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Research article

Escherichia coli expressing endoglucanase gene from Thai higher termite bacteria for enzymatic and microbial hydrolysis of cellulosic materials

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ABSTRACT

Background: Endoglucanase plays a major role in initiating cellulose hydrolysis. Various wild-type strains were searched to produce this enzyme, but mostly low extracellular enzyme activities were obtained. To improve extracellular enzyme production for potential industrial applications, the endoglucanase gene of *Bacillus subtilis* M015, isolated from Thai higher termite, was expressed in a periplasmic-leaky *Escherichia coli*. Then, the crude recombinant endoglucanase (EglS) along with a commercial cellulase (Cel) was used for hydrolyzing celluloses and microbial hydrolysis using whole bacterial cells.

Results: E. coli Glu5 expressing endoglucanase at high levels was successfully constructed. It produced EglS (55 kDa) with extracellular activity of 18.56 U/mg total protein at optimal hydrolytic conditions (pH 4.8 and 50°C). EglS was highly stable (over 80% activity retained) at 40–50°C after 100 h. The addition of EglS significantly improved the initial sugar production rates of Cel on the hydrolysis of carboxymethyl cellulose (CMC), microcrystalline cellulose, and corncob about 5.2-, 1.7-, and 4.0-folds, respectively, compared to those with Cel alone. *E. coli* Glu5 could secrete EglS with high activity in the presence of glucose (1% *w*/*v*) and Tween 80 (5% *w*/*v*) with low glucose consumption. Microbial hydrolysis of CMC using *E. coli* Glu5 yielded 26 mg reducing sugar/g CMC at pH 7.0 and 37°C after 48 h.

Conclusions: The recombinant endoglucanase activity improved by 17 times compared with that of the native strain and could greatly enhance the enzymatic hydrolysis of all studied celluloses when combined with a commercial cellulase.

1. Introduction

Cellulosic biomass obtained from agricultural residues offers a higher potential than grains and edible feedstocks to serve as a sustainable resource with lower production costs for alternative liquid fuel production in the future [1]. The conversion of cellulose to ethanol is typically accomplished through the enzymatic hydrolysis of cellulosic materials to reducing sugars, followed by fermentation of the produced sugars to ethanol [2]. The enzymatic hydrolysis of cellulose involves synergistic actions of endoglucanases, exoglucanases, and β-glucosidases. Endoglucanases play a major role in initiating cellulose hydrolysis by randomly cleaving internal glucosidic bonds in cellulose fibers and creating free chain ends; exoglucanases release cellobiose either from reducing or non-reducing chain ends; and β-glucosidases finally hydrolyze the cellobiose to glucose [3].

The use of cellulolytic bacteria for the large-scale production of enzymes has increased in recent years because of their short doubling times and ease of cultivation at high cell densities using inexpensive carbon and nitrogen sources [4]. A number of cellulolytic bacteria from termite gut and other sources have been studied and continually developed by genetic engineering techniques to achieve desired properties and high productivity of endoglucanase for industrial applications [5,6,7,8,9,10].

Because of the high cost of enzyme production, biofuel production from cellulose is not feasible at present [11,12]. A consolidated bioprocess, combining cellulase production and conversion of cellulose into desired products as a single step, has been the subject of increased research efforts in recent years [13]. The absence of cellulase whole cell separation step can lower the overall process cost [14]. The term "microbial hydrolysis" referred to cultures of cellulolytic bacteria growing in the presence of cellulose substrate, which is to be

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hydrolyzed, without added enzyme [15]. Currently, there is little research available on cellulose hydrolysis using whole cells [14,15]. Hence, a study on this issue is of great interest because it will lead to potential applications for large-scale biofuel production from lignocellulosic materials.

In this study, an endoglucanase gene from *Bacillus subtilis* M015, isolated from Thai higher termites (*Microcerotermes* sp.) [5], was cloned into a periplasmic-leaky *Escherichia coli* strain. The crude recombinant endoglucanase produced by *E. coli* expressing the endoglucanase gene was characterized. A mixture of the recombinant endoglucanase and commercial cellulase was tested for their hydrolytic activity on different cellulosic materials such as soluble cellulose, insoluble microcrystalline cellulose, and corncob compared with the commercial enzyme alone. The recombinant bacteria were also studied for glucose consumption and microbial hydrolysis. Moreover, the effects of Tween 80 on bacterial growth and endoglucanase production were determined.

2. Materials and methods

2.1. Bacterial strains, vectors, and culture conditions

B. subtilis M015 (GenBank accession number **KP192484**), isolated from Thai higher termites (*Microcerotermes* sp.) by our research group [5], was preserved by lyophilization and grown in a 65 modified Deutsche Sammlung von Mikroor Ganismen und Zellkulturen (DSMZ) medium containing 5 g/L of carboxymethyl cellulose (CMC), 4 g/L of yeast extract, and 10 g/L of malt extract at pH 7.0. *E. coli* JE5505 (carrying an *lpp*-deletion), obtained from the Coli Genetic Stock Center (CGSC), Yale University, was maintained in a lysogeny broth (LB; 10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of sodium chloride at pH 7.0) for use as an expression host in this study. The expression plasmid, pFLAG-CTS, carrying an OmpA secretion signal for the periplasmic expression of recombinant proteins, and ampicillin sodium salt (100 μg/mL), used as the selection agent, were purchased from Sigma-Aldrich. Isopropyl β-D-thiogalactopyranoside (IPTG, Invitrogen Life Technologies) at 1 mM concentration was added to the culture to induce protein expression.

2.2. Amplification, cloning, and expression of endoglucanase gene

To obtain the DNA template for gene amplification, *B. subtilis* M015 cells were harvested in the mid-exponential growth phase and separated by centrifugation, and genomic DNA was extracted from the cell pellets using the DNeasy Blood and Tissue Kit (QiaGen, USA). For the amplification of the gene coding for endoglucanase by polymerase chain reaction (PCR), two primers were designed on the basis of the reference sequence of the endoglucanase (*egl*S) gene of *B. subtilis* subsp. *subtilis* 168 (GenBank accession number **BSU18130**); forward primer: 5′-GATCCAAGCTTCTATGAAACGGTCAATCTCTAT-3′, reverse primer: 5′-CTCACTCGAGTTACTAATTTGGTTCTGTTCCC-3′. Two restriction sites, *Hin*dIII and *Xho*I, were incorporated to allow directional cloning of the PCR products into the expression vector pFLAG-CTS. The *egl*S gene was amplified using Phusion Hot Start II polymerase (Thermo Scientific, USA) under the following PCR conditions: 2 min of initial denaturation at 98°C, 30 cycles of 30 s denaturation at 98°C, 30 s of annealing at 55°C, and 30 s of extension at 72°C, followed by 5 min of final extension at 72°C.

PCR products were double-digested with *Hin*dIII and *Xho*I and cloned into the plasmid pFLAG-CTS, which was also digested with the same restriction enzymes. The ligation products (pCTS-*egl*S) were transformed into *E. coli* JE5505 by electroporation. Positive clones were confirmed by recovering plasmid DNA using the QIAprep Spin Miniprep Kit (QiaGen, USA) and running on 0.8% agarose gel. The presence of the endoglucanase gene was confirmed by DNA sequencing. The transformed *E. coli* containing pCTS-*egl*S was named *E. coli* Glu5. *E. coli* carrying an empty plasmid was included as a control in the study. All molecular biology studies were performed by following standard techniques [16].

