaOH), for 1 h, and destained in 1 M NaCl for 30 min. Clear bands indicated the presence of β-1,3-glucanase activity [16-18].

RESULTS

Transformation of Escherichia coli

The β-1,3-glucanase gene of *C. cellulans* was cloned into pUC18 and thus recombinant plasmids pTEG5 and pTEG11 were created *(Fig. 1)*.

The pTEG5 and pTEG11 recombinant plasmids were then transformed into *E. coli* DH5α strain to express the β-1,3-glucanase gene. Intracellular supernatant of recombinant *E. coli* strains showed β-1,3-glucanase activity on LB-agar plate supplemented with ampicillin (50 μg mL^{-1}) and laminarin (0.1% wt/v) by producing clear zones *(Fig. 2)*.

Recombinant pTEG5 and pTEG11 plasmids were isolated from *E. coli* cells. They were, then subjected to restriction fragment length analysis together with PCR amplified DNA fragment on agarose gel electrophoresis (0.8% wt/v) encoding the gene. β-1,3-Glucanase gene fragment (~1.9 kbp) amplified by PCR and restriction endonuclease digested recombinant plasmids confirmed the success of the cloning experiments *(Fig. 3)*. Recombinant plasmids digested with *Eco*RI were both yielded the same DNA fragments consisting of pUC18 and β-1,3-glucanase gene (both are the same plasmid).

Culture supernatants of *C. cellulans*, recombinant *E. coli*/pTEG5, and non-recombinant *E. coli* DH5α strains were applied to SDS-PAGE and SDS-Laminarin-PAGE to visualize total proteins and zymogram analysis, respectively. For

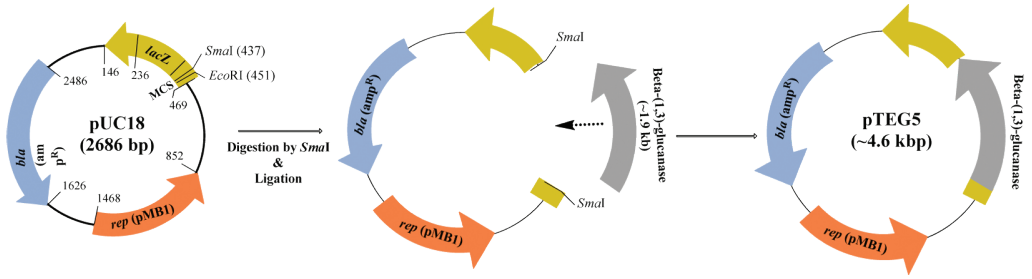

Fig 1. Construction of pTEG5 plasmid (~4.6 kbp) from β-1,3-glucanase gene (~1.9 kbp) and pUC18 plasmid (~2.7 kbp)
Şekil 1. β-1,3-glukanaz geni (~1.9 kbç) ve pUC18 plazmidinden (~2.7 kbç) pTEG5 plazmidinin (~4.6 kbç) oluşturulması

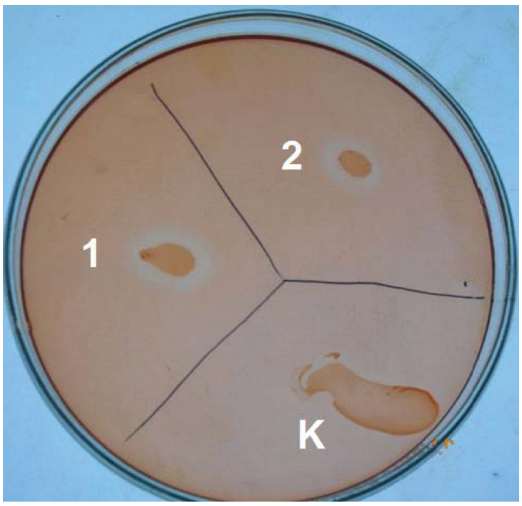

Fig 2. Intracellular supernatant of recombinant *E. coli* strains showing clear β-1,3-glucanase enzyme activity with Congo red staining (1: *E. coli*/pTEG5, 2: *E. coli*/pTEG11, K: *E. coli* as control)

Şekil 2. Rekombinant *E. coli* suşlarına ait hücre içi süpernatantların Congo-red boyaması ile β-1,3-glukanaz aktivitesi göstermesi (1: *E. coli*/pTEG5, 2: *E. coli*/pTEG11, K: *E. coli* kontrol)

zymogram analysis denaturated proteins were renaturated on SDS-Laminarin-PAGE after removing denaturating agents from the gel and then allowing to the enzyme to digest substrate, thereby producing clear zones on the gel. On zymogram analysis, only β-1,3-glucanase protein band of *C. cellulans* with 54.5 kDa in size was showed a clear zone together with intracellular protein counterparts of all other recombinant *E. coli* strains *(Fig. 4)*.

