

HOSTED BY



Contents lists available at ScienceDirect

## Journal of Genetic Engineering and Biotechnology

journal homepage: [www.elsevier.com/locate/jgeb](http://www.elsevier.com/locate/jgeb)

Full length article

Improvement of cellulose degradation by cloning of endo- $\beta$ -1, 3-1, 4 glucanase (*bgls*) gene from *Bacillus subtilis* BTN7A strainWafaa K. Hegazy<sup>a</sup>, Mohamed S. Abdel-Salam<sup>a,\*</sup>, Azhar A. Hussain<sup>b</sup>, Hoda H. Abo-Ghalia<sup>b</sup>, Safa S. Hafez<sup>b</sup><sup>a</sup> Microbial Genetics Department, National Research Centre, P.O. 12622, Dokki, Giza, Egypt<sup>b</sup> Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams Univ., Egypt

## ARTICLE INFO

## Article history:

Received 8 January 2018  
 Received in revised form 14 May 2018  
 Accepted 22 June 2018  
 Available online 28 June 2018

## Keywords:

*Bacillus subtilis*  
 Endo- $\beta$ -1, 3-1, 4 glucanase  
 PCR  
 Gene cloning  
 Gene expression  
 Cellulolytic activity

## ABSTRACT

The aim of this study is to construct a new recombinant strain able to degrade cellulose efficiently. The endo- $\beta$ -1, 3-1, 4 glucanase (*bgls*) gene was cloned from *Bacillus subtilis* BTN7A strain by using PCR technique. The specific primers of *bgls* gene were deduced. Optimization of PCR mixture and program were identified. The nucleotide sequence of *bgls* was placed in the public domain (GenBank accession number KM009051.1). The obtained *bgls* DNA was cloned with pGEM<sup>®</sup>-T Easy Vector. The recombinant plasmid designated as Bgls-NRC-1 was transformed into *E. coli* DH5 $\alpha$ . The successful cloning of the *bgls* gene was tested either by PCR or by evaluating its expression in its new bacterial host. The *bgls* gene was expressed efficiently in *E. coli* and the enzyme activity of the transformant was compared to the enzyme activity of the donor bacterial strain. The new constructs produce much higher enzyme yields than the donor bacterial strain, they produce about 29% and about 57% higher cellulase specific activity at 37 °C and 55 °C respectively. Optimization of cellulolytic activity of the new recombinant strain were described. The effect of minimal medium supplemented with CMC or cellulose, or complete medium (LB) on *bgls* expression were tested, the order of cellulase activity production was CMC27.2 > cellulose 21.9 > LB 19.8 U/mg protein, respectively at 24 h. CMC was proved to be the best medium for cellulase production. Results also showed that double the initial inoculum resulted in more cellulase activities in all media.

© 2018 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## 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- $\beta$ -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  $\beta$ -glucan endohydrolase able to degrade 1, 3-1, 4- $\beta$ -glucans but distinguished by fine differences in substrate specificity are known. Two of them, endo-1,4- $\beta$ -glucanase or carboxymethyl cellulase (CMCase, cellulase, EC 3.2.1.4) and endo-

1,3-1,4- $\beta$ -glucanase or lichenase (EC 3.2.1.73) are secreted by *Bacillus subtilis*. The third  $\beta$ -glucan endohydrolase (endo-1,3(4)- $\beta$ -glucanase, EC 3.2.1.6) able to hydrolyse laminarin and barley glucan was detected only in *Rhizopus arrhizus* [4]. The  $\beta$ -1,3-1,4-glucanase activity was assayed using 1% (w/v) barley  $\beta$ -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  $\beta$ -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].

Peer review under responsibility of National Research Center, Egypt.

\* Corresponding author at: 33 EL Bohouth st. (former EL Tahrir st.), P.O. 12622, Dokki, Giza, Egypt.

E-mail address: [sass-one@hotmail.com](mailto:sass-one@hotmail.com) (M.S. Abdel-Salam).<https://doi.org/10.1016/j.jgeb.2018.06.005>1687-157X/© 2018 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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. Different 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 concerning with cloning of endo- $\beta$ -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* DH5 $\alpha$  was used for transformation.

### 2.2. Media

Luria-Bertani agar medium (LB) was used for bacterial growth. Bunsell 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<sup>®</sup> 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](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<sup>™</sup> plasmid DNA purification Kit (iNtRON BIOTECHNOLOGY).

### 2.4. Amplification of endo- $\beta$ -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 for overnight at 37 °C. Two colonies were suspended in 100  $\mu$ l sterile distilled water and boiled for 10 min, and then were centrifuged for five minutes at 10,000 rpm. The supernatant was used as DNA template in PCR amplification.

PCR amplification was carried out using Go Taq<sup>®</sup> Flexi DNA Polymerase Kit (Promega Co, Madison, USA). PCR amplification was performed in a thermal cycler Amplitronyx<sup>™</sup> (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 extension then reaction mixtures were held at 4 °C. The selected primers were *bgls* reverse primer (ATTG CAGCAGGCTCTTTCAC) and *bgls* forward primer (AATGAAAGGGGAATGCCAAT).

After the program was completed, 10  $\mu$ l 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 (iNtRON BIOTECHNOLOGY). *Bgls* gene was then cloned with pGEM<sup>®</sup>-T Easy Vector (Promega Co. Madison, USA). Five  $\mu$ l of ligated DNA were used to transform *E. coli* DH5 $\alpha$  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-dinitrosalicylic 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 dinitrosalicylic (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  $\mu$ mol 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 ( $\mu$ mol reducing sugars/min) expressed as units by the total protein (mg) of the sample.

## 3. Results

### 3.1. Isolation of endo- $\beta$ -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.

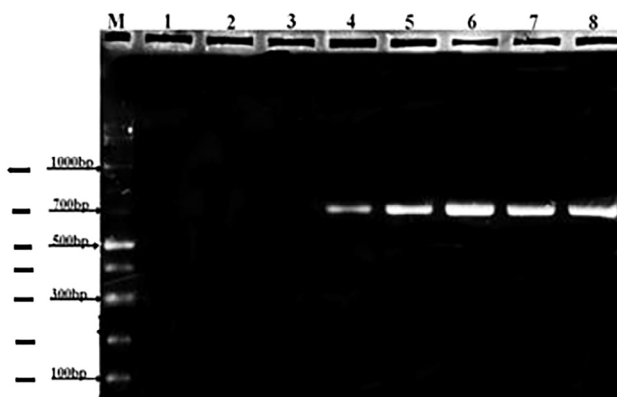
The amplified PCR product of *bgls* gene (777 bp) obtained after PCR amplification was represented in Fig. 1. However the highest *bgls* DNA concentration produced followed the addition of 6  $\mu$ l of 25 mM MgCl<sub>2</sub> in PCR program (Fig. 1, lane 6).

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 contains 729 bp which encodes for 243 amino acids.

### 3.2. Cloning of *bgls* gene

The *bgls* gene was ligated with pGEM<sup>®</sup>-T Easy and the recombinant plasmid, named Bgls-NRC-1 (3794 bp) (Fig. 3).



**Fig. 1.** An agarose gel (1%) electrophoresis showing the amplification of *bglS* gene from *Bacillus subtilis* subsp. *subtilis* BTN7A. Lane M, 100 bp DNA ladder; lane 1, no MgCl<sub>2</sub> in the PCR reaction mixture; lane 2, 2 μl MgCl<sub>2</sub>; lane 3, 3 μl MgCl<sub>2</sub>; lane 4, 4 μl MgCl<sub>2</sub>; lane 5, 5 μl MgCl<sub>2</sub>; lane 6, 6 μl MgCl<sub>2</sub>; lane 7, 7 μl MgCl<sub>2</sub> and lane 8, 8 μl MgCl<sub>2</sub>.

### 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-*bglS*1 to T-*bglS*7 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-*bglS*1) or *E. coli*

```

atgccttatctgaaacgagtggttgctgcttcttgctcactggattgtttatgagtttg
tttgcagtcactgctactgcctcagctcaaacaggtggatcgttttttgaccctttt
aacggctataactccgggtttttggcaaaaagcagatgggttattcgaatggaaatatg
ttcaactgcacgtggcgggctaataacgtatcagtgacgtcattgggtgaaatgcgt
ttagcgctaacaagccatcttataacaagtttgactgcggggaaaaacggtctgtt
caaacatatggctatggactttatgaagtcagaatgaaaccagctaaaaacacaggg
atcgtttcatcgttcttacttacacaggtccaacagatggaactccttgggatgag
attgatatcgaattttttaggaaaagatacaacaaaggttcaatttaactattataca
aatgggtgcaggaaccatgagaagattggtgatctcgggtttgatgcagccaatgcc
tatcatacgtatgcattcgattggcagccaaactctattaatggatgctgcagcggg
caagtaaaacatactgcaacaaacaaattccgacaacacctggaaagatcatgatg
aatttggtgaatggtacgggtgctgatgaatggcttggctcctacaatggtgtaaat
ccgctatacgcctcattatgactgggtgocgtatacaaaaaataa
    
```