2.3. Preparation of secreted recombinant endoglucanase and size verification

E. coli Glu5 was cultured overnight in 2 mL LB-ampicillin as an inoculum in an orbital shaker at 180 rpm and 37°C under aerobic conditions and then diluted at a ratio of 1:400 into a 250-mL Erlenmeyer flask containing 100 mL of fresh LB-ampicillin medium. The flask was incubated with constant shaking. At the mid-exponential phase of the culture, when the optical density (OD_{600}) reached 0.5, endoglucanase (EglS) expression was induced with 1 mM IPTG. The optimal culturing time of *E. coli* Glu5 for secreted recombinant EglS production was determined during 40 h post-induction by the DNS assay (explained below) and further used for producing enzyme stock. The secreted EglS used as crude form was obtained by centrifuging the cell culture at 11000 × g for 10 min at 4°C and filtered through a 0.22-μm membrane. To verify the molecular size of the secreted mature protein, the secreted EglS was concentrated 10-fold by ultrafiltration (10 kDa MW membrane cut-off, Amicon, Beverly, MA, USA). The protein profile of the concentrated enzyme was determined by SDS-PAGE (12% (*w*/*v*) acrylamide resolving gel) and native-PAGE (containing 1% (*w*/*v*) CMC) for zymogram analysis [17]. The cell-free culture supernatant of *E. coli* carrying the empty plasmid was prepared in the same manner and used as a control. The theoretical molecular weight of EglS was calculated using the online tool available at Georgetown University Protein Information Resources Website (http://pir.georgetown.edu/cgi-bin/comp_mw.pl).

2.4. Hydrolytic ability assay

The hydrolytic ability of *E. coli* Glu5 was tested by the Congo red assay [18]. The inoculum (5 μL) was spotted on LB-ampicillin agar plates containing 1% (*w*/*v*) CMC and 1 mM IPTG. For comparison with the cloned strain (*E. coli* Glu5), *B. subtilis* M015 was assayed on 65 modified DSMZ agar plates at 37°C for 24 h, and then hydrolysis zones were visualized by 1% (*w*/*v*) Congo red reagent staining for 10 min and destaining with a 1 M sodium chloride solution. The presence of a clear zone around the colonies indicated the hydrolysis of cellulose by the secretion of active endoglucanase. The hydrolysis capacity value was calculated from the clear zone diameter divided by the colony diameter.

2.5. Enzymatic activity and protein determination

Endoglucanase activity was determined from crude enzyme by the standard DNS method using glucose as the standard reference [19]. The assays were conducted in triplicates in 96-well-microplates. Each diluted crude enzyme sample was incubated with 2% (*w*/*v*) CMC solubilized in 50 mM sodium citrate buffer (pH 4.8) at an equivalent ratio (total volume 60 μL). The mixture was incubated at 50°C for 30 min. The reaction was stopped by adding an equal volume of a DNS solution (1.4% (*w*/*v*) 3,5-dinitrosalicylic acid, 0.28% phenol, 1.4% sodium hydroxide, 28% sodium potassium tartrate, and 0.07% sodium sulfite) and heating at 95°C for 5 min and, finally, quickly cooled to room temperature. The degree of enzymatic hydrolysis of the soluble cellulose (CMC) was determined spectrophotometrically by measuring the absorbance at 540 nm in a VersaMax Microplate Reader (Molecular Probes, USA). One Unit (U) of endoglucanase activity was defined as the amount of enzyme producing 1 μmol of reducing sugars from CMC per min under the assay conditions. The total protein concentration was determined by the Bradford method (Bio-Rad) using bovine serum albumin standards. The specific activity was

calculated from the number of units of enzyme activity per mg of total proteins.

The hydrolytic mixture of added EglS enzyme at a volume ratio of 1:40 in 50 mM sodium citrate buffer containing 1% (*w*/*v*) CMC (9.4 U/g) was incubated at the optimum pH (4.8) and temperature (50°C) for 30 min and then analyzed for sugar products by high-performance liquid chromatography (HPLC) equipped with a refractive index detector (6040 XR, Spectra-Physics, USA) and an Aminex HPX-87H column (Bio-Rad Lab, USA). Sulfuric acid (H_2SO_4) at 5 mM concentration was used as a mobile phase at a flow rate of 0.6 mL/min, and the column temperature was controlled at 60°C. The supernatant of the cultured *E. coli* carrying the empty plasmid was used as control.

2.6. Characterization of crude endoglucanase

To determine the optimum pH for maximum endoglucanase activity of crude EglS, samples were diluted with citrate and phosphate buffers of various pH (50 mM sodium citrate buffer for pH 3.0–6.0 and 50 mM sodium phosphate buffer for pH 7.0–8.0), with 2% (*w*/*v*) CMC at an equivalent volumetric ratio, and incubated at 50°C. The effect of temperature on the endoglucanase activity was determined by incubating the diluted samples with 2% (*w*/*v*) CMC in 50 mM sodium citrate buffer (pH 4.8) at an equivalent volumetric ratio under temperatures ranging from 25°C to 80°C. The enzymatic hydrolytic reactions were stopped after 30 min by adding the DNS solution, and the endoglucanase activities were assessed as previously described. Thermal stability was determined by pre-incubating the diluted EglS in the 50 mM sodium citrate buffer (pH 4.8) at different temperatures of 40°C, 50°C, and 60°C. The residual endoglucanase activity of samples was determined as the percentage of the initial activity during an incubation period of 100 h.

The kinetic parameter values of the fresh crude EglS on CMC hydrolysis in terms of Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) were determined using the Lineweaver-Burk plots. The reaction was initiated by adding and mixing the enzyme (0.715 U/mL of reaction mixture) to CMC solutions of various initial CMC concentrations (2.5–47 mg/mL). The initial reaction velocity of the enzyme that produced reducing sugars was determined under the optimal conditions (pH 4.8 and 50°C) within 10 min of reaction time.

2.7. Enzymatic hydrolysis studies with various cellulosic substrates

Crude EglS, commercial cellulase (Cel), and a mixture of the two enzymes were tested for their hydrolytic abilities on various cellulosic substrates including a soluble cellulose (CMC), an insoluble microcrystalline cellulose (Avicel PH-101, Sigma-Aldrich), and a milled corncob (CC) as lignocellulose. The activity of Cel exoglucanase, obtained from *Trichoderma reesie* ATCC 26921 (Sigma Aldrich), was determined to be 62 FPU/mL by the DNS method. Both CMC and Avicel substrates were used as received without any treatment. A corncob sample, obtained from Kanchanaburi province, Thailand, was dried overnight at 105°C, milled by a hammer mill (Fitzmill Fitzpatrick DAS06 5HP), and sieved to obtain a particle size range of 60–80 mesh. The CC, composed of 44 ± 2% cellulose, 34 ± 1% hemicellulose, 12 ± 1% lignin, and 10 ± 1% extractives on dry weight basis, was used in the experiment without any treatment [20]. The properties of all test cellulosic substrates are summarized in Table 1 [20,21,22,23]. The enzymatic hydrolysis tests were conducted with 100 mL of total reaction volume in Erlenmeyer flasks. The enzyme solutions of EglS (50 U), Cel (50 FPU), and the mixture of EglS and Cel (50 U and 50 FPU, respectively) were loaded into separately into flask containing 0.5 g of each dried substrate in the 50 mM sodium citrate buffer (pH 4.8). Moreover, 0.01% sodium azide reagent was added to prevent microbial contamination. For controls, all the studied substrates were suspended in the buffer solution without any added enzymes. All flasks were incubated at 50°C in a shaking incubator (180 rpm).

