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High Ability of Cellulose Degradation by Constructed *E. coli* Recombinant Strains

Mohamed S. Abdel-Salam[1,*] , Wafaa K. Hegazy[1], Azhar A. Hussain[2], Hoda H. Abo-Ghalia[2] and Safa S. Hafez[2]

[1]*Microbial Genetics Department, National Research Centre, 33 EL Bohouth St. – Dokki- Giza- P.O. 12622;* [2]*Botany Department, Faculty of Women for Arts, Science and Education, Ain Shams University, Egypt.*

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ABSTRACT

Endo-β-1, 4 glucanase gene (*eglS*) from local isolate *Bacillus subtilis* BTN7A was isolated, sequenced and cloned by using PCR technique. *EglS*-primers were designed. Optimization of both PCR program and mixture content was identified. The *eglS* sequence was submitted into the GenBank. The gene expressed efficiently in *E.coli* DH5α. The recombinant *E.coli* harboring recombinant vector pGEM® –T Easy /eglS produced much higher enzyme yields than its parental strain which has non-detectable cellulase activity. The new construct also had about 78%-98% higher activity than *B. subtilis* BTN7A donor strain. For enhancing recombinant enzyme expression, different optimum parameters (temperature, inoculum level, incubation time, and carbon source of the growth media) were investigated. Maximum enzyme production of the recombinant strain was 37.9 U/ml obtained after 24h grown on the growth media, supplemented with CMC as a sole source of carbon at 37°C. The data demonstrated that the presence of endo-β-1, 4 glucanase (EGL) with endo-β-1, 3-1, 4 glucanase; (BGL) showed a highly synergistic effect in hydrolyzing cellulose. The specific activities were increased up to 60.6 U/ml when mixed culture of both recombinant *E. coli* harboring *eglS* gene and *E. coli* harboring *bgls* gene, inoculated in CMC medium. The constructed strain can be used for biodegradation of cellulosic wastes as an essential step for their conversion to economic valuable products.

Keywords: *Bacillus subtilis*; Endo-β-1,4 glucanase; Endo-β-1, 3-1, 4 glucanase; Cellulase; Gene cloning; PCR

1. Introduction

Cellulose is the most abundant polymer in the biosphere and most preeminent waste on earth (Moo-Young *et al.,* 1987). This polymer is built up from units of glucose attached by β-1, 4 linkages. This agriculture waste is considered as a huge renewable bioresource (Jarvis, 2003 and Zhang &Lynd 2004), where it has a high potential to be used as an inexpensive feedstock for bioconversion to important products such as acetone and ethanol.

The biological conversion of lignocellulosic wastes requires the use of cellulolytic and hemicellulolytic enzymes (Jourdier *et al.,* 2012). Degradation of cellulose occurred by glycosyl hydrolase enzymes families (Bayer *et al.*, 1998). Cellobiohydrolase, endoglucanase (Carboxy-methyl-celllulase), and β-glucosidases are three types of enzymes required for cellulosic hydrolysis (Bhat, 2000). Cellulases depolymerize cellulose as environmental friendly process (Karmakar and Ray, 2011 and Kuhad *et al.*, 2011).

A variety of *Bacillus* species produce cellulases, including strains of *B. cereus* (Thayer and David 1978) *Bacillus subtilis*, (Hussain *et al.,* 2017), *B. licheniformis* (Dhillon *et al.,* 1985), *Bacillus* sp. KSM-330 (Ozaki and Ito, 1991), *Bacillus thuringiensis* strains (Lin *et al.,* 2012) and alkaliphilic *Bacillus* (Horikoshi, 1997). Bacterial cellulase complexes act as synergic multi enzyme systems. However; most of these bacteria produce mainly endoglucanases (Wood, 1985). By contrast, there are reports of certain *Bacillus* endoglucanases (CMCase) that have shown detectable activity on microcrystalline cellulose (Kim, 1995).

In addition, application of cellulases commercially has different obstacles including their high prices and the poor knowledge of their interactive actions and their mechanisms (Juturu and Wu 2014).

Open-air agriculture-waste-burning is the most remarkable source of pollution. For many decades, different places in Egypt including Cairo suffered from a serious environmental problem of air pollution mostly because of burning rice husks. This behavior is responsible for extremely increasing air pollution over the limits set by World Health Organization.

The objective of this study was to clone endo-β-1, 4 glucanase (*eglS*) gene from *B. subtilis* subsp. *subtilis* BTN7A and optimize its expression under control of different growth conditions, aiming to develop a recombinant strain with a high ability of cellulose degradation, where the end product of the hydrolysis may be identified as a cheap source of essential material for second generation of ethanol production and other important economical products. The ultimate aim of this

* Corresponding author e-mail: sass-one@hotmail.com.

research was to reduce pollution in our country through biodegradation of cellulosic wastes and converting them into useful materials.

2. Materials and Methods

2.1. Bacterial strains

Bacillus subtilis subsp. *subtilis* BTN7A is a highly cellulolytic indigenous strain isolated from Egypt (Hussain *et al*., 2017); it was used as a source of endo-β-1, 4 glucanase (*eglS*) gene. *E. coli* DH5α was used as a recipient in transformation. Plasmid pGEM®-T Easy Vector (Promega Co., Madison, USA) was used for gene cloning. T-*Bgls*-1 is a recombinant *E. coli* strain harbouring endo-β-1, 3-1, 4 glucanase (*bgls*) gene from *B. subtilis* subsp. *subtilis* BTN7A (Hegazy *et al*., 2018).

2.2. Media

Luria-Bertani agar medium (LB) (Bertani 1952), and Bushnell- Haas medium (BHM) (Bushnell and Haas 1941) supplemented with carboxymethyl cellulose (CMC) or cellulose as a sole carbon source, were used as bacterial growth media.

2.3. Bioinformatics

Different web-based tools, including The National Center for Biotechnology Information (NCBI), webcutter 2.0 software, primer design (Primer3), Plasmid Mapping (Dong *et al*., 2004), and SnapGene®Viewer program, have been used throughout this study.

All molecular biology manipulations were performed according to standard protocols (Sambrook and Russell, 2001) and kits suppliers' instructions unless specified otherwise.

Agarose gel electrophoresis (1%) was used 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 (INTRON BIOTECHNOLOGY).

2.4. Isolation of eglS gene

Primers to amplify *eglS* CDS and the flanking region were designed based on sequence in GeneBank database using Primer3. The eglS reverse primer was (AATGATGCGAAGGAGGAAAA) and eglS forward primer was (TTACTGATGTCCGCCAAAAA), and the expected *eglS* fragment was 1557 bp.

Total genomic DNA was extracted according to (Dashti *et al*., 2009) from *B. subtilis* BTN7A strain. PCR amplification of *eglS* gene was carried out using Go Taq® Flexi DNA Polymerase Kit (Promega Co., Madison, USA). It was done in a total volume of 50µL containing: 10 µL of 5x Green Go Taq® Flexi buffer, (2-8) µL of 25 mM of $MgCl_2$, 1 µL of 10mM dNTPs, 0.75 µL of forward *eglS* primer, 0.75 µL of reverse *eglS* primer, 1.5 µL of template DNA, 0.25 µL of Go Taq DNA polymerase (5 unites/ µL) and then volume was adjusted to 50 µL with water (nuclease free).

PCR amplification was performed in the thermal cycler programmed for one cycle at 95ºC for 5 minutes, then 30 cycles as follows: one minute at 95ºC for denaturation, one minute at 50ºC for annealing, 90 seconds at 72ºC for elongation then 5 minutes at 72ºC for final extension, and the reaction mixtures were held at 4ºC.

The PCR products were analyzed by 1% agarose gel electrophoresis. The DNA band containing *eglS* gene was purified from gel, and sent to Macrogen Co., Korea for sequencing**.**

2.5. Cloning of eglS gene

EglS gene was cloned with pGEM®-T Easy Vector. It was done as follows: Five µL of 2x Rapid Ligation Buffer, 1-3 µL PCR product, and the volume was adjusted to 8 µL with water (nuclease free). 1 µL pGEM®-T Easy Vector and 1 µL T4 DNA Ligase were added, briefly mixed together by pipetting, and the tube was incubated overnight at 4ºC.

Five µL of ligated DNA were used to transform *E. coli* DH5α by heat-shock treatment 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 measured in the supernatant or in the cell lysate using (3, 5-dinitrosalisylicacid) DNS which measures the amount of reducing sugar liberated from CMC or cellulose, according to (Miller, 1959).

Total protein concentration in the intracellular (pellets) and in extracellular (supernatant) was determined according to (Bradford 1976). Tested isolates were incubated for 24 h then centrifuged at 13,000 rpm for 10 min. One ml of Bradford reagent was added to 50µL supernatant for 10 min. The optical density was measured at 595 nm against blank using spectrophotometer (SHIMADZU UV-Vis spectrophotometer 1201).

Intracellular soluble protein was done as follows: 1.5mL from bacterial culture were harvested by centrifugation, the pellet was resuspended in 350µL Smart™ Bacterial Protein Extraction solution (INTRON BIOTECHNOLOGY) by vigorously vortexing for 1 min. Sample was centrifuged for 5 min at 13,000 rpm. The supernatant was used for intracellular protein determination using Bradford reagent as described above for extra-cellular protein determination.

Protein standard curve was generated using different concentrations of bovine serum albumin (BSA) ranging from 10 to 100 µg/mL distilled water.

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 and Discussion

Interactive cooperation between cellulases enzymes is essential process for cellulose depolymerization to glucose. The current study focused on the development of a recombinant strain with a high ability of cellulose degradation; to this goal the two cellulases enzymes endo-β-1, 4 glucanase (EGL) and endo-β-1, 3-1, 4 glucanase (BGL) were manipulated and expressed under the effect of different environmental factors. Synergistic effect of the two enzymes in hydrolyzing cellulose was studied.

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

The *eglS* coding sequence was amplified from *B. subtilis* BTN7A by PCR using the designed primers. A 1557 bp DNA band containing the *eglS* gene was successfully obtained after PCR amplification. Results

indicated that addition of 5 µL of 25mM $MgCl_2$ produced the highest *eglS* DNA concentration.

The obtained DNA band was purified using MEGAquick-spin™ Total Fragment DNA purification kit and sequenced by (Macrogen Co., Korea). The complete CDS sequence of *eglS* was deposited in GenBank (Accession number KM009052.1). The *eglS* –CDS (1500 bp) which encodes for 500 amino acids was shown in (Figure 1).

atgaaacggtcaatctcgatttttattacgtgtttattgattacgttattgacaatgggcggcatgctggcttcgccggcatcagcagcagggacaaaaacgccagtagccaagaatggccagctt
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gcaaacgaaccaaacggtgatgtgaactggaagcgtgatattaaaccgtatgcggaagaagtgatttccgttatccgcaaaaatgatccagacaacatcatcattgtcggaaccggtacatgga
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agattcgacgaaggacattcctgaaacgccagcaaaagataaacccacacaggaaaacggtatttctgtacaatacagagcaggggatgggagtatgaacagcaaccaaatccgtccgcagc
ttcaaataaaaaataacggcaataccacggttgatttaaaagatgtcactgcccgttactggaataaagcgaaaaacaaaggccaaaactttgactgtgactacgcgcagattggatgcggcaa
tgtgacacacaagtttgtgacgttgcataaaccaaagcaaggtgcagatacctatctggaacttgggtttaaaaacggaacgctggcaccgggagcaagcacagggaatattcagctccgtctt
cacaatgatgactggagcaattatgcacaaagcggcgattattcctttttcaaatcaaatacgtttaaaacaacgaaaaaaatcacattatatgatcaaggaaaactgatttggggaacagaacc
aaatttag

