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Cloning, Fusion Expression, and Enzymatic Characterization of Two Cellulase Genes from Bacillus subtilis: Postprint

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Date: 2017-10-11T00:00:00+00:00

Abstract

This study aimed to construct a fusion expression system for different cellulases and investigate the enzymatic properties of the fused cellulase. Using PCR technique, two cellulase genes, Cel42 and Cel22, were amplified from Bacillus subtilis previously isolated in our laboratory, a flexible linker (GSGGGS) was designed, and the two cellulase genes were assembled within a single open reading frame (ORF) through restriction enzyme digestion and ligation, inserted into pET32a(+) to construct the recombinant expression vector pET32a(+)-Cel42-Cel22, transformed into Escherichia coli BL21(DE3) for induced expression, and its enzymatic properties were investigated. The results showed that this study successfully cloned the two cellulase genes Cel42 and Cel22 and constructed the recombinant expression system BL21(DE3)/pET32a(+)-Cel42-Cel22; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) estimated its molecular mass to be approximately 101 kDa, the endoglucanase activity in the crude enzyme solution was 57.62 U/mL, and the exoglucanase activity was 32.57 U/mL. The optimal reaction temperature of the fused cellulase Cel42-Cel22 obtained in this study was 50 °C, and the optimal reaction pH was 6.0; the cellulase activity could be maintained above 70% within a temperature range of 30–70 °C, and above 75% within a pH range of 4.0–9.0. Except for Mn2+, other metal ions exhibited certain inhibitory effects on cellulase activity, among which Hg2+ and Cu2+ showed relatively significant inhibition. Thus, this study successfully expressed the fused cellulase Cel42-Cel22 in Escherichia coli BL21(DE3), and the enzyme exhibited certain activity, could adapt to a relatively broad temperature and pH range, and was sensitive to metal ions.

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Full Text

Cloning, Fusion Expression, and Enzymatic Characterization of Two Cellulase Genes from *Bacillus subtilis*

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Abstract: This study aimed to construct a fusion expression system for different cellulases and investigate the enzymatic properties of the resulting fusion enzyme. Two cellulase genes, *Cel42* and *Cel22*, were amplified via PCR from *Bacillus subtilis* strains previously isolated in our laboratory. A flexible linker peptide (GSGGGS) was designed to fuse the two genes into a single open reading frame (ORF), which was then inserted into the pET32a(+) vector to construct the recombinant expression plasmid pET32a(+)-*Cel42*-*Cel22*. The recombinant plasmid was transformed into *Escherichia coli* BL21(DE3) for induced expression, and the enzymatic properties of the fusion cellulase were characterized. The results demonstrated successful cloning of both *Cel42* and *Cel22* genes and construction of the recombinant expression system BL21(DE3)/pET32a(+)-*Cel42*-*Cel22*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis estimated the molecular mass of the fusion protein to be approximately 101 kDa. The crude enzyme extract exhibited endoglucanase activity of 57.62 U/mL and exoglucanase activity of 32.57 U/mL. The optimal reaction temperature for the fusion cellulase Cel42-Cel22 was 50 °C, with the enzyme maintaining over 70% of its activity between 30–70 °C. The optimal pH was 6.0, with over 75% activity retained across pH 4.0–9.0. Except for Mn^2 , all tested metal ions inhibited enzyme activity to varying degrees, with Hg^2 and Cu^2 showing the most pronounced inhibitory effects. These findings indicate that the fusion cellulase Cel42-Cel22 was successfully expressed in *E. coli* BL21(DE3), possesses significant enzymatic activity, and demonstrates broad temperature and pH adaptability, though it remains sensitive to metal ions.

Keywords: cellulase; *Bacillus subtilis*; cloning; fusion expression; enzymatic properties

CLC number: S852.2
Document code: A

Received: 2017-02-08
Funding: National Natural Science Foundation of China (31101744); Major

Science and Technology Project of Henan Provincial Science and Technology Department (131100110300)

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Introduction

Cellulose is a high-molecular-weight compound polymerized from 800–1,200 glucose molecules and represents the most abundant renewable organic resource worldwide. As a major agricultural country, China produces approximately 20% of the world' s crop straw [1-2], yet only about 10% is utilized as ruminant feed due to its polysaccharide structure [3]. Conventional straw utilization requires physical or chemical pretreatment, which suffers from low efficiency and secondary environmental pollution, limiting widespread application [4-5]. Consequently, research has increasingly focused on biological degradation using microbial cellulases. Cellulases are classified into three major categories: endo-1,4- -D-glucanases, exo-1,4- -D-glucanases, and -1,4-glucosidases [6-7], with applications in alcohol production, papermaking, animal husbandry, food processing, and textiles [8-10]. Therefore, cellulase research holds significant promise for both industrial applications and societal benefit.

Natural microbial cellulase production is limited by low yields and single enzyme types, while synergistic degradation by different cellulase-producing microorganisms is difficult to optimize, making natural biodegradation too slow for industrial demands. Molecular biology offers an ideal solution by integrating different cellulase genes into efficient microbial expression systems. Although previous studies have cloned and expressed cellulase genes from various sources in bacteria, fungi, and yeast [10-14], most have focused on single enzymes, which exhibit poor individual performance and require precise 配比 for effective application. Fusion expression technology has matured to create multifunctional composite proteins, simplifying downstream purification and processing [15]. While *E. coli* pET vectors have been used for cellulase expression [12,16-17], reports on fusion expression of different cellulases in *E. coli* remain scarce.

In our previous work, we isolated two cellulose-degrading *Bacillus subtilis* strains (N042 and N22) from soil and preliminarily identified two distinct cellulases. To investigate their synergistic effects, this study cloned both cellulase genes, designed a flexible linker based on the flanking sequences, fused them into a single ORF, and inserted the construct into the *E. coli* expression vector pET32a(+). We evaluated expression efficiency in *E. coli* BL21(DE3) and characterized the resulting fusion cellulase to establish a foundation for efficient cellulose degradation.

Materials and Methods

Strains and Plasmids *Bacillus subtilis* N42 and *Bacillus subtilis* N22 were isolated and identified in our laboratory. Competent *E. coli* DH5 and BL21(DE3) cells, plasmids pMD18-T and pET32a(+), and recombinant strains BL21(DE3)/pET32a(+)-*Cel42* and BL21(DE3)/pET32a(+)-*Cel22* were constructed and stored in our laboratory.

Enzymes and Reagents Pyrobest™ DNA Polymerase, T4 DNA ligase, restriction enzymes BamH , Xho , Nco , DNA markers, and protein markers were purchased from TaKaRa. Isopropyl- -D-thiogalactoside (IPTG), X-gal, Goldview nucleic acid stain, and Ni-NTA Sefinose™ Resin Kit were obtained from Beijing Solarbio Science & Technology. Plasmid purification and DNA gel extraction kits were purchased from Beijing Tiangen Biotech.

Cloning of Target Genes Based on *Bacillus subtilis* endoglucanase (KF240848.1) and -1,3-1,4-glucanase (KM009051.1) sequences from GenBank, two primer pairs were designed using Primer Premier 5.0: F1: 5' -CATGCCATGGGAGTGCAGATGAAAC-3', R1: 5'-GGATCCACCGCCAGATCCATTTG-3'; F2: 5'-CGGGATCCATGCCTTATCTGAAACG-3', R2: 5'-CCGCTCGAGTTATTTTTTTGTATAGCGC-3' . Underlined sequences indicate restriction sites, and boxed sequences in R1 and F2 encode the linker peptide for gene fusion. Primers were synthesized by Sangon Biotech (Shanghai). Using genomic DNA from *B. subtilis* N42 and N22 as templates, PCR amplification with F1/R1 and F2/R2 yielded products named *Cel42* and *Cel22*, respectively. These were cloned into pMD18-T to generate pMD18-T-*Cel42* and pMD18-T-*Cel22*, which were sequenced by Sangon Biotech.

Construction of Fusion Expression Vector Plasmids pMD18-T-*Cel42* and pMD18-T-*Cel22* were double-digested with BamH /Xho . The target fragments were recovered using a gel extraction kit, ligated with T4 DNA ligase at 16 °C overnight, and transformed into *E. coli* DH5 competent cells. Positive clones were screened and identified by PCR and double digestion to obtain pMD18-T-*Cel42*-*Cel22*. This plasmid and the expression vector pET32a(+) were digested with Nco /Xho , ligated, transformed into *E. coli* DH5 , and positive clones were identified by PCR, double digestion, and sequencing to yield pET32a(+)-*Cel42*-*Cel22*.