Table 1
Properties of cellulosic substrates as crystallinity index (CrI), fraction of β-glucosidic bond accessible to cellulase (F_a), average degree of polymerization (DP_n), degree of substitution (DS), and fraction of reducing ends (F_{Re}) [20,21,22,23].

Substrate	Size (μm)	CrI	F_a (%)	DP_n	DS	F_{Re} (%)
Soluble						
Carboxymethyl cellulose (CMC)	–	–	100	400	0.65–0.9	0.05–1
Insoluble						
Microcrystalline cellulose (Avicel)	50	0.7–0.9	0.6	90–220	–	0.2–0.67
Corncob (CC)	300	0.57	–	–	–	–

The samples were collected every 20 h for 100 h and boiled for 5 min to stop the hydrolytic reaction. Subsequently, the samples were centrifuged and filtered through a 0.22-μm filter for sugar analysis by HPLC. The initial and overall sugar production rates of each hydrolytic reaction were calculated from the total concentration of glucose, cellobiose, and xylose produced in the first 20 h and the entire range of 100 h, respectively.

2.8. Study on the effect of glucose on bacterial growth and endoglucanase production

To determine the effects of glucose on the growth profile and endoglucanase production of *E. coli* Glu5 compared with *B. subtilis* M015, glucose (1% *w*/*v*) was supplemented in the medium (pH 7.0). *E. coli* Glu5 was inoculated (1:400) in LB-ampicillin medium (100 mL) with glucose and without glucose as a control. One mM of IPTG was used to induce protein expression at 4 h post inoculation. *B. subtilis* M015 was diluted at the same ratio (1:400) in LB with and without glucose. CMC (1% *w*/*v*) was used as the enzyme-induced substrate. All flasks were incubated at 37°C and 180 rpm for 48 h. The absorbance of samples obtained from the cultures was measured at OD_{600} to determine cell concentration. The endoglucanase activity of cell-free supernatant samples was determined by the DNS assay. The concentration of un-metabolized glucose was determined by HPLC. All experiments were performed in triplicate.

2.9. Microbial hydrolysis experiments of CMC using E. coli Glu5

Microbial hydrolysis of CMC using *E. coli* Glu5 was investigated by growing *E. coli* Glu5 in medium containing CMC as substrate to determine whether the secreted EglS from bacterial cells could directly hydrolyze the substrate present in the medium and produce sugar products. Various concentrations of CMC (10–50 g/L) were added to the LB-ampicillin medium (pH 7.0). *E. coli* Glu5 inoculum was then added into each medium at a volumetric ratio of 1:400, and the entire experiment was performed at 37°C and 180 rpm for 48 h. The reducing sugar amount in the culture solution was determined using the DNS reagent.

2.10. Study of toxicity of Tween 80 on E. coli Glu5 growth and activity of the secreted enzyme

Polyoxyethylenesorbitan monooleate (Tween 80, Sigma-Aldrich), a nonionic surfactant, was added to the LB-ampicillin medium at different concentrations (0.1–10% (*w*/*v*)). The OD_{600} of cultures and EglS activities of the supernatants obtained from the stationary phase were measured to determine the toxicity of the added surfactant.

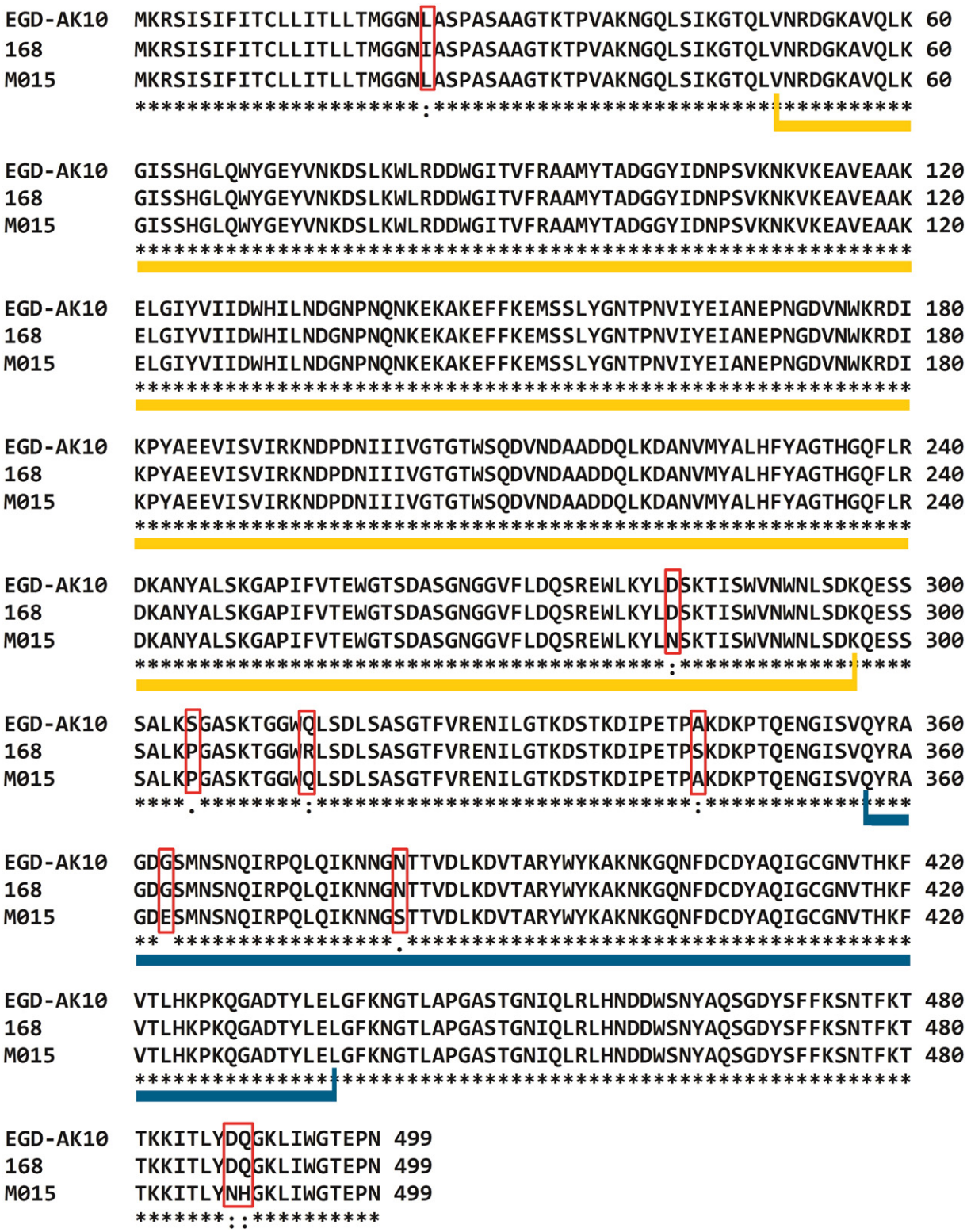

Fig. 1. Sequence alignment of *B. subtilis* M015 endoglucanase (**KP223322**) to that of *B. subtilis* ssp. *subtilis* 168 and EGD-AK10 (**CAB13696**, **WP021479778.1**). The differences between sequences are framed in red. Conserved domains are shown by straight colored bars; yellow bar indicates residues 50 to 296 corresponding to cellulase-encoding sequence (glycosyl hydrolase family 5) and blue bar indicates residues 357 to 436 corresponding to cellulose-binding domain (CBM3) (determined by NCBI Protein Blast).