A

MKRSISIFITCLLITLLTMGGMLASPASAAGTKTPVAKNGQLSIKGTQLVNRDGKAVQLKGISSHGLQWYGEYVNKDSLKW
LRDDWGITVFRAAMYAADGGYIDNPSVKNKVKEAVEAAKELGIYVIIDWHILNDGNPNQNKEKAKEFFKEMSSLYGNTPN
VIYEIANEPNGDVNWKRDIKPYAEEVISVIRKNDPDNIIIVGTGTWSQDVNDAADDQLRDANVMYALHFYAGTHGQFLRDK
ANYALSKGAPIFVTEWGTSDASGNGGVFLDQSREWLKYLDSKTISWVNWNLSDKQESSSALKPGASKTGGWQLSDLSASG
TFVRENILGTKDSTKDIPETPAKDKPTQENGISVQYRAGDGSMNSNQIRPQLQIKNNGNTTVDLKDVTARYWNKAKNKGQ
NFDCDYAQIGCGNVTHKFVTLHKPKQGADTYLELGFKNGTLAPGASTGNIQLRLHNDDWSNYAQSGDYSFFKSNTFKTTK
KITLYDQGKLIWGTEPN

B

Figure 1. *Bacillus subtilis* subsp. *subtilis* strain BTN7A endo-β-1,4 glucanase (*eglS*) gene, complete CDS (A) and its deduced amino acids sequence (B).

3.2. Cloning of eglS gene

A 1557 bp band of endo-β-1,4glucanase (*eglS*) gene obtained after PCR was purified and ligated with pGEM®-T Easy Cloning Vector using T4 DNA ligase. The recombinant plasmid was named EglS-nrc-1 (4573bp) (Figure 2). *E. coli* DH5α was used as heterologous new host for the recombinant plasmid (EglS-nrc-1) by transformation.

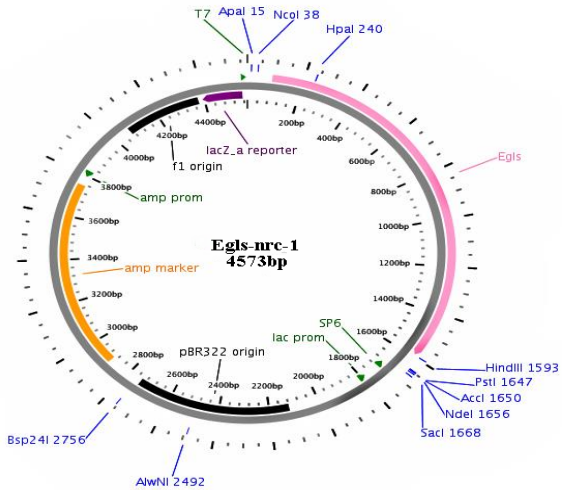

Figure 2. EglS-nrc-1 recombinant plasmid containing *B. subtilis* subsp. *subtilis* BTN7A *eglS* gene.

3.3. Detection of eglS gene in pGEM®-T Easy /eglS (EglS-nrc-1) plasmid

Blue/white colonies screening technique and ampicillin resistant were used to select *E. coli* DH5α EglS - transformants. To confirm the existence of *eglS* gene in *E. coli* transformants, nine ampicillin resistant and white colonies were randomly selected, named T-eglS1 to T-eglS9. They were grown in LB broth at 37°C for 24h and their plasmids were isolated. The occurrences of *eglS* within these plasmids were tested using PCR amplification and the designed *eglS*-primers. All tested nine transformants showed one DNA band of 1557 bp corresponding to *eglS* gene (Figure 3).

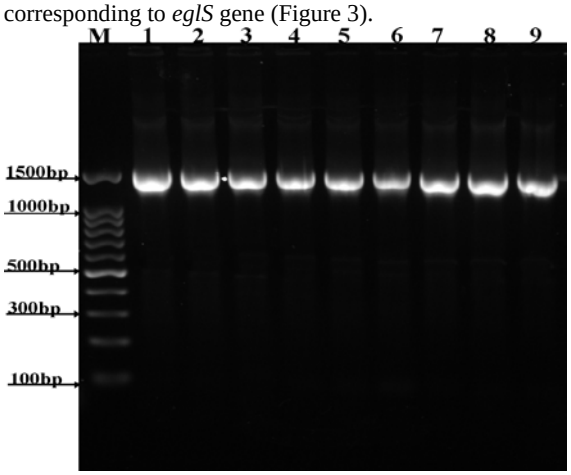

Figure 3. An agarose gel (1 %) electrophoresis of the amplification of *eglS* gene of nine *E. coli* DH5α (EglS-nrc-1) transformants. Lane M: 100bp DNA ladder, lane1: T-eglS1; lane2: T-eglS2; lane3: T-eglS3; lane4: T-eglS4; lane5: T-eglS5; lane6: T-eglS6; lane7: T-eglS7; lane8: T-eglS8 and lane9: T-eglS9 transformants.

