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Cloning, expression and characterization of the endoglucanase gene from *Bacillus subtilis* UMC7 isolated from the gut of the indigenous termite *Macrotermes malaccensis* in *Escherichia coli*

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ABSTRACT

Background: *Bacillus subtilis* UMC7 isolated from the gut of termite *Macrotermes malaccensis* has the ability to secrete a significant amount of extracellular endoglucanase, with an enzyme activity of 0.12 ± 0.01 μmol/min/mL. However, for economically viable industrial applications, the enzyme needs to be expressed in a heterologous host to overcome the low enzyme production from the wild-type strain.

Results: The endoglucanase gene from *B. subtilis* UMC7 was successfully cloned and expressed. A higher enzyme activity was observed in the intracellular fraction of the recombinant clone (0.51 ± 0.02 μmol/min/mL) compared with the cell-bound fraction (0.37 ± 0.02 μmol/min/mL) and the extracellular fraction (0.33 ± 0.01 μmol/min/mL). The recombinant endoglucanase was approximately 56 kDa, with optimal enzyme activity at 60°C and pH 6.0. The activity of the enzyme was enhanced by the addition of Ca^{2+} . However, the enzyme was inhibited by other metal ions in the following order: $Fe^{3+} > Ni^{2+} > Cu^{2+} > Mn^{2+} = Zn^{2+} > Mg^{2+} > Cd^{2+} > Cr^{2+}$. The enzyme was able to hydrolyze both low- and high-viscosity carboxymethyl-cellulose (CMC), avicel, cotton linter, filter paper and avicel but not starch, xylan, chitin, pectin and p-nitrophenyl α-D-glucopyranoside.

Conclusions: The recombinant endoglucanase showed a threefold increase in extracellular enzyme activity compared with the wild-type strain. This result revealed the potential of endoglucanase expression in *E. coli*, which can be induced for the overexpression of the enzyme. The enzyme has a broad range of activity with high specificity toward cellulose.

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1. Introduction

A termite gut is comprised of a complex microbial community of fungi and bacteria that has the ability to degrade complex cellulose [1,2,3]. This ability has attracted much attention for potential industrial application in the hydrolysis of cellulosic biomass to monosaccharides and their subsequent bioconversion to biofuels and value-added chemical products [4]. Both the fungi and bacteria of the termite gut have been exploited for cellulase production, but most studies have focused on fungal cellulases. Of late, the focus of studies have shifted from fungi to bacteria cellulases due to the higher growth rate of bacteria, their production of multi-enzyme complexes and their

ability to survive in harsh conditions [5]. Cellulolytic bacteria produce a cocktail of enzymes consisting of endoglucanases, exoglucanases and β-glucosidases [6,7]. Endoglucanase functions by cleaving intermolecular β-1,4-glycosidic bonds within the cellulose chain to release oligosaccharides for further hydrolysis by exoglucanase and β-glucosidase [8]. Endoglucanase and exoglucanase work synergistically on cellulose to produce cello-oligosaccharides and cellobiose, which are later converted to glucose by β-glucosidase [9].

Over the years, a number of cellulolytic bacteria have been successfully isolated from the gut of termites [2,10,11,12], and advanced molecular techniques, such as metagenomic analysis, have been used for cellulolytic bacterial identification [1,13]. A key issue is the need for the identification of efficient endoglucanase-producing bacteria. The identification of such a bacterium and its genetic manipulation is of great importance in our effort to produce sufficient endoglucanase for the global biotechnological industry, which is essentially based on cellulosic biomass.

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Table 1
Bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Description	Source
<i>Strains</i>		
<i>B. subtilis</i> UMC7	Wild type, able to degrade cellulose	Isolated from indigenous termite gut
<i>E. coli</i> JM109	Cloning host	Purchased from Promega (USA)
<i>E. coli</i> BL21 (DE3)	Expression host	Purchased from Novagen (Germany)
<i>Plasmids</i>		
pGEM-T easy	Cloning vector	Purchased from Promega (USA)
pET-30a(+)	Expression vector	Purchased from Novagen (Germany)
pGEM-EG1	Recombinant cloning vector, EG1 gene cloned into the pGEM-T easy vector	Developed in this study
pET-EG1	Recombinant expression vector, EG1 gene cloned into the pET-30a vector	Developed in this study

In this study, we cloned and expressed an endoglucanase gene (EG1) obtained from *Bacillus subtilis* UMC7 and characterized the resultant recombinant endoglucanase as part of our effort to search for an efficient biocatalyst for the hydrolysis of cellulosic biomass.

2. Materials and methods

2.1. Bacterial strains, plasmids, chemical reagents and culture conditions

The bacteria strains and plasmids used in this study are listed in Table 1. Nutrient broth (NB) was used to culture *B. subtilis* UMC7, and Luria–Bertani (LB) broth was used to culture *Escherichia coli*. 5-Bromo-4-chloro-indolyl- β -galactopyranoside (X-Gal), isopropyl thiogalactoside (IPