Expression of the Endoglucanase Gene in *Escherichia coli* for the Second-generation Bioethanol Production

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Abstract

The endoglucanase enzyme in *Escherichia coli* was expressed to improve enzymatic activities at low temperature and facilitate large-scale enzyme production. The endoglucanase gene (GenBank CAE51308.1) encoding endoglucanase enzyme (EC 3.2.1.4) was selected from *Clostridium thermocellum*, a major component in the cellulosome complex and effective in cellulose hydrolysis. The mature endoglucanase gene was cloned into pET28a (+) vector and expressed in *E. coli* BL21 (DE3) pLysS. SDS-PAGE revealed the molecular weight of recombinant endoglucanase enzyme at around 72 kDa. Carboxymethylcellulose (CMC) was used as a substrate to determine endoglucanase enzyme activity. Under various combinations of pH, temperature and time results showed that the recombinant enzyme reached maximum activity (3.05 U/mL) at pH 10, 40 °C for 10 min. Further study of large-scale enzyme production together with exoglucanase and β-glucosidase enzymes is planned for biomass hydrolysis in second-generation bioethanol production.

Keywords: Bioethanol, Biomass, Endoglucanase, Cellulose

1. Introduction

Cellulase enzymes play an important role in biomass degradation with potential applications in various industries including animal feed and biofuel production. Development of renewable energy sources has become increasingly important to keep pace with the ever-increasing global population and limited crude oil supplies (Schiffer, 2008). One important issue in renewable energy is the conflict between foods versus fuel. Lignocellulosic biomass is an excellent choice of feedstock for renewable energy since it is cheap and better suited to the production of biofuel than as a food. Lignocellulosic biomass can be turned into biofuel through an enzymatic conversion process (Maki *et al.*, 2011). Studies on cellulose bioconversion indicated that cellulase enzymes play important roles in extracting fermentable sugars from lignocellulosic biomass (Liang *et al.*, 2014).

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Cellulase enzymes, as exoglucanase, endoglucanase and β-glucosidase, are used in lignocellulosic biomass hydrolysis. Exoglucanase (EC 3.2.1.91) degrades cellobiosyl units from the ends of the cellulose polymer, while endoglucanase (EC 3.2.1.4) randomly degrades internal 1,4-β-glycosidic bonds into cello-oligosaccharides of various lengths. β-glucosidase (EC 3,2,1,21) is used in the saccharification process, whereby glucose is produced from cleavage of cello-oligosaccharides (Ueda *et al.*, 2014; Gupta *et al.*, 2013). Endoglucanase (EglC) can release smaller cellulose fragments of random length and has wide applications in biomass waste management, improvement in beer brewing and decreasing viscosity of β-glucan solutions (Kuhad *et al.*, 2011; Sweeney and Xu, 2012). Bai *et al*. (2016a) showed that endoglucanase (EglC) hydrolyzes glycosidic linkages and releases oligosaccharides of different lengths.

Here, the endoglucanase gene was expressed in *E. coli* which is considered one of the most appropriate hosts for the production of recombinant proteins, secreting protein into soluble fractions and ease of genetic manipulation. The selected gene was codon optimized compatible with *E. coli* codon usage to improve enzymatic activities at a lower temperature than its counterpart enzyme.

2. Materials and Methods

2.1 Endoglucanase gene, bacterial strains, plasmids, and chemicals

The endoglucanase gene named *EndoF* (GenBank CAE51308.1) encoding endoglucanase enzyme was selected from *Clostridium thermocellum* in the NCBI database. This gene was synthesized and ligated into the pET28a (+) vector by GenScript Corporation (GenScript, USA). The recombinant vector was then named pET28a (+)-EndoF. *E. coli* DH5α and *E. coli* BL21 (DE3) pLysS (Invitrogen, USA) were used for gene cloning and protein expression. Restriction enzymes as *NcoI* and *XhoI*, T4 DNA ligase, DNA polymerase, proteinase inhibitor, and GeneJET Plasmid Minprep Kit were supplied by Thermo Fisher Scientific (USA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich (USA).

2.2 Construction of expression system

The pET28a (+)-EndoF recombinant vector was transformed into *E. coli* DH5α by heat shock method (Sambrook *et al.*, 1989) and colonies were selected on LB agar (Difco, USA) containing 50 µg/mL kanamycin. All selected clones were checked by colony PCR using specific EndoF-F primer (5'-CATGCCATGGCATGCCATGGATTATAACTATGGCGAA-3') and
NcoI
EndoF-R primer (5'-CCGCTCGAGGCTGTTGCCCGGAATGGTCGGAAT-3').
XhoI

A mature endoglucanase gene named EndoM was generated from pET28a (+)-EndoF by DNA amplification using primers EndoF-F and EndoF-R. The EndoM gene was then cloned into pET28a (+) vector and transformed into *E. coli* BL21 (DE3) pLysS. Colonies were selected on LB agar containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. Restriction enzyme digestion and DNA sequencing (GenScript, USA) were carried out to verify the EndoM clones.

2.3 Determination of recombinant endoglucanase activity by Congo red assay

E. coli BL21 (DE3) pLysS harboring the full endoglucanase gene named EndoF and mature endoglucanase gene named EndoM were streaked on LB agar plates containing 50 µg/mL kanamycin, 34 µg/mL chloramphenicol, 1% (w/w) CMC, and 1 mM IPTG. The plates were then incubated at 37 °C for 48 h and hydrolysis zones were visualized by staining with 1% (w/v) Congo red for 10 minutes and destaining with 1 M NaCl solution (Li *et al.*, 2008). A clear zone around the colonies indicated that the endoglucanase gene was successfully expressed.

2.4 Expression of the mature endoglucanase gene (EndoM) in *E. coli*

Expression of the EndoM gene in *E. coli* BL21 (DE3) pLysS was done in 50 mL of LB broth containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol. The culture was then incubated in a shaking incubator at 37 °C with 200 rpm. Then, 10 mL of overnight culture was inoculated into 100 mL of LB broth containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and incubated at 30 °C in a shaking incubator with 200 rpm. When optical density of the culture reached OD_{600} 0.4–0.6, 1 mM IPTG was added into the medium to induce endoglucanase expression. After 3 hours, *E. coli* cells were harvested by centrifugation at 8,000 rpm for 5 min, resuspended in 30 mL of phosphate buffer saline (PBS, pH 7.0), and centrifuged at 10,000 rpm for 15 min. The pellet was then added with 4.8 mL of lysozyme solution (100 mg/mL) and proteinase inhibitor (1 tablet/50 mL of extract) and the solution was incubated at 37 °C for 3 h. A total of 800 µL of $MgCl_2$ solution was then added and incubation continued for another 30 min. Finally, the cells were collected by centrifugation at 10,000 rpm for 30 min at 4 °C.

Protein concentration was determined by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard. Protein molecular weight was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

2.5 Characterization of enzymatic activities

Enzyme activity was determined by the amount of reducing sugar released during enzyme incubation with carboxymethyl cellulose (CMC), using the 3, 5-dinitrosalicylic (DNS) method as described by Miller (Miller, 1959). A solution containing 100 µL of diluted enzyme and 400 µL of 1% (w/v) CMC in 50 mM sodium acetate buffer (pH 5), was incubated at 50 °C

for 30 min. Enzyme activity was stopped by adding 500 µL of DNS reagent to the solution, boiling for 10 min and then placing on ice for a further 10 min. Absorbance was measured at 540 nm using a microplate reader (Infinite F50, Tecan Trading AG, Switzerland). One unit (U) of endoglucanase activity was defined as the amount of enzyme required to release 1 µmol of glucose per minute at the assay temperature. The activity of recombinant endoglucanase enzyme was determined over 5 to 60 min at 30 °C using glucose (Wako, Japan) as the standard.

To determine optimal pH, the enzyme was separately incubated with 1% (w/v) CMC in 50mM sodium acetate buffer (pH 3–6) and 50 mM sodium phosphate buffer (pH 7–11) at 50 °C. Temperature was varied from 30 to 80 °C at the optimal pH and time, respectively. All measurements were performed in duplicate.

3. Results and Discussion

3.1 Construction of recombinant plasmid

The mature endoglucanase gene (EndoM) was amplified from the recombinant pET28a (+)-EndoF vector using EndoF-F and EndoF-R primers. Based on primer design, *NcoI* and *XhoI* restriction sites were incorporated at the 5' and 3' end, respectively. Results suggested that the PCR product was approximately 2,127 bp as expected (Figure 1).

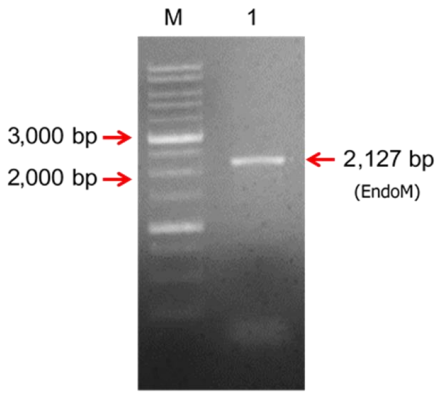

Figure 1 Agarose gel electrophoresis of PCR products (EndoM gene) amplified using EndoF-F and EndoF-R primers. Lane M represents the DNA ladder, lane 1 is the EndoM gene at around 2,127 bp

After DNA amplification, the EndoM gene was purified and ligated into pET28a (+) vector. The recombinant vector was then transformed into *E. coli* BL21 (DE3) pLysS. Clones containing the EndoM gene were preliminarily verified by restriction enzyme digestion with *NcoI* and *XhoI*. Results showed that the EndoM gene was successfully cloned into pET28a (+) vector (Figure 2). To confirm the correction of DNA sequence, clones containing the EndoM gene were subjected to further DNA sequence analysis.

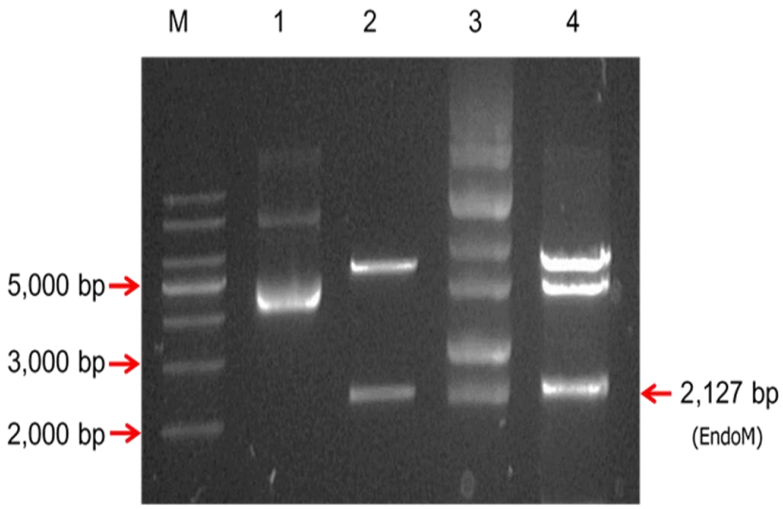

Figure 2 Agarose gel electrophoresis of recombinant plasmid digestion with *NcoI* and *XhoI* restriction enzymes. Lane M is the DNA marker, lane 1 is undigested pET28a (+)-EndoF recombinant plasmid, lane 2 is digested pET28a (+)-EndoF recombinant plasmid, lane 3 is undigested pET28a (+)-EndoM recombinant plasmid, lane 4 is digested pET28a (+)-EndoM recombinant plasmid. Arrows indicate the EndoM gene, 2,127 bp

3.2 Determination of recombinant endoglucanase activity on CMC agar plates

Results showed a clear zone around the clones containing the EndoM gene (mature gene), whereas no clear zone was found around clones containing the EndoF gene (full-length gene). Thus, clones containing the EndoM gene were selected and tested for protein expression and characterization (Figure 3).

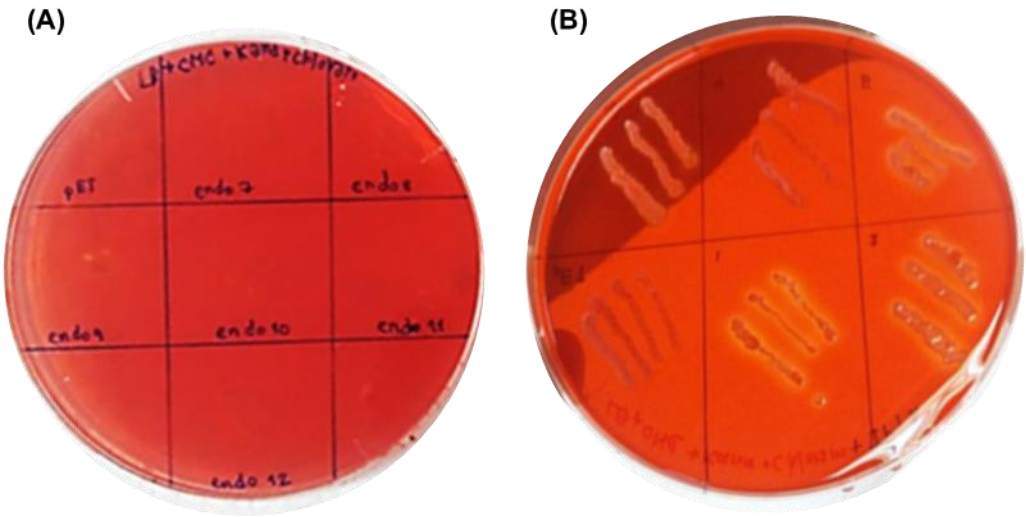

Figure 3 Cellulase activity was determined by incubating selected clones on CMC agar plates at 37 °C for 48 h and evaluated by clear zones after staining with 1% (v/w) Congo red solution. (A) *E. coli* BL21 (DE3) pLysS containing the EndoF gene (full-length gene), (B) *E. coli* BL21 (DE3) pLysS containing the EndoM gene (mature gene)

3.3 Expression of endoglucanase enzyme in *E. coli* BL21 (DE3) pLysS

Clones containing the EndoM gene were induced with 0.1 mM IPTG at 30 °C for 3 h. After cell disruption, the induced protein was then evaluated by SDS-PAGE analysis for both the supernatant and pellet fractions. SDS-PAGE showed that the recombinant protein, 72 kDa, was found mostly in the supernatant fraction (Figure 4). Therefore, this fraction was selected for enzyme activity evaluation.

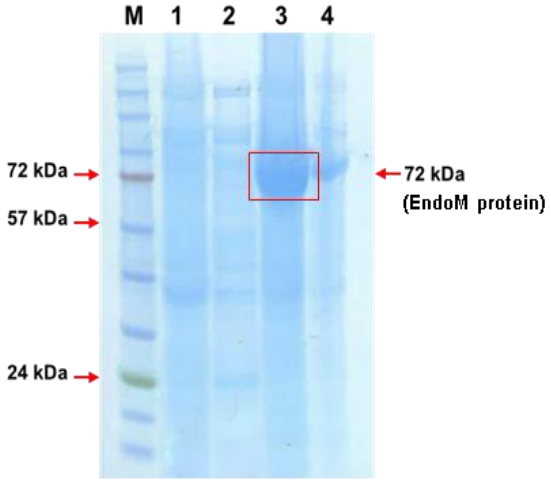

Figure 4 SDS-PAGE analysis of recombinant protein comparing supernatant and pellet fractions. Lane M is the protein marker, lane 1 is the supernatant fraction without IPTG induction, lane 2 is the pellet fraction without IPTG induction, lane 3 is the supernatant fraction with 1 mM IPTG induction, and lane 4 is the pellet fraction with 1 mM IPTG induction. The square indicates EndoM protein with molecular weight of about 72 kDa

3.4 Characterization of enzymatic activities

Activity of the recombinant enzyme was assayed in 1% (w/v) CMC at different pH, time and temperature. Results gave optimal pH at 10 (Figure 5), optimal temperature at 40 °C (Figure 6), and optimal incubation time of 10 min (Figure 7).

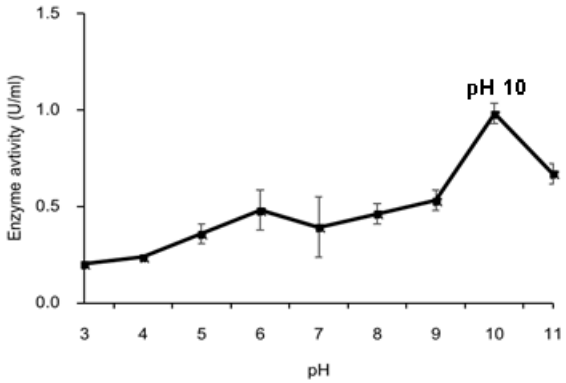

Figure 5 Effect of pH on recombinant endoglucanase enzyme activity

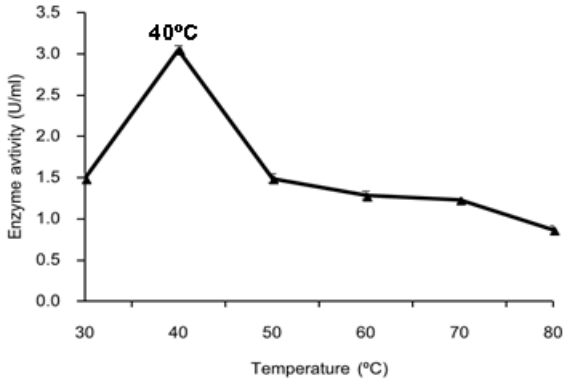

Figure 6 Effect of temperature on recombinant endoglucanase enzyme activity

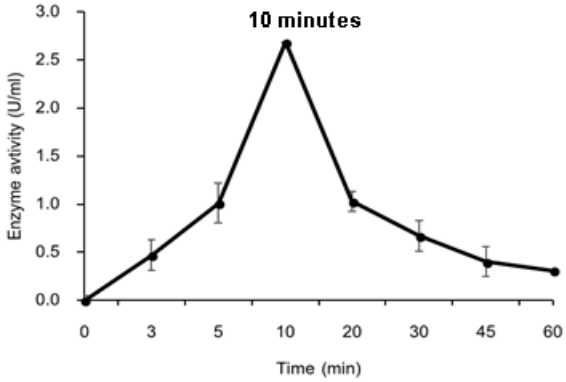

Figure 7 Effect of time on recombinant endoglucanase enzyme activity

Results showed that the endoglucanase gene selected from *C. thermocellum* was successfully cloned and expressed in *E. coli*. The recombinant enzyme had maximum activity of about 3.09 U/mL and was active at 40 °C. Optimal temperature of enzyme activity was lower than the original endoglucanase enzyme from *C. thermocellum* that showed activity at 78.5 °C (Zverlov *et al.*, 2005). These results concurred with previous reports that cold-active endo-1,4-β-glucanase (EglC) showed optimal temperature for activity at 30–40 °C. This cold-active enzyme offers high potential for biotechnology as improved activity at lower temperature will decrease energy costs for biomass hydrolysis (Bai *et al.*, 2016b).

4. Conclusions

The mature endoglucanase gene (EndoM) from *C. thermocellum* was codon optimized, cloned into pET28a (+), and expressed in *E. coli* BL21 (DE3) pLysS to improve enzyme activity at low temperature and facilitate large-scale enzyme production. This recombinant endoglucanase enzyme gave optimal pH of 10 and optimal temperature at 40 °C when incubated with substrate for 10 min. Maximum enzyme activity was 3.05 U/ml. These results concurred with an earlier report which found that a cellulase of bacterial origin could effectively

ensure proper folding and secretion in *E. coli* (Adlakha *et al*., 2011). Further research is required to focus on large-scale enzyme production for other cellulase enzymes, i.e. exoglucanase and β-glucosidase, for biomass hydrolysis in second-generation bioethanol production.

Acknowledgements

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