3.4. Optimization of Carboxymethylcelllulase (CMCase) activity

3.4.1. Temperature effect

Cellulase activity of three stains; *E. coli* T-*eglS*1 (transformant), *B. subtilis* BTN7A (donor) and *E. coli* DH5α (recipient) were measured at 37°C and 55°C. Crude

enzyme preparations from bacterial cultures were used to degrade CMC, and reducing sugar was determined after 30 min of incubation with substrate at 37°C or 55°C.

The results presented in Figure 4 indicated that the two temperatures used; 37°C and 55°C were suitable for enzyme production; however, there was about 30.4% reduction in their activities at 55°C compared to their activities at 37°C. The effect of temperature was previously studied by many reports; some observed that the optimum temperature of *B. subtilis* -endoglucanase production ranged from 35 °C to 39 ° C (Deka *et.al*, 2013; Gautam and Sharma, 2014; Lee *et al.*, 2010), while Wei *et.al* (2015) concluded that it was 60 ° C. Chan and Au (1987) reported that it might be even up to 65 °C.

The present data (Figure 4) also revealed that *eglS* gene expressed successfully in *E. coli* DH5α, and the enzyme activity of the constructed strain *E. coli* DH5α (T-eglS1) was much higher than the enzyme activity of its parental strain which has low constitutive level of celulases activity (negligible value). Moreover the new construct T-eglS1 produced much higher enzyme yield than its donor strain *B. subtilis* BTN7A. It produced 27 U/ mg at 37°C which was about 78% more than *B. subtilis* BTN7A, and 20.88 U/ mg at 55°C which was about 98% more than *B. subtilis* BTN7A. Similar result on *blgs* production has been previously reported (Hegazy *et al.*, 2018). The data obtained was also in agreement with Pandey *et al.*, (2014) who demonstrated that up to four times increase in cellulase production had been obtained by cloning *B. subtilis* IARI-SP-1-*eglS* gene in the expression vector pET-28a and over expressed in *E. coli* BL21 DE3.

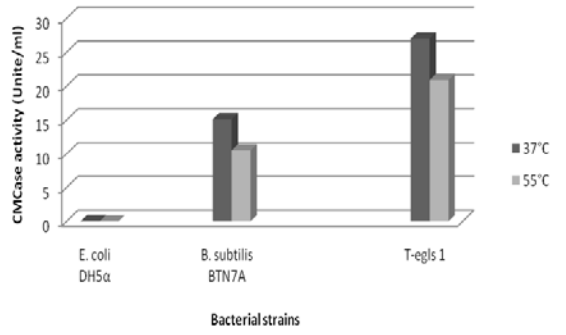

Figure 4 Effect of temperature on CMCase activity of *E. coli* DH5α, B.*subtilis* BTN7A (donor) and T-eglS-1(transformant)*

*The selected bacterial strains were inoculated into flasks containing 20 mL LB broth medium and incubated at 37°C for 24h under shaking (120 rpm). Enzyme activity was calculated at 37°Cor 55°C.

3.4.2. Inoculum size effect

Two different inoculum sizes (X and 2X) of *E. coli* TeglS-1 cultures were inoculated into three types of media; LB, BHM broth medium supplemented with CMC or cellulose as a sole carbon source. The three culture sets were incubated at 37 °C up to 3 days with shaking (120 rpm), and CMCase activity was determined daily. Data presented in Figure 5 indicated that the cellulolytic activity was increased by doubling the inoculum size. And the highest activity produced after 24 h of incubation in BHM medium supplemented with CMC.