3. Results and discussion

3.1. Cloning and sequencing results

Amplification of the endoglucanase gene of *Bacillus subtilis* M015 yielded a 1520-bp fragment, which was subsequently cloned into pFLAG-CTS and sequenced. DNA sequencing of the cloned fragment yielded a 1497-bp long open reading frame (ORF) encoding for a 499-amino acid protein with a calculated molecular weight of 55,294 Da. Further investigation of the sequence across GenBank database revealed that the amino acid sequence of *B. subtilis* M015 endoglucanase (accession number **KP223322**) is 99% identical to that of *B. subtilis* EGD-AK10 (**WP021479778.1**) and E1 (**CCU58432.1**), 98% identical to that of *B. subtilis* subsp. *subtilis* 168 (**CAB13696**) and *B.* sp. HY2-3 (**AAV34758.1**), and 93% identical to that of *B.* sp. NK2 (**ADO85705.1**). The alignment of *B. subtilis* M015 endoglucanase with the two reference sequences is shown in Fig. 1. These findings confirm that endoglucanase belongs to glycoside hydrolase (GH) family 5.

3.2. Secretory expression and high activity of recombinant endoglucanase

To verify the expression and secretion of the recombinant endoglucanase (EglS) from periplasmic-leaky *E. coli*, the supernatant samples were visualized on 12% SDS-PAGE gel stained with Coomassie Blue (Biorad) (Fig. 2a). The band corresponding to recombinant endoglucanase was observed at 55 kDa (lane 2). Two additional proteins around 41 and 37 kDa were also observed on the gel, which are most likely the truncated products of EglS due to proteolysis. A barely visible band of approximately 37 kDa was also present in the concentrated supernatant samples of induced *E. coli* carrying empty plasmid (lane 4), which possesses no detectable cellulase activity. The CMC-zymogram of EglS (lane 5) showed two bands consistent with those in lane 2, which confirms that the smaller band does correspond to truncated EglS with some preserved cellulase activity.

The secretion of the recombinant EglS was further verified by the Congo red assay. The presence of a clear zone around *E. coli* Glu5 colony indicated hydrolytic activity on the added CMC by the secreted extracellular enzyme under aerobic incubation, whereas no clear zone was found around the control colonies. The clear zone of *E. coli* Glu5 was significantly larger than that of the native strain *B. subtilis* M015 (Fig. 2b) with hydrolysis capacities of 2.66 ± 0.34 and 1.63 ± 0.02, respectively, corresponding to a 63% increase due to enhanced expression and secretion of the recombinant EglS enzyme by *E. coli*.

From the crude enzyme production profile (Fig. 2c), it was determined that *E. coli* Glu5 secreted crude EglS with a maximum specific endoglucanase activity of 18.56 U/mg total protein (an endoglucanase activity of 3.68 U/mL and a total protein of 0.198 mg/mL) at 16 h post induction. This level is approximately 17 times higher than that of the native strain *B. subtilis* M015 (1.098 U/mg total protein) [5]. This high activity of crude EglS toward CMC, achieved by *E. coli* JE5505-based expression system, also appears to be superior to other crude endoglucanases from *Bacillus velesensis* P3-1 and P4-6 (0.015 U/mL) [10] and other purified recombinant endoglucanases produced by other *E. coli* strains: *E. coli* harboring endoglucanase gene from *Ruminococcus flavefaciens* strain 186 (0.183–0.455 U/mg protein under the studied conditions) [24], *E. coli* BL21 (DE3) harboring cellulase *Cel*DR of *B. subtilis* DR (0.82 U/mL) [18], and *E. coli* harboring the endoglucanase gene of *B. subtilis* UMC7 (0.73 ± 0.002 U/mL) [6]. In 2015, Gao et al. [25] reported an effort to produce extracellular cellulase by using *E. coli* expressing heterologous cellulase with N20 from Cel-CD. Their expression system produced higher amount of extracellular protein (514 mg/L), however, with lower CMCase activity (558.4 U/L), compared to our results. The results revealed that *E. coli* Glu5 has great ability to produce highly active extracellular endoglucanase without a need for downstream purification steps that would be very cost-effective for the cellulosic biofuel production industry.

Fig. 3a shows the sugar end products of the enzymatic hydrolysis of CMC using crude EglS as compared to the control. The broad CMC peak (peak 4) of the control (RT 6.34 min) represents the distribution of CMC degree of polymerization ($DP_n \geq 3$), indicating that it was not hydrolyzed. In contrast, the narrower but much higher CMC peak (peak 4) of the *E. coli* Glu5 system shifted to RT 6.20 min, confirming that CMC was partially hydrolyzed by EglS and still retained the gluco-oligomer portion (peaks 4 and 5). Fig. 3b, the enlarged version of Fig. 3a, shows the presence of three small peaks of glucose (peak 1), cellobiose (peak 3), and remaining gluco-oligomers (peak 5) because endoglucanases randomly cleave the internal bonds of the polymer and not necessarily result in direct soluble sugars [26]. The results suggest that a combination of EglS with exoglucanase and β-glucosidase will be needed to complete the hydrolysis of CMC into glucose. In future, a further attempt will be made to insert both exoglucanase and β-glucosidase genes in *E. coli* Glu5 for complete hydrolysis of cellulosic materials by using a mixed solution of these three enzymes.

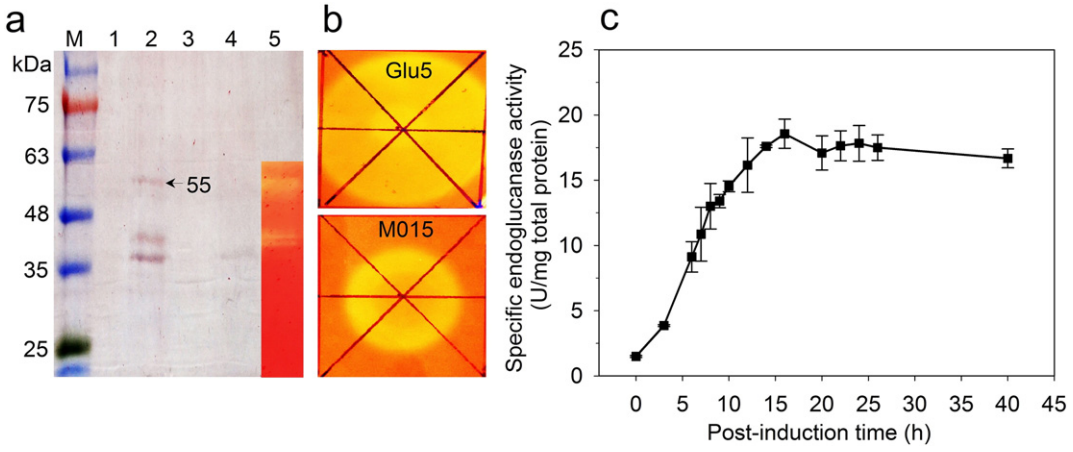

Fig. 2. (a) SDS-PAGE analysis of endoglucanase (EglS) secreted into the culture medium by the recombinant *E. coli*. Lane M: Protein marker, lane 1: uninduced *E. coli* Glu5 (before adding IPTG), lane 2: induced *E. coli* Glu5 (after adding IPTG for 16 h), lane 3: uninduced *E. coli* carrying empty plasmid, lane 4: induced *E. coli* carrying empty plasmid, and lane 5: CMC-zymogram of EglS. (b) Congo red plate assay of *E. coli* Glu5 and native strain *B. subtilis* M015. (c) Crude EglS production rate of *E. coli* Glu5 cultured at 37°C and 180 rpm.