DISCUSSION

With this study, β-1,3-glucanase gene of *C. cellulans* was cloned and expressed in *E. coli*. The enzyme secreted from recombinant *E. coli* strains were found to be active, showing intracellularly clear zones on LB-agar plate containing laminarin. On the other hand, zymogram analysis clearly indicated that activity bands surrounded with clear zones confirming renaturation of denatured enzyme.

β-1,3-Glucanase gene of *C. cellulans* (ATCC 21606) was well studied and enzymatic properties were revealed [19].

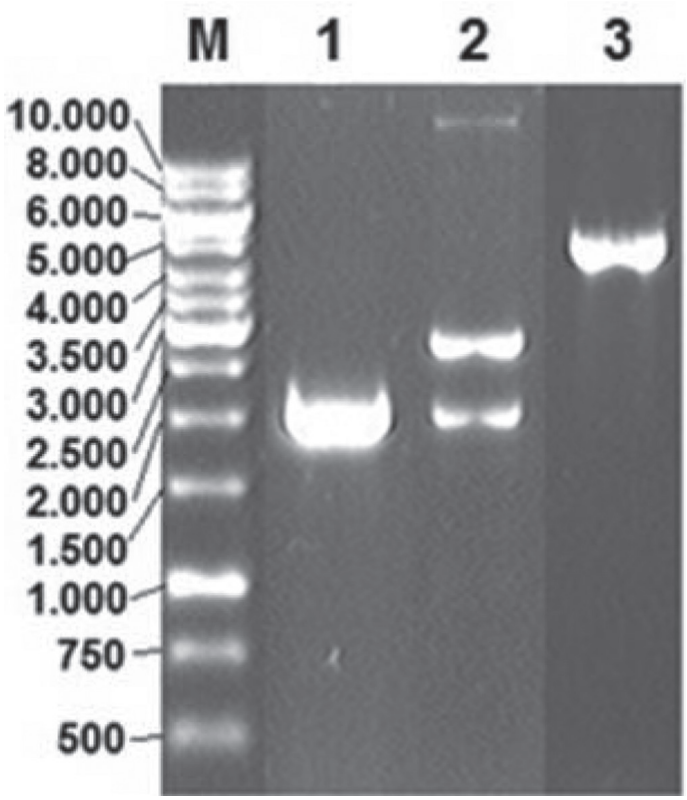

Fig 3. Insert and PCR analysis of *E. coli*/pTEG5 (M: 1 kbp DNA markers, 1- PCR amplified fragment of β-1,3-glucanase gene from pTEG5, 2- pTEG5/*Sac*I, 3- pTEG5/*Eco*RI)

Şekil 3. *E. coli*/pTEG5'e ait insört ve PCR analizleri (M: 1 kbç DNA markır, 1- pTEG5'den PCR ile amplifiye edilmiş β-1,3-glukanaz genine ait fragment, 2- pTEG5/*Sac*I, 3- pTEG5/*Eco*RI)

