Full length article

Improvement of cellulose degradation by cloning of endo-β-1, 3-1, 4 glucanase (bgls) gene from Bacillus subtilis BTN7A strain

Wafaa K. Hegazy a, Mohamed S. Abdel-Salam a,⁎, Azhar A. Hussain b, Hoda H. Abo-Ghalia b, Safa S. Hafez b

a Microbial Genetics Department, National Research Centre, P.O. 12622, Dokki, Giza, Egypt
b Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams Univ., Egypt

1. Introduction

The major part of plant cell walls are cellulose and hemicelluloses and the major constituents of cell walls of cereals consisting of a mixed linked 1, 3-1, 4-β-glucans. The bioconversion of lignocellulosic feedstock to bioethanol has been a global interest as alternative to petroleum fuels. For commercializing this approach, several technical improvements are needed [16,17]. The high cost of enzymes involved in the conversion of the cellulose component into fermentable sugars is one of the major limitations for lignocellulosic-base bioethanol processes [16]. Cellulose is degraded by enzymes belonging to the glycosyl hydrolase families, which hydrolyze oligosaccharides and polysaccharides [17].

Three types of β-glucan endohydrolase able to degrade 1, 3-1,4-β-glucans but distinguished by fine differences in substrate specificity are known. Two of them, endo-1,4-1,β-glucanase or carboxymethyl cellulase (CMCase, cellulase, EC 3.2.1.4) and endo-1,3-1,4-β-glucanaseorlichenase (EC 3.2.1.73) are secreted by Bacillus subtilis. The third β-glucan endohydrolase (endo-1,3(4)-β-glucanase, EC 3.2.1.6) able to hydrolyse aminarin and barley glucan was detected only in Rhizopus arrhizus [4]. The β-1,3-1,4-glucanase activity was assayed using 1% (w/v) barley β-glucan, 1% (w/v) lichenan, and 1% (w/v) CM-cellulose as the substrate [24].

Lichenase encoded by the bgls gene is restricted in its substrate range to mixed linked β-glucans. Only 1,4-linkages adjacent to 1,3-linkages are hydrolysed. The bgls gene has been isolated from B. subtilis strains and the amino acid sequences of their products have been deduced [18].

Different cellulolytic bacterial strains have been collected and isolated including different B. subtilis strains among them B. subtilis subsp. subtilis BTN7A strain which was isolated from Egypt environment had the highest cellulase activity [10,11]. Among the possible ways to produce high production of cellulases, different attempts have been made to clone and express the genes encoding for cellulases in a heterologous host, E. coli [13,1]. Extracellular production of the recombinant protein in E. coli, could allow the potential to develop E. coli system as a cell factory for extracellular production of bacterial enzyme [2].

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The air pollution in Cairo is a matter of serious concern. In 2007 the World Bank ranked Cairo’s air worst in the world for pollution by particulates, the tiny fragments of soot or dust that are most damaging to human lungs. One of the most notable sources of pollution is open-air waste-burning. A black cloud over Cairo has been noticed each year for many decades during harvest time where farmers burn leftover rice husks at the end of the growing season. The black cloud brings pollution levels up to ten times the limits set by the World Health Organization, and can persist for days or weeks at a time. It sends people to the hospital with exacerbated lung infections and asthma attacks at unusually high rates, and contributes to cancer and other long-term health problems. Differ-ent strategies have been planned to overcome this problem including using rice husks instead of them.

Our research group aimed to solve this problem by biodegradation of plant wastes and use them for production of economic value products using biotechnological approach. The present study con-cerning with cloning of endo-β-1, 3-1, 4 glucanase (bgls) gene from B. subtilis BTN7A strain, and optimize its expression in cellulose degradation, as an essential step to accomplish this goal.

2. Materials and methods

2.1. Bacterial strains

Bacillus subtilis subsp. subtilis BTN7A is a highly cellulolytic strain isolated by the research team from Egypt [11]. GenBank accession number KC438368. E. coli DH5α was used for transformation.

2.2. Media

Luria-Bertani agar medium (LB) was used for bacterial growth. Bunshell Haas medium (BHM) containing carboxymethyl cellulose (CMC) or cellulose as a sole carbon source [6].

All molecular biology manipulations were performed according to standard protocols [22] and kit’s suppliers’ instructions unless specified.