A

```

MPYLKRVLLLLVTGLFMSLFAVTATASAQTTGGSFFDPFNGYNSGFWQKADG
YSNGNMFNCTWRANNVSVTSLGEMRLALTPSYNKFDCGENRSVQTYGYG
LYEVRMKPAKNTGIVSSFFTYTGPTDGPWDEIDIEFLGKDTTKVQFNYYTNG
AGNHEKIVDLGFDAANAYHTYAFDWQPNSIKWYVDGQVKHTATNQIPTTPG
KIMMNLWNGTGVDEWLGSYNGVNPLYAHYDWVRYTKK
    
```

B

**Fig. 2.** *Bacillus subtilis* subsp. *subtilis* strain BTN7A endo-beta-1,3-1,4 glucanase (*bglS*) gene, complete CDS (A) and its deduced amino acids sequence (B).

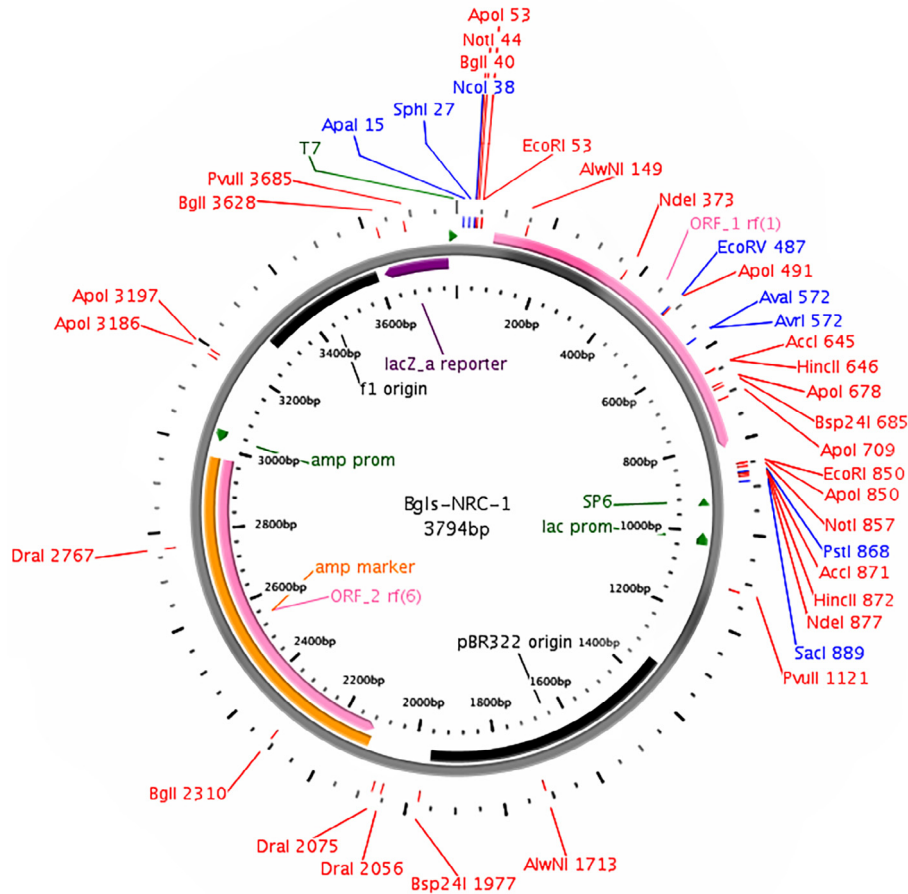


Fig. 3. Bgls-NRC-1 recombinant plasmid containing *B. subtilis* subsp. *subtilis* BTN7Abgls gene.