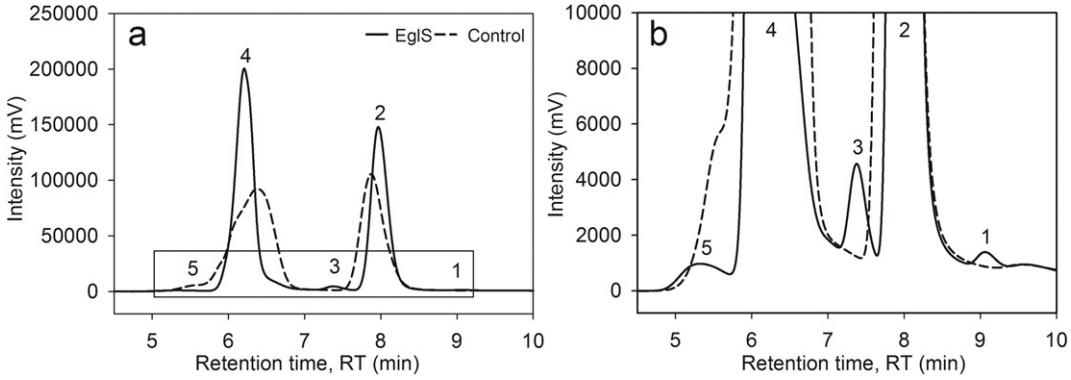

Fig. 3. HPLC chromatograms of sugar end products from the hydrolysis of CMC (1% *w*/*v*) by recombinant endoglucanase (EglS) and control in 50 mM sodium citrate buffer (pH 4.8) at 50°C for 30 min, peak 1: glucose, 2: sodium citrate, 3: cellobiose, and 4–5: gluco-oligomers of CMC. The rectangular area in chromatogram (a) is enlarged in (b).

3.3. EglS enzyme characterization results

Crude EglS showed maximum activity at pH 4.8 in the sodium citrate buffer and could retain over 70% of its maximum activity across a broad pH range of 4.0–8.0 in sodium citrate/phosphate buffer (Fig. 4a). Similarly, the EglS activity at pH 5.0 was insignificantly different (only 0.2%) from that at the optimum pH 4.8. The maximum endoglucanase activity at pH 5.0 was also found for other cellulolytic enzymes such as CMCase from *Bacillus* sp. DUSELR13 [8] and cel28a from *E. coli* DH5α expressing *cel28a* with high activity retained in a narrower pH range than crude EglS [7].

As shown in Fig. 4b, maximum endoglucanase activity was observed at 50°C, with 50% of its maximum activity in the temperature range of 30–60°C. This is consistent with other endoglucanases from *B. subtilis* DR, *E. coli* BL21 (DE3) [18], and *B. velesensis* [10], tested at slightly different pH values (6.5, 6.0, and 7.0, respectively). When the temperature increased to 75°C, the EglS activity approached zero, which is most likely due to denaturation of the enzyme.

The thermal stability of crude EglS sample in the 50 mM sodium citrate buffer (pH 4.8) was determined at different temperatures (40°C, 50°C, and 60°C) for 100 h. The crude enzyme showed high thermal stability at 40°C and 50°C, with residual activities of 98% and 82%, respectively, after 100 h of incubation (Fig. 4c), which is similar to that of non-specific endoglucanase (cel28a) tested at a much shorter incubation time (1 h) [7]. EglS lost almost all of its total enzymatic activity (94%) after 1 h of incubation at 60°C. High stability of EglS during extended incubations of up to 100 h will be beneficial for enzymatic processes that are generally carried out at 50°C for

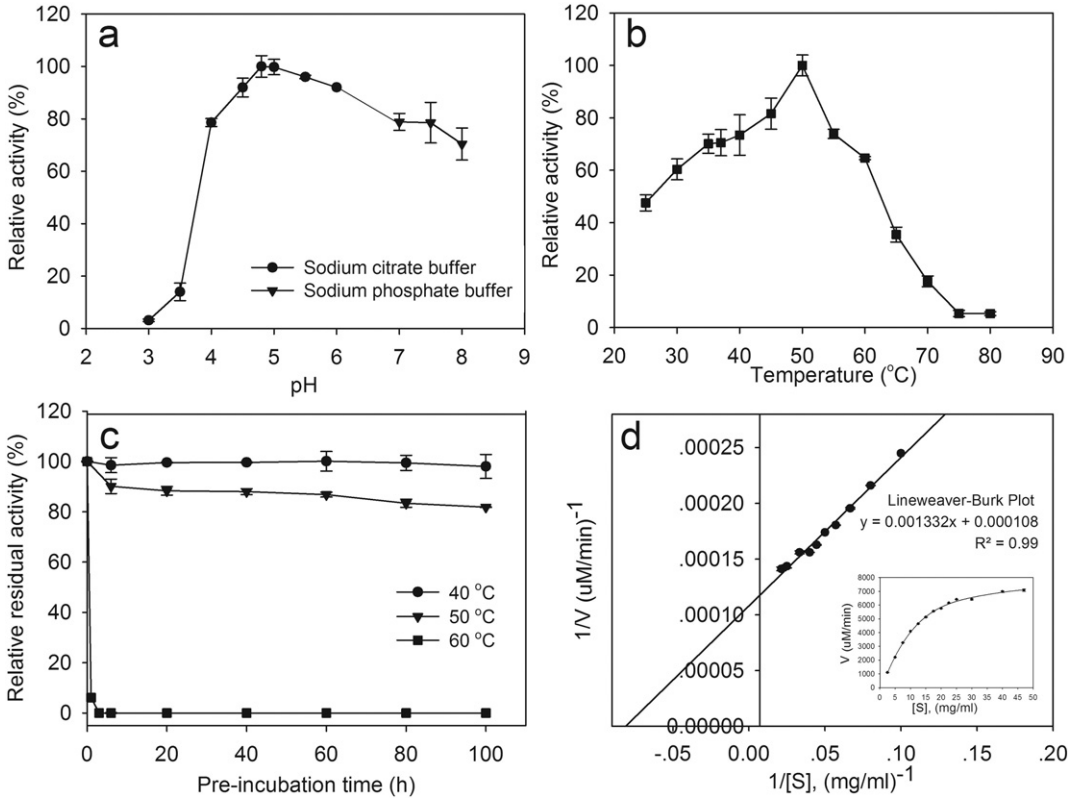

Fig. 4. (a) The effect of pH on endoglucanase activity measured at 50°C for 30 min using buffers at various pH; 50 mM sodium citrate buffer for pH 3.0–6.0; 50 mM sodium phosphate buffer for pH 7.0–8.0. (b) The effect of temperature on endoglucanase activity measured in 50 mM sodium citrate buffer (pH 4.8) for 30 min. (c) Thermal stability of recombinant EglS at different temperatures in 50 mM sodium citrate buffer (pH 4.8) for 100 h measured from residual endoglucanase activity. (d) Kinetic analysis of crude EglS toward various concentrations of CMC using Lineweaver-Burk plot.

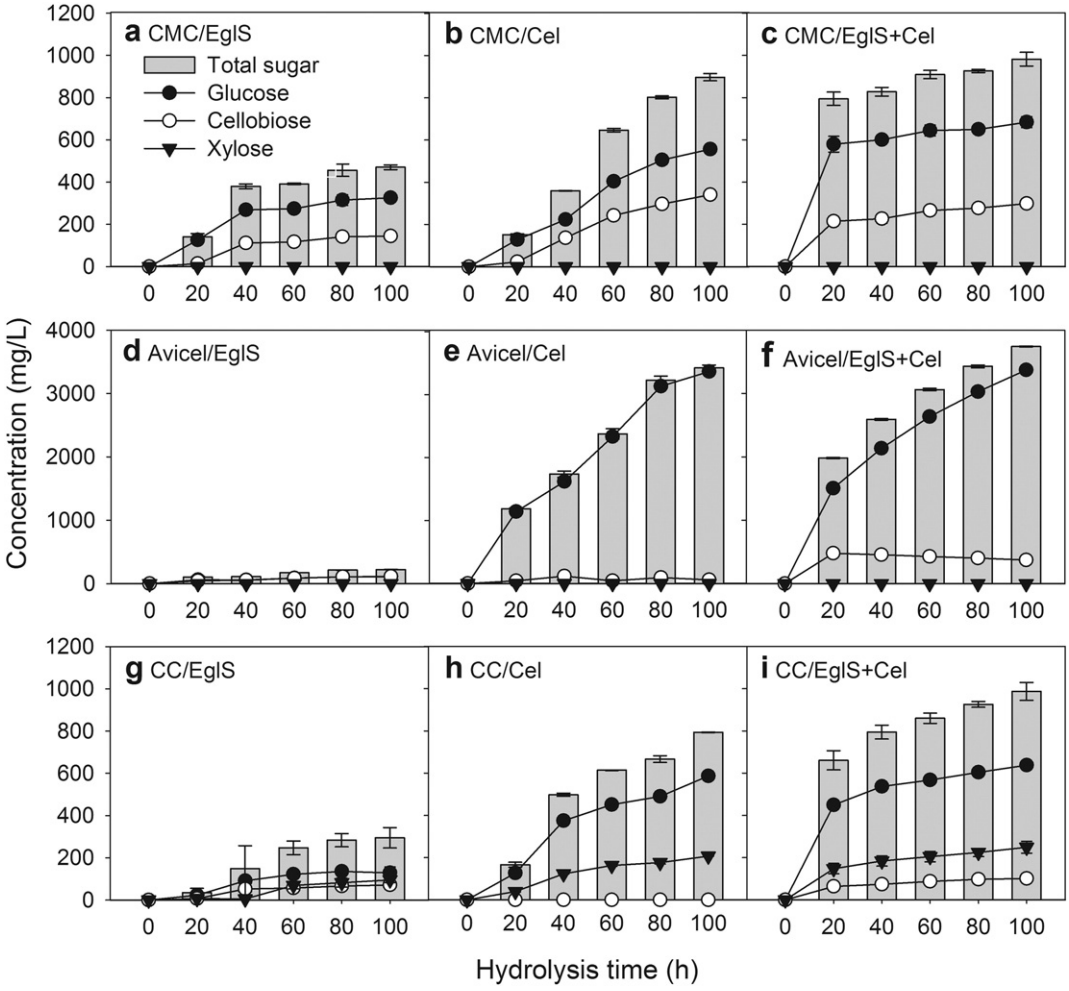

Fig. 5. Sugar products from enzymatic hydrolysis of different cellulosic substrates (5 g/L); (a–c): carboxymethyl cellulose (CMC), (d–f): microcrystalline cellulose (Avicel), and (g–i): corncob (CC) using crude recombinant EglS with 25 U/g substrate, commercial cellulase (Cel) with 25 FPU/g substrate, and enzyme mixture of EglS and Cel with 25 U/g substrate and 25 FPU/g substrate, respectively. The reactions were conducted at 50°C and 180 rpm for 100 h. Bar charts represent total sugar concentration based on glucose, cellobiose, and xylose.

3 days or longer. For industrial applications, material costs can be reduced significantly by using more stable enzymes like EglS [27].

The kinetic parameter values of crude EglS toward CMC were calculated using the Lineweaver–Burk plots (Fig. 4d). The K_m and V_{max} values were 12 mg/mL and 9259 μM/min (0.83 mg/min), respectively. In principle, K_m is independent of the enzyme concentration [28]. Lower K_m values refer to higher affinity between enzyme and substrate, indicating that crude EglS had higher affinity for CMC than purified endoglucanase (Thcel9A) from *E. coli* BL 21(DE) expressing endoglucanase gene from *Thermobifida halotolerans* YIM 90462 (K_m of 37 mg/mL) [9] and purified endoglucanase from *Aspergillus niger* B03 (K_m of 21.01 mg/mL) [29] but lower than the purified cellulase from *Bacillus* sp. MSL2 (K_m of 0.8 mg/mL) [30]. In addition, the K_m value of crude enzyme is normally higher than that of purified enzyme because of the kinetic efficiency of the enzyme, which might be retarded by the complex mixture of crude enzyme in medium and other available proteins [8,31].

3.4. Enzymatic hydrolysis of various cellulosic substrates

Fig. 5 shows all hydrolysis products of three different cellulosic materials over time (soluble cellulose, CMC; insoluble microcrystalline cellulose, Avicel; and 60–80 mesh milled corncob, CC, as a representative of agricultural residue) using crude EglS and the commercial enzyme (Cel) with and without EglS addition. Both EglS and Cel were tested to ensure the enzyme stability after 100 h of incubation at 50°C, and it was found that they were highly stable with 18% and 4% loss of their initial activity, respectively. The hydrolysis results of the three substrates showed that glucose was the main component of sugar products, followed by cellobiose. A small concentration of xylose was found in CC, which contains hemicellulose, but not in CMC and Avicel. EglS displayed much lower hydrolytic ability with much lower sugar production because EglS has only endoglucanase activity, while Cel has multifunctional cellulolytic ability of endoglucanase, exoglucanase, and β-glucosidase. Interestingly, the use of Cel with added crude EglS showed great

Table 2
The summary of initial and overall sugar production rate from enzymatic hydrolysis at 50°C and pH 4.8.