Transformation and SDS-PAGE Analysis The recombinant plasmid pET32a(+)-*Cel42*-*Cel22* was transformed into *E. coli* BL21(DE3). Positive clones were inoculated into LB medium containing 100 μg/mL ampicillin and cultured at 37 °C, 220 rpm overnight. Cultures were diluted 1:50 into 200 mL fresh LB with the same antibiotic concentration and grown to OD = 0.6. IPTG was added to a final concentration of 0.5–2.0 mmol/L, and induction proceeded at 37 °C, 180 rpm for 12 h. Cells were harvested by centrifugation

at 4 °C, 3,500 rpm for 30 min, washed with PBS (pH 7.0) at a 1:20 volume ratio, and centrifuged at 12,000 rpm for 10 min. The pellet was resuspended in 6 mL PBS, subjected to 3–5 freeze-thaw cycles at -80 °C, sonicated on ice, and centrifuged at 12,000 rpm for 10 min. The supernatant was analyzed by SDS-PAGE.

Enzyme Activity Assay of Fusion Cellulase Cel42-Cel22 Glucose standard solutions (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 mg/mL) were prepared to generate a standard curve by measuring OD nm. The recombinant culture was centrifuged at 4 °C, 12,000 rpm for 30 min, and the supernatant after sonication served as the crude enzyme extract.

Endoglucanase Activity: Determined by the 3,5-dinitrosalicylic acid (DNS) method [18]. One milliliter of crude enzyme was mixed with 2 mL 1.0% sodium carboxymethylcellulose (pH 4.8), incubated at 50 °C for 30 min, followed by addition of 2 mL DNS reagent and boiling for 10 min. After cooling and dilution to 5 mL, OD nm was measured to calculate glucose content. One unit (U) of endoglucanase activity was defined as the amount of enzyme producing 1 µg glucose per minute under these conditions.

Exoglucanase Activity: One milliliter of crude enzyme was mixed with 2 mL 1.0% microcrystalline cellulose sodium (pH 4.8), incubated at 50 °C for 30 min, centrifuged, and the supernatant analyzed by DNS method. One unit (U) of exoglucanase activity was defined as the amount of enzyme producing 1.0 µg reducing sugar per minute.

Optimal Temperature and Thermal Stability To determine optimal temperature, 25 µL purified fusion cellulase Cel42-Cel22 (pH 4.0–5.0) was mixed with 25 µL 1% sodium carboxymethylcellulose (pH 4.8) and incubated at 30, 35, 40, 45, 50, 55, 60, and 65 °C for 60 min. For thermal stability assessment, 25 µL purified enzyme was pre-incubated at 30–90 °C for 60 min, immediately cooled on ice, then mixed with 25 µL 1% substrate and assayed as above.

Optimal pH and pH Stability The purified fusion cellulase Cel42-Cel22 was adjusted to pH 3.0–12.0 using appropriate buffers: 100 mmol/L sodium citrate (pH 3.0), acetate (pH 4.0–5.0), phosphate (pH 6.0–7.0), Tris-HCl (pH 8.0–9.0), sodium bicarbonate/NaOH (pH 10.0–11.0), and KCl/NaOH (pH 12.0). Twenty-five microliters of enzyme at each pH was mixed with 25 µL 1% substrate at the corresponding pH and incubated at optimal temperature for 60 min. For pH stability, enzyme was held at various pH values at room temperature for 2 h before substrate addition and activity measurement.

Effect of Metal Ions on Fusion Cellulase Activity Various metal ions (Na , K , Mg^{2} , Fe^{2} , Cu^{2} , Mn^{2} , Ni^{2} , Hg^{2} , Co^{2} , Al^{3} , Fe^{3}) were added to the reaction system at 1 mmol/L final concentration. After incubation at room temperature for 60 min, residual cellulase activity was measured.

Results

Cloning of Cellulase Genes *Cel42* and *Cel22* PCR amplification with specific primers yielded two DNA fragments [Figure 1: see original paper] matching the expected sizes. Sequencing revealed lengths of 1,515 bp and 729 bp, which were submitted to GenBank (accession numbers KJ130416 and KJ130415). BLAST analysis showed *Cel42* shared high homology with *Bacillus subtilis* -1,4-endoglucanase, while *Cel22* was highly homologous to *Bacillus subtilis* -1,4-exoglucanase, confirming successful amplification.

PCR Amplification and Restriction Digestion of pMD18-*Cel42-Cel22* Using pMD18-*Cel42-Cel22* as template, PCR with primers F1/R1 and F2/R2 produced fragments of approximately 1.50 kb and 0.75 kb, corresponding to *Cel42* and *Cel22*, respectively. BamH single digestion yielded a ~4.95 kb band; Nco /BamH double digestion produced ~3.45 kb and ~1.50 kb bands; Nco /Xho digestion gave ~2.70 kb and ~2.25 kb bands; and BamH /Xho digestion generated two bands [Figure 2: see original paper].

PCR Amplification and Restriction Digestion of pET32a(+)-*Cel42-Cel22* The recombinant plasmid pET32a(+)-*Cel42-Cel22* digested with Nco /Xho produced ~5.90 kb and ~2.24 kb bands, while Xho single digestion yielded an ~8.10 kb band. PCR with F1/R1 primers amplified a ~1.50 kb fragment, all consistent with expected results [Figure 3: see original paper].

Expression of Fusion Protein Under induction conditions of 25 °C and 1 mmol/L IPTG, SDS-PAGE analysis of BL21(DE3)/pET32a(+)-*Cel42-Cel22* showed a prominent band near 101 kDa, absent in the control strain BL21(DE3)/pET32a(+), matching the predicted molecular mass of the fusion protein [Figure 4: see original paper]. This confirmed successful expression of the Cel42-Cel22 fusion cellulase in *E. coli*.

Enzyme Activity of Fusion Cellulase Cel42-Cel22 Based on the glucose standard curve [Figure 5: see original paper] and activity calculations, the crude enzyme extract exhibited endoglucanase activity of 57.62 U/mL and exoglucanase activity of 32.57 U/mL.

Effect of Temperature on Activity and Stability The purified fusion cellulase showed optimal activity at 50 °C [Figure 5A: see original paper]. Thermal stability tests revealed that over 70% activity was retained after 60 min incubation at 30–70 °C, with activity declining sharply above 70 °C [Figure 5B: see original paper].

Effect of pH on Activity and Stability The enzyme displayed optimal activity at pH 6.0 [Figure 5C: see original paper]. pH stability tests showed that over 75% activity was maintained across pH 4.0–9.0 after 2 h incubation [Figure 5D: see original paper].

Effect of Metal Ions on Activity Metal ion analysis [Figure 6: see original paper] demonstrated that, except for Mn^{2} , all tested ions inhibited enzyme activity to varying degrees. Hg^{2} exhibited the strongest inhibition (relative activity 28.94%), followed by Cu^{2} (41.53%). Between iron species, Fe^{2} showed higher biological activity than Fe^{3} .

Discussion

Cellulose degradation is a complex process requiring synergistic action of multiple cellulase types [18]. While mixed microbial consortia can enhance degradation efficiency, strain selection and optimization complexities limit large-scale industrial application [19]. Genetic engineering enables integration of different cellulase genes into a single strain, facilitating process control and simplification. Successful gene fusion depends critically on appropriate linker peptides to maintain stable spatial structure and biological activity. Common linker amino acids include proline, glycine, serine, threonine, and alanine [20]. In this study, we designed a nucleotide sequence (GGATCTGGCGGT) encoding Gly-Ser-Gly-Gly, combined with the BamH site (GGATCC) encoding Gly-Ser, to create a six-amino-acid flexible linker (GSGGGS). This linker fused two cellulase genes within a single ORF, generating the fusion gene *Cel42-Cel22* inserted into pET32a(+). PCR and restriction analyses confirmed successful construction of pET32a(+)-*Cel42-Cel22*. The recombinant strain BL21(DE3) expressed the fusion cellulase with endoglucanase activity of 57.62 U/mL and exoglucanase activity of 32.57 U/mL, demonstrating advantages over previously reported *E. coli* expression systems [16] though lower than some other systems [21]. The pET system' s tendency to form inclusion bodies may partially explain the moderate activity [22].

Enzymatic characterization revealed optimal conditions of 50 °C and pH 6.0, consistent with cellulase from *Bacillus pumilus* S124A [23]. The fusion enzyme maintained >70% activity at 30–70 °C and >75% activity at pH 4.0–9.0, indicating broad temperature and pH tolerance suitable for practical applications. Since complete cellulose degradation requires endoglucanases, exoglucanases, and -glucosidases, future studies could integrate additional cellulase genes to further improve yield and activity. Moreover, developing recombinant lactic acid bacteria expressing multiple cellulase genes as probiotic preparations would enhance practical application value and warrants further investigation.

Conclusions

1. This study successfully cloned the *Bacillus subtilis* -1,4-endoglucanase gene *Cel42* and -1,4-exoglucanase gene *Cel22*, fused them within a single ORF using a designed linker peptide, and achieved functional expression of the fusion cellulase Cel42-Cel22 in *E. coli*. The crude enzyme exhibited endoglucanase activity of 57.62 U/mL and exoglucanase activity of 32.57 U/mL.

2. The fusion cellulase Cel42-Cel22 displayed optimal activity at 50 °C and pH 6.0, maintained >70% activity between 30–70 °C, and retained >75% activity across pH 4.0–9.0. Metal ions Hg^2 and Cu^2 significantly inhibited enzyme activity.

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