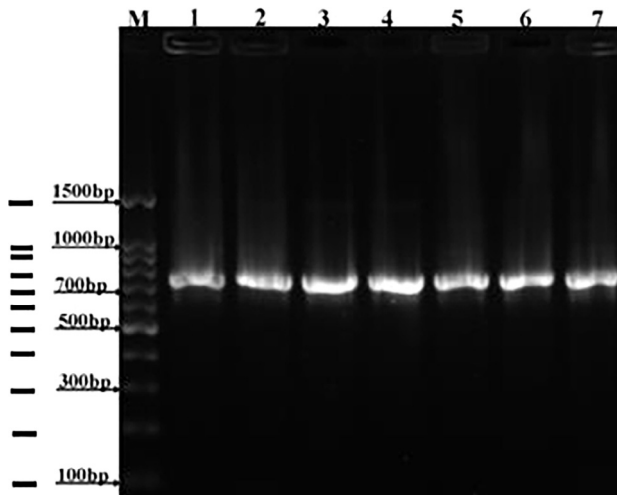


Fig. 4. Agarose gel (1%) electrophoresis of the amplification of *bgls* gene from seven *E. coli* DH5 $\alpha$  (Bgls-NRC-1) transformants. Lane M: 100 bp DNA ladder, lane1: T-bgls1; lane2: T-bgls2; lane3: T-bgls3; lane4: T-bgls4; lane5: T-bgls5; lane6: T-bgls6 and lane7: T-bgls7 transformants.

Table 1

Cellulase specific activity of *E. coli* DH5 $\alpha$ , *B. subtilis* BTN7A, and *E. coli* DH5 $\alpha$  (T-bgls1).

Bacterial strains	Cellulase specific activity (U/mg protein) at	
	37 °C	55 °C
<i>E. coli</i> DH5 $\alpha$	0.05 c <sup>*</sup>	0.01 c
<i>B. subtilis</i> BTN7A	15.11 b	10.51 b
<i>E. coli</i> DH5 $\alpha$ (T-bgls1)	19.50 a	16.47 a

<sup>\*</sup> Means not followed by the same letter are significantly different by Duncan's Multiple Range Test [9] ( $P \leq 0.05$ ).

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

DH5 $\alpha$  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

**Table 2**  
Medium effects on *bgls* expression.

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
<i>E. coli</i> DH5 $\alpha$	0.51 <sup>c</sup> *	0.14 <sup>b</sup>	0.081 <sup>c</sup>	0.41 <sup>c</sup>	0.029 <sup>c</sup>	0.037 <sup>c</sup>	0.061 <sup>c</sup>	0.042 <sup>c</sup>	0.03 <sup>c</sup>
T- <i>bgls</i> 1(X)**	27.20 <sup>b</sup>	25.80 <sup>a</sup>	23.53 <sup>b</sup>	21.93 <sup>b</sup>	21.01 <sup>b</sup>	19.51 <sup>b</sup>	19.83 <sup>b</sup>	15.90 <sup>b</sup>	15.11 <sup>b</sup>
T- <i>bgls</i> 1(2X)	35.10 <sup>a</sup>	29.43 <sup>a</sup>	82.53 <sup>a</sup>	30.20 <sup>a</sup>	28.83 <sup>a</sup>	26.90 <sup>a</sup>	25.36 <sup>a</sup>	21.51 <sup>a</sup>	20.10 <sup>a</sup>

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

\*\* X = Bacterial inoculum corresponding to OD<sub>620</sub> = 0.01 in 20 ml medium.

hence multi gene copies per cell. Another possible explanation of the higher activity of the constructed plasmid may also be due to the promoter effect where the cloned gene was under the control of T7 promoter of the pGEM<sup>®</sup>-T Easy (Fig. 3). Similar results have been reported by [12] who cloned *bgls* gene from *Bacillus circulans* and transfer it to each of *B. subtilis* and *B. megaterium*. The *B. subtilis* enzyme was 83 folds more active than that of the gene donor cells. The *B. megaterium* enzyme was 7 folds more active than that of the gene of donor cells. While *E. coli* secreted only about 10% of the produced enzyme, *B. subtilis* excreted the enzyme completely into the medium and *B. megaterium* by about 98%. A four folds increase in cellulase production by the recombinant cell to that of wild type suggests the applicability of the *E. coli* system as a cell factory for cellulase over-production due to the higher level of recombinant expression as mentioned by [25]. *E. coli* is one of the most widely used host for the production of recombinant proteins as it is the best characterized host with many available expression systems [20].