Substrate	Enzyme	Total sugar production (mg/L)	Sugar production rate (mg/L/h)	
			Initial rate (20 h)	Overall rate (100 h)
CMC	EglS	470 ± 11	7.02 ± 0.80	4.70 ± 0.11
	Cel	897 ± 17	7.59 ± 0.20	8.97 ± 0.17
	EglS + Cel	982 ± 32	39.71 ± 1.58	9.82 ± 0.32
Avicel	EglS	222 ± 03	5.19 ± 0.55	2.22 ± 0.03
	Cel	3424 ± 46	59.20 ± 0.84	34.24 ± 0.46
	EglS + Cel	3743 ± 07	99.10 ± 0.49	37.43 ± 0.07
CC	EglS	294 ± 47	1.75 ± 1.00	2.94 ± 0.48
	Cel	793 ± 01	5.72 ± 0.40	7.93 ± 0.01
	EglS + Cel	987 ± 42	33.02 ± 2.26	9.87 ± 0.42

enhancement on the initial sugar production rate in the first 20 h for any studied substrate (Table 2). The initial sugar production rates from CMC, Avicel, and CC using a mixture of Cel and EglS were higher than those of the Cel alone by 5.23, 1.67, and 5.77 times, respectively. This can be reasonably explained as follows: EglS possessing endoglucanase activity cleaves cellulose at random sites to shorter cellulose chains and increases the number of active sites or reducing ends for subsequent enzymes, specifically exoglucanase to act on [3]. The enzyme mixture could hydrolyze CMC, Avicel, and CC to produce maximum total sugar concentrations of 196, 749, and 197 mg/g substrate, respectively, at 100 h. The sugar level from Avicel hydrolysis was much higher than those from CMC and CC hydrolysis, because of the exoglucanase activity and specificity of Cel toward Avicel. Conversely, CMC, which is a modified cellulose with a degree of substitution of 0.65–0.9, has a very low accessibility (only non-substituted glucose units are accessible for the enzyme) with a high DP_n [21]. CC is a lignocellulosic material that contains not only cellulose but also hemicellulose and lignin as inhibitors for enzymatic hydrolysis. Moreover, the average particle size of corncob was relatively high, and consequently, its available surface area was much lower than that of Avicel.

Comparisons between this work and other previous reports are not straightforward because of differences in the studied conditions such as substrate type, enzyme concentration, reaction volume, pH, temperature, reaction time, and purity of the enzyme used. However, in comparison with other published work performed under relatively similar conditions, the use of enzyme mixture in this study could produce relatively higher reducing sugar content: a purified enzyme mixture (CelZ and CelY) from *Erwinia chrysanthemi* produced 105.12 mg reducing sugar/g CMC [32], and a purified cellulase mixture (EG II, CBH I, CBH II, and β-glucosidase) produced approximately 430 mg glucose/g Avicel [33]. The hydrolysis of Avicel using an enzyme mixture of commercial cellulase and β-glucosidase (Novozymes) provided a lower carbohydrate conversion of 69.6% at 96 h [34] compared to that of our enzyme mixture (75% conversion at 100 h). Hydrolysis of milled corncob using the purified enzyme mixture of endoglucanase and endoxylanase from *A. niger* B03 resulted in 540 mg/L reducing sugar [29], which is lower than that in the present work (987 mg/L). These results reveal that crude EglS has great potential to be utilized for the enhancement of commercial cellulase, especially for the enzymatic hydrolysis of agricultural residues.

3.5. Effects of glucose on bacterial growth and endoglucanase production

In the microbial hydrolysis of cellulose, bacterial cells play a role in cellulolytic enzyme production for hydrolyzing the cellulose mixed in the liquid medium. Simultaneously, the hydrolysis products, i.e., reducing sugars, mainly glucose, can be further utilized as a carbon source for bacterial growth, leading to a reduction in the sugar yield. A lower sugar level can directly decrease the ethanol production rate during downstream fermentation [35]. Moreover, glucose at high concentrations can inhibit bacterial growth and enzyme activity [36]. Therefore, the effects of glucose concentration in the culture media of *E. coli* Glu5 and *B. subtilis* M015 on bacterial growth and endoglucanase activity were investigated by adding glucose (1% *w*/*v*) in both systems.

Fig. 6a shows that the addition of 1% glucose can inhibit the growth of *E. coli* Glu5 up to 40% when compared to the growth without added glucose at 48 h. The specific growth rate (μ) obtained from its exponential phase decreased from 0.177 h^{-1} in the absence of glucose to 0.087 h^{-1} in the presence of glucose. The pH of the 48-h culture with added glucose was found to dramatically decrease (from the initial value of 7.0 to 5.0), whereas that of the 48-h culture without added glucose slightly increased (7.5). The results imply that the presence of glucose in the medium causes acid formation, resulting in

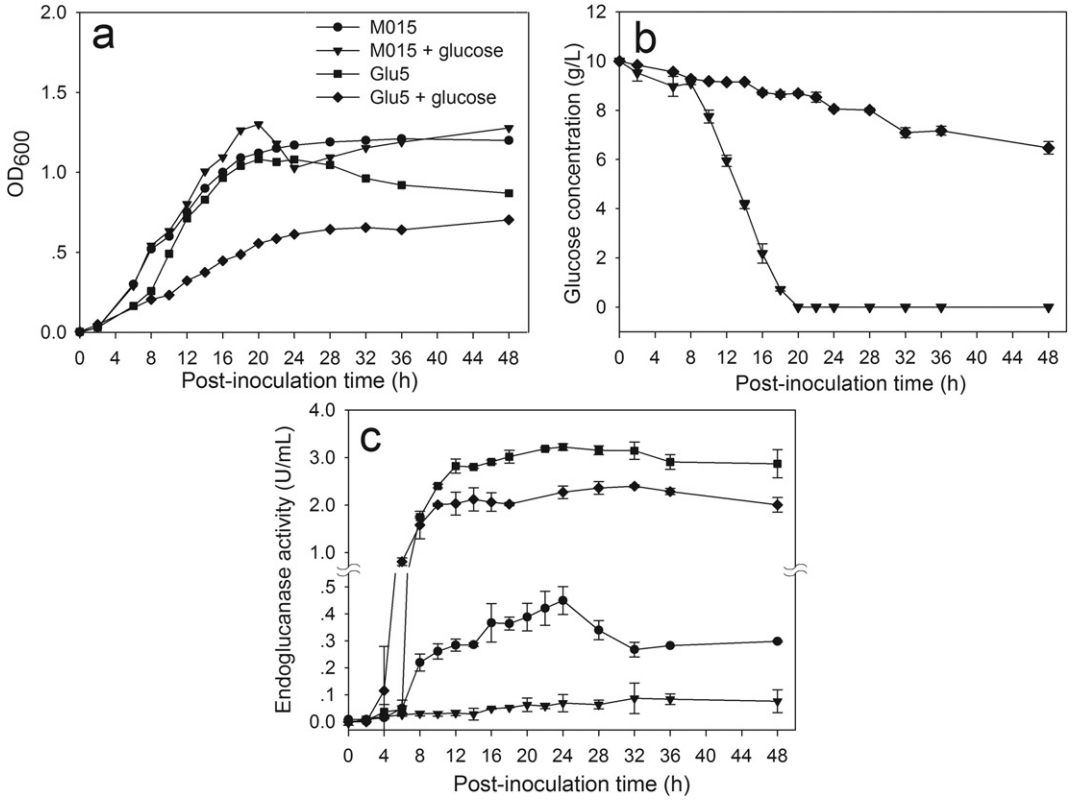

Fig. 6. Effects of glucose (1% *w*/*v*) on bacterial growth and endoglucanase production. (a) Growth, (b) glucose consumption, and (c) endoglucanase activity of *B. subtilis* M015 in LB with (*triangle*) and without glucose (*circle*) and *E. coli* Glu5 in LB-ampicillin with (*diamond*) and without glucose (*square*). IPTG (1 mM) and CMC (1% (*w*/*v*)) were used to induce enzyme synthesis in *E. coli* Glu5 and *B. subtilis* M015, respectively, at 37°C and 180 rpm for 48 h.

lower pH, and consequently, both lower cell density and growth rate were observed [36]. For *B. subtilis* M015, glucose supplemented in LB medium with CMC (1% *w*/*v*) as endoglucanase inducer caused a biphasic growth, resulting from the two carbon sources (glucose and CMC) available in the medium. Glucose was preferentially utilized as a carbon source for *B. subtilis* M015 and rapidly consumed during 8–20 h (Fig. 6b), corresponding to the first exponential phase observed in Fig. 6a. After 20 h of incubation, the growth of *B. subtilis* M015 slightly dropped, with a leveling off of glucose. The new exponential phase started at 24 h, suggesting that *B. subtilis* M015 started to utilize CMC as a carbon source. Interestingly, glucose was found to be slowly consumed by *E. coli* Glu5, with almost 65% of the initial glucose remaining unutilized after 2 days of culturing. This is most likely because LB medium contains abundant catabolizable amino acids as a carbon source for *E. coli* [37].