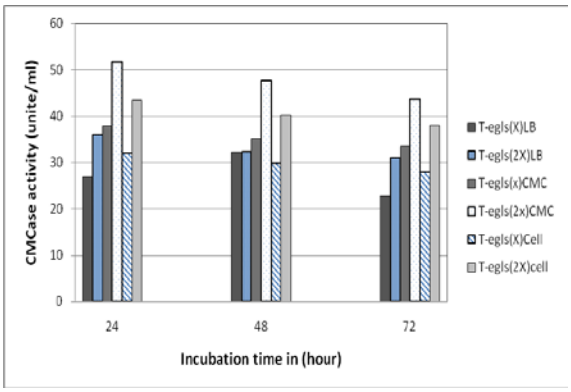

Figure 5 Effect of inoculum size, carbon source of the growth medium and incubation time on enzyme activity*

X = bacterial inoculum corresponding to OD_{620} = 0.01

2X = bacterial inoculum corresponding to OD_{620} = 0.02

* Bacterial cell were grown into complete LB medium and two minimal media of BHM amended with carboxymethyl cellulose (CMC) or cellulose powder as a sole carbon source at 37°C up to 3 days under shaking (120 rpm) and the enzyme activity of the culture supernatant was determined.

3.4.3. Synergising effect of co-culture of E. coli –eglS with E. coli – bgls and carbon source of the growth media

E. coli clone containing *eglS* gene designated as T-eglS and a mixture culture of *E. coli eglS*1 and *E. coli bgls*1 were grown into LB or BHM broth medium supplemented with CMC or cellulose as a sole carbon source, at 37 °C for 3 days shaking incubator, and then their enzymes activities in cell –free culture supernatant were daily measured.

Results presented in Figure 6 revealed that the highest cellulolytic activity was observed after 24 h and by increasing the incubation time the enzyme activity was decreased. It was also noticed that Carboxymethyl cellulose (CMC) was the best carbon source regulating both *eglS* and *bgls* expressions. This finding is in agreement with (Azadian *et al.*, 2017, Gautam and Sharma 2014, Hegazy *et al.*, 2018, Sadhu *et al.*, 2013 and Shaikh *et al.*, 2013) and with Sreena and Sebastian (2018) who found that CMC among the most important parameter had positive effect on cellulase production by *B. subtilis* MU.

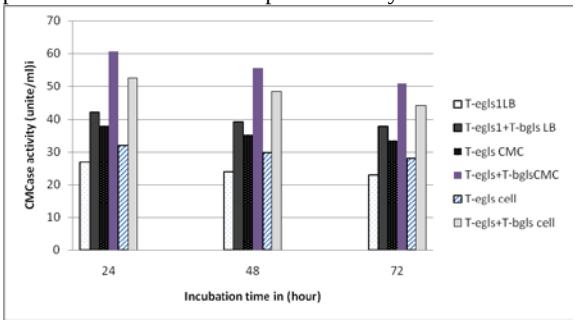

Figure 6. Synergizing effect of co-culture of *E.coli* –eglS with *E.coli* -bgls and carbon source of the growth media with incubation time*

* Bacterial cell were grown into LB or BHM broth medium supplemented with CMC or cellulose as a sole carbon source, at 37 °C for 3 days in shaking incubator, and then their enzymes activities in cell –free culture supernatant were daily measured.

By inoculating a mixture of both T-*eglS* and T-*bgls*, the cellulase activity reached the maximum value among all tested inoculation conditions. It reached up to 60.6 U/ mL at 24 h the occurrence of the two endoglucanase enzymes; *eglS* plus *bgls* suggesting synergic cellulolytic system in bacterial strain that play a role in complete cellulose hydrolysis. The previous data indicated that endoglucanase expression in *E coli* showed a high potential for enhancement of the enzyme production.

4. Conclusions

A successful endo-β-1, 4 glucanase (*eglS*) gene cloning method was described using the PCR technique. The new constructed plasmid was transformed into *E. coli*, and *eglS* expression was studied in the new exogenous bacterial host. Optimum enzyme activity was identified including temperature, inoculum size, carbon source of the growth media and incubation time. CMC had positive regulation effect in *eglS* expression. The present study illustrates the synergistic cooperative interactions between both of the endo-β-1, 4 glucanase and the endo-β-1, 3-1, 4 glucanase enzymes on cellulose degradation. Further studies are planned to use the identified optimum conditions for biodegradation of cellulolytic-waste biomass. New cellulolytic microorganisms could be developed to gain such economical properties for cellulose degradation by introducing the two endoglucanase genes, *eglS* and *bgls*, into new bacterial hosts.

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