2.3. Bioinformatics

Different internet sites have been used through this study. They included, The National Center for Biotechnology Information (NCBI), Webcutter 2.0 software, primer design (Primer3), Plasmid Mapping [8], and SnapGene® Viewer program. The Sequence Similarity Search was done using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

Agarose gel electrophoresis (1%) was used in the present study for DNA analysis. The obtained DNA bands were visualized using UV transilluminator, and then photographed for analysis. Plasmid DNA was isolated using DNA-spin™ plasmid DNA purification Kit (INRION BIOTECHNOLOGY).

2.4. Amplification of endo-β-1,3-1,4 glucanase (bgls) gene

Total genomic DNA was extracted using fresh crude extract method [7] Bacillus subtilis subsp. subtilis BTN7A was grown on LB agar medium overnight at 37 °C. Two colonies were sus-pended in 100 μl sterile distilled water and boiled for 10 min, and then centrifuged for five minutes at 10,000 rpm. The super-natant was used as DNA template in PCR amplification.

PCR amplification was carried out using Go Taq® Flexi DNA Polymerase Kit (Promega Co, Madison, USA). PCR amplification was performed in a thermal cycler AmpliTaq™ (NYXTECHNIK, USA) programmed for one cycle at 95 °C for two minutes, then 30 cycles were performed as follows: one minute at 95 °C for denaturation, one minute at 52 °C for annealing, one minute at 72 °C for elongation and 5 min at 72 °C for final elongation then reaction mixtures were held at 4 °C. The selected primers were bgls reverse primer (ATGCGACAGGCTTTCAC) and bgls forward primer (AATGAAAGGGGAATGCCAAT).

After the program was completed, 10 μl of amplified bgls gene were analyzed by 1% agarose gel electrophoreses.

2.5. Cloning of bgls gene

BgLs gene was purified from gel using MEGAquick-spin TM Total Fragment DNA purification kit (INRION BIOTECHNOLOGY). BgLs gene was then cloned with pGEM®-T Easy Vector (Promega Co, Madison, USA). Five μl of ligated DNA were used to transform E. coli DH5α using heat shock technique and transformants were selected using ampicillin resistance and white/blue screening method (i.e., IPTG/X-gal).

2.6. Cellulase activity assay

Cellulase activity was determined using (3,5-dinitrosalisylic acid) DNS which measures the amount of reducing sugar liberated from CMC or cellulose, according to [19]. The tested bacterial strain was grown in BHM minimal medium or LB broth medium up to three days at 37 °C, and then centrifuged at 13,000 rpm for 5 min. One ml of the supernatant (enzyme solution) was mixed with one ml of CMC solubilized in phosphate buffer (1%) and incubated at 37 °C for 30 min under shaking (120 rpm). One ml dinitrosalisylic (DNS) acid reagent was added and the mixture was boiled for 5 min, then the absorbency was measured at 540 nm. One unit of enzyme activity was defined as μmol substrate consumed or product formed per minute.

Total protein concentration was determined according to [5] and absorbance was measured at 595 nm. Different concentrations of Bovine serum albumin (BSA) were conducted for plotting standard curve according to [15], Cellulase specific activity was calculated by dividing the end product concentration (μmol reducing sugars/ min) expressed as units by the total protein (mg) of the sample.

3. Results

3.1. Isolation of endo-β-1,3-1,4 glucanase (bgls) gene

B. subtilis bgls sequencing available data presented in GenBank was used by Primer3 software to select the appropriate primers which amplify the bgls complete CDS and few surrounding nucleotides.

The amplified PCR product of bgls gene (777 bp) obtained after PCR amplification was represented in Fig. 1. However the highest bgls DNA concentration produced followed the addition of 6 μl of 25 mM MgCl2 in PCR program (Fig. 1, lane 6).

The product band of PCR was purified from the gel by using MEGAquick-spin TM Total Fragment DNA purification kit and sequenced by Macrogen Co., Korea and then analyzed using different softwares. The nucleotide sequence of bgls was placed in the public domain (GenBank accession number KM009051.1).

Nucleotide sequence of B. subtilis subsp. subtilis BTN7A bgls CDS and their deduced amino acid were presented in Fig. 2. It was contains729 bp which encodes for 243 amino acids.

3.2. Cloning of bgls gene

The bgls gene was ligated with pGEM®-T Easy and the recombi-nant plasmid, named Bgls-NRC-1 (3794 bp) (Fig. 3).
3.3. Detection of bgls gene in Bgls-NRC-1 plasmid

To detect the presence of *bgls* gene in the transformants colonies, seven white colonies were selected, named T-bgls1 to T-bgls7 and grown in LB broth containing ampicillin at 37°C for 24 h. The recombinant plasmid Bgls-nrc-1 was isolated and then PCR amplification was carried out using the designed pair of *bgls* primers. Fig. 4 shows the amplified fragments from the seven chosen transformants. All lanes contained one band about 777 bp *bgls* gene as an evidence of its existence in the recombinant plasmid.