The presence of glucose in the culture medium resulted in a lower endoglucanase production (activity) in both strains than that in the absence of glucose (Fig. 6c). For *E. coli* Glu5, the lower endoglucanase production (26% relative activity) was consistent with the lower cell density due to acid formation from glucose. This was confirmed by measuring the endoglucanase activities of *E. coli* Glu5 grown at pH 5.0, which was approximately 25% relative activity, compared to that at pH 7.0. In contrast, the lower endoglucanase production in *B. subtilis* M015 might be caused by catabolite repression, preventing the expression of catabolic systems (endoglucanase activity) by preferred carbon sources (glucose) that enable the use of secondary substrates (CMC) [38].

From our findings, it was determined that *E. coli* Glu5 produced endoglucanase with higher activity than that of *B. subtilis* M015 and consumed less glucose. The effect of glucose on lowering endoglucanase production can be reduced by controlling the pH of the culture.

3.6. Microbial hydrolysis activity of CMC by E. coli Glu5

An investigation of microbial hydrolysis using whole cells of *E. coli* Glu5 was conducted at different initial CMC concentrations for 48 h. As shown in Fig. 7, an increase in CMC concentration increases the reducing sugar concentration and decreases the sugar yield slightly. A compromise of the two was found to be at a CMC concentration of 40 g/L, resulting in a reducing sugar concentration of 1060 mg/L or a reducing sugar yield of 27 mg/g CMC. The reducing sugar concentration produced in this study is comparable to that from the hydrolysis of CMC (10 g/L) with an excess of purified endoglucanase Hi Cel5A at room temperature for 48 h (reducing sugar concentration of 5.9 mM or 1062 mg/L); however, the sugar yield in this study was approximately four times lower than that of Hi Cel5A [39]. Zhou et al. (2000) reported that enzymatic hydrolysis of CMC (20 g/L) with two endoglucanases of CelY and CelZ provided a reducing sugar concentration of 3.83 mM (689 mg/L) and 3.98 mM (716 mg/L), respectively, at 35°C for 4 h [32]. The report showed that the sugar product concentration obtained from purified enzyme (CelY and CelZ) is similar to that obtained from the microbial hydrolysis in the present study but with a shorter time. The use of whole cells of *E. coli* Glu5 for microbial hydrolysis is technically feasible for cellulosic conversion processes. The direct use of *E. coli* Glu5 will reduce the cost of enzyme production resulting from both separation and purification steps. However, reducing sugars produced can be further consumed by bacteria, as mentioned in Section 3.5, resulting in the formation of lesser sugar product. Hence, the use of a two-step process (enzyme production at a lower temperature and enzymatic hydrolysis step at a higher temperature) can improve the sugar concentration [14].

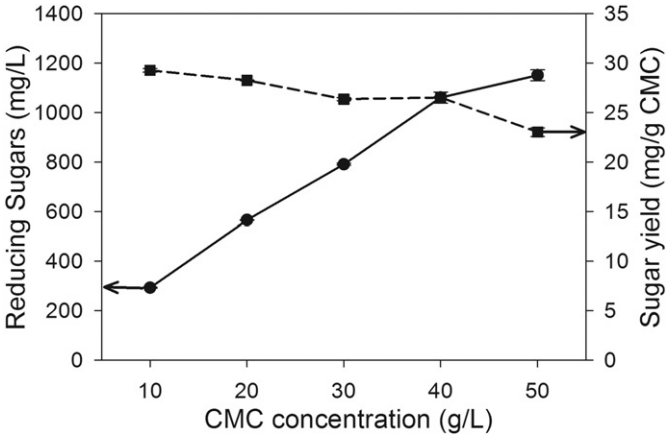

Fig. 7. Reducing sugar profile from microbial hydrolysis of various concentrations of CMC (10–50 g/L) by *E. coli* Glu5 culturing in LB-ampicillin (pH 7.0) at 37°C and 180 rpm for 48 h.

3.7. Toxicity results of Tween 80

Tween 80, a nonionic surfactant, has received great interest to be utilized in the enhancement of enzymatic hydrolysis of lignocellulosic materials because the hydrophobic interaction of the surfactant with lignin can prevent enzyme loss from adsorption onto the lignin surface, leading to higher availability of enzyme [40]. It can also stimulate microbial enzyme production by increasing cell permeability [10]. However, a high concentration of Tween 80 can inhibit bacterial growth [41]. Therefore, the effect of Tween 80 concentration on cell growth rate and activity of the enzyme secreted from *E. coli* Glu5 was investigated. The results indicated that Tween 80 did not affect the cell growth rate in the concentration range of 0.1–0.5% (*w*/*v*) and enhanced EglS secretion of *E. coli* Glu5 by approximately 10% at 0.2% (*w*/*v*) (data not shown). Another report showed that CMCase activity of enzyme secreted from *Isoptericola variabilis* sp. IDAH9 was increased approximately 36% with the addition of 0.6% Tween 80 [42]. The effect of Tween 80 on enhancing enzyme secretion of *E. coli* Glu5 was smaller than that on the wild-type strain *I. variabilis* sp. IDAH9, which is most likely due to the fact that the cell wall of *E. coli* strain used in this study was already permeable for recombinant proteins to exit. After 16 h of cultivation, *E. coli* Glu5 could grow in 5% (*w*/*v*) Tween 80, with 35% reduction in cell density and 18% reduction in enzymatic activity. Compared to another report, *E. coli* (ATCC 11303) growth was inhibited at 4% Tween 80, with only 50% viable cells remaining in nutrient broth [41]. The results reveal that *E. coli* Glu5 has a robust ability to both grow and secrete enzymes under the presence of a high concentration of Tween 80, which will be useful for the microbial hydrolysis process of lignocellulose with Tween 80 treatment.

4. Conclusions

In this study, crude recombinant endoglucanase (EglS) was successfully produced by *E. coli* Glu5 with high activity. The addition of EglS could greatly enhance the initial sugar production rate of commercial cellulase by the hydrolysis of soluble cellulose (CMC), microcrystalline cellulose (Avicel), and corncob (CC). Remarkably, *E. coli* Glu5 had lower glucose consumption than the native strain (*B. subtilis* M015) and retained high enzyme activity at high sugar concentrations, making it a potential candidate for the microbial hydrolysis process. Furthermore, *E. coli* Glu5 also showed a high tolerance to Tween 80. These findings can help in developing an economical biofuel production process in the future.

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