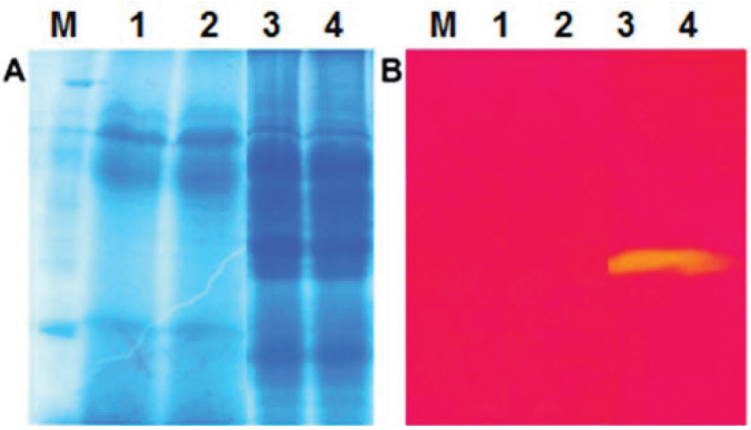

Fig 4. SDS-PAGE (A) and SDS-Laminarin-PAGE (B) analysis of recombinant and non-recombinant bacterial proteins (M: Marker, 1- *E. coli*, 2- *E. coli*/pTEG5 (extracellular proteins), 3- *E. coli*/pTEG5 (intracellular proteins), 4- *C. cellulans* culture supernatant)

Şekil 4. Rekombinant ve rekombinant olmayan bakterilere ait proteinlerin SDS-PAGE (A) ve SDS-Laminarin-PAGE (B) analizleri (M: Markır, 1- *E. coli*, 2- *E. coli*/pTEG5 (hücre dışı proteinler), 3- *E. coli*/pTEG5 (hücre içi proteinler), 4- *C. cellulans* kültür süpernatantı)

Herbal β-1,3-glucanase genes from soybean [20], jujube fruit [21] and rice [22] were cloned in different organisms. The *C. cellulans* β-1,3-glucanase gene was also cloned and expressed in *Bacillus subtilis* and *E. coli* [2-5,23]. In one of these studies, 75% of the recombinant protein was released to the extracellular space [3]. On the other hand, in another study, it has been reported that *E. coli* cells secreted the recombinant β-1,3-glucanase into the periplasm as a mature enzyme [2].

Although restriction digestion and PCR analysis showed the β-1,3-glucanase gene was cloned into *E. coli* DH5α, no lytic activity was observed on LB-laminarin-agar plates with Congo-red staining. But revealing of β-1,3-glucanase activity on LB-laminarin-agar plates, after dropping of extracellular compounds of disrupted bacteria and Congo-red staining, showed that the enzyme was produced by recombinant bacteria intracellularly. It was noted that, compared to other hosts, *E. coli* does not naturally secrete high amounts of proteins [24,25]. Nevertheless, protein secretion in *E. coli* is a complex process [26-28] and so secretion of recombinant proteins can face several problems [28]. Among these problems, incomplete translocation across the inner membrane [28,29], insufficient capacity of the export machinery [30,31], and proteolytic degradation [32] are the most frequently problems encountered [28]. Additionally, protein size may influence secretion efficiency [28,33] and large cytoplasmic proteins may be physically impossible to translocate [28,29]. The amino acid composition of the leader peptide also could be important for segregation of proteins [28,34]. We observed intracellular activity of β-1,3-glucanase on the agar plates containing laminarin. On the other hand, the recombinant enzyme is 54.5

kDa in size and there are several studies demonstrated extracellularly secretion of recombinant proteins with similar size in *E. coli* [35-37]. In one of these studies, endo-1,4-β-glucanase gene of *Bacillus licheniformis* subcloned and 52.2 kDa corresponding enzyme was extracellularly secreted by *E. coli* strain [35]. Therefore, we do not consider the proteolytic degradation and protein size about the enzyme secretion. Generally, premature proteins found in the outer membrane or periplasmic space contain a short specific amino acid sequence (signal sequence) that allows proteins to be exported outside the cytoplasm and during transportation, the signal sequence is cleaved by signal peptidase to yield a mature protein product [38]. Proteins without a signal peptide are made within the cell cytoplasm. Our results most likely indicate that signal peptide of the enzyme can not be recognized by the *E. coli* secretion machinery. It was reported that, the *rsda* gene of a *Cytophaga* sp. was cloned and expressed in *E. coli* DH5α and the enzyme was produced intracellularly by the transformant [39]. Similarly, the *Bacillus subtilis* β-1,4-glucanase gene (a similar enzyme hydrolyzing β-1,4-linkages present in noncrystalline cellulosic substrates such as carboxymethyl cellulose and trinitrophenyl carboxymethyl cellulose) was cloned in *E. coli* expressing the intracellularly [40]. These reported results are supporting our thesis.

As a conclusion, the β-1,3-glucanase gene was isolated from *C. cellulans* genome and cloned in *E. coli*. Our results indicate that, recombinant *E. coli* strains secreted the enzyme intracellularly.

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