3.4. Cellulase specific activity of bgls gene

Cellulase specific activity of *bgls* clones was determined. Bgls - transformants, *E. coli* DH5α and *B. subtilis* BTN7A (donor strain) were grown in LB broth medium at 37°C for 24 h under shaking conditions (120 rpm). After incubation period, cultures were centrifuged. The cellulase specific activity was assayed using the supernatant at temp. 37 and 55°C and the protein concentration was measured in both supernatant and pellets. Cellulase specific activity was then calculated.

It is noted that *B. subtilis* BTN7A (the donor strain of *bgls* gene) cellulase enzyme was active at 37°C and 55°C, where cellulase specific activities were 15.11 U/mg protein and 10.51 U/mg protein at 37°C and 55°C, respectively (Table 1). In this case the cellulase activity was reduced by about 44% at 55°C than at 37°C. It is also observed that, although *E. coli* DH5α had negligible cellulase activity, the recombinant *E. coli* DH5α, which contains the *bgls* gene, showed high cellulase activities. The results also revealed that *bgls* gene has the ability to hydrolyse CMC at 37°C and 55°C. Moreover, *bgls* constructed plasmid had more cellulase activity, at both temperatures than the donor *B. subtilis* BTN7A strain, in spite of the donor strain had different cellulase enzymes, the recombinant strain contain *Bgls*-NRC-1 plasmid had 19.5 U cellulase activities at 37°C which was about 29% more than *B. subtilis* BTN7A donor strain, and 16.47 U/mg at 55°C which was about 57% more than *B. subtilis* BTN7A donor strain.

3.5. Medium effects on cellulase activity of bgls

To determine the medium effect on cellulase activity by time, overnight cultures of *bgls* transformant (i.e., T-bgls1) or *E. coli* DH5α were grown in LB broth medium containing ampicillin at 37°C for 24 h. The recombinant plasmid Bgls-nrc-1 was isolated and then PCR amplification was carried out using the designed pair of *bgls* primers. Fig. 4 shows the amplified fragments from the seven chosen transformants. All lanes contained one band about 777 bp *bgls* gene as an evidence of its existence in the recombinant plasmid.
DH5α were grown in minimal media supplemented with CMC or cellulose and in LB broth at 37°C up to 3 days with shaking (120 rpm).

The cellulase activities presented in Table 2 showed different indications. First, bgls gene had more reducing sugars when using CMC than cellulose powder. Second, reducing sugars were decreased by increasing the incubation period. Third, double the initial inoculums resulted in more cellulase activities, e.g., after 24 hrs bgls had 15.1 U/mg comparing with 27.2 U/mg.

On the other hand, another three indications were observed in (Table 2): Firstly, the successful bgls gene expression by using complex medium (i.e., LB medium) up to three days. Secondly, bgls gene expression was less than those expressed when CMC or cellulose was used as sole carbon sources. Thirdly, double initial inoculum resulted in more cellulase activities e.g., after 24 h bgls had 25.36 U/mg comparing with 19.83 U/mg.

### 4. Discussion

The obtained data revealed that in spite of the donor B. subtilis BTN7A strain, had bgls gene beside different other cellulase genes, it had less cellulase activity than constructed plasmid, the higher cellulase activities of the cloned gene may be probably due to one or both of the high copy number of the cloning vectors and decreased by increasing the incubation period. Third, double the initial inoculums resulted in more cellulase activities, e.g., after 24 hrs bgls had 15.1 U/mg comparing with 27.2 U/mg.

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### Table 2

| Bacterial strains | Cellulase specific activity (U/mg protein) |
|-------------------|------------------------------------------|
|                   | BHM medium + CMC | BHM medium + cellulose | LB Medium |
|                   | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| **E. coli DH5α**  | 0.51 b | 0.14b | 0.081b | 0.41b | 0.029b | 0.037b | 0.061b | 0.042b | 0.03b |
| T-bgls1(X)        | 27.20b | 25.80b | 23.53b | 21.93b | 21.01b | 19.51b | 19.83b | 15.90b | 15.11b |
| T-bgls1(2X)       | 35.10b | 29.43b | 62.53b | 30.20a | 28.83b | 26.90b | 25.36a | 21.51a | 20.10a |

* Means not followed by the same letter are significantly different by Duncan's Multiple Range Test [9] (P ≤ 0.05).

**X** = Bacterial inoculum corresponding to OD620 = 0.01 in 20 ml medium.

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