Maximum *bgls* production was obtained when using CMC (Table 2) this result confirmed the recently reported that *Bacillus* sp. produced maximum cellulases production in the presence of CMC as a carbon source [3,21,14,23]. This finding indicates the positive regulation effect of CMC in cellulases gene expression in *Bacillus*.

## 5. Conclusions

In conclusion in the present work, endo- $\beta$ -1, 3-1,4glucanase (*bgls*) gene was cloned from *Bacillus subtilis* strain BTN7A using PCR. The optimized conditions for amplifying *bgls* gene was identified including DNA, primers and MgCl<sub>2</sub> concentrations, and PCR program. The obtained recombinant plasmid was tested for its new constructs. The *bgls* gene was intact physically in the recombinant plasmid as confirmed by PCR and by its expression in the new bacterial host. Further studies are planned to use the cellulase-efficient *bgls* clone in degradation of different celluloses

waste matters. The obtained degradation products will be used to produce economical products such as alcohol and single cell protein when using yeasts.

## References

- [1] Aftab S, Aftab MN, Haq I, Javed M, Zafar A, Iqbal I. Afr J Biotechnol 2012;11(12):2846–54.
- [2] Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V. Microbial Cell Factories 2012;11:164–76.
- [3] Azadian F, Badoei-dalfard A, Namaki-Shoushtari A, Karami Z, Hassanshahian M. JGEB 2017;15:187–96.
- [4] Behera BC, Sethi BK, Mishra RR, Dutta SK, Thatoi HN. JGEB 2017;15:197–210.
- [5] Bradford MM. Annal Biochem 1976;72:248–54.
- [6] Bushnell LD, Haas HF. J Bacteriol 1941;41(5):653–73.
- [7] Dashti AA, Jadaon MM, Abdulsamad AM, Dashti HM. Kuwait Med J 2009;41(2):117–22.
- [8] Dong X, Stothard P, Forsythe IJ, Wishart DS. Nucl Acids Res 2004;32 (Web Server issue): W660–W664.
- [9] Duncan DB. Biometrics 1955;11:11–24.
- [10] S.S. Hafez, Ph. D. Thesis, Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Egypt; 2015.
- [11] Hussain AA, Abdel-Salam MS, Abo-Ghalia HH, Hegazy WK, Hafez SS. JGEB 2017;15:77–85.
- [12] Kim JY. Biotechnol Lett 2003;25:1445–9.
- [13] Li W, Zhang WW, Yang MM, Chen YL. Mol Biotechnol 2008;40:195–201.
- [14] Lin L, Kan X, Yan H, Wang D. Biotechnol J 2012;15:1–7.
- [15] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. J Biol Chem 1951;193:265–75.
- [16] Lynd LR, Laser MS, Bransby D, Dale BE, Davison B, Hamilton R, Himmel M, Keller M, McMillan JD, Sheehan J, Wyman CE. Nat Biotechnol 2008;26:169–72.
- [17] Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS. Microbiol Mol Biol Rev 2002;66(3):506–77.
- [18] Masilamani R, Sharma OP, Muthuvel SK, Natarajan S. Bioinformatics 2013;9(19):958–62.
- [19] Miller GL. Anal Chem 1959;31:426–8.
- [20] Pandey S, Kushwaha J, Tiwaria R, Kumar B, Somvanshi VS, Naina L, Saxena AK. Microbiol Res 2014;169:693–8.
- [21] Sadhu S, Ghosh PK, De TK, Maiti TK. J Microbiol 2013;3:280–8.
- [22] Sambrook J, Russell DW, editors. Molecular cloning: a laboratory manual. N. Y: Cold Spring Harbor Laboratory Press; 2001.
- [23] Shaikh NM, Patel AA, Mehta SA, Patel ND. Environ Sci Technol 2013;1:39–49.
- [24] Teng D, Wang JH, Fan Y, Yang YL, Tian ZG, Luo J, Yang GP, Zhang F. Appl Microbiol Biotechnol 2006;72:705–12.
- [25] Zou G, Shi S, Jiang Y, van den Brink J, de Vries RP, Chen L, Zhang J, Ma L, Wang C, Zhou Z. Microb Cell Fact 2012;11(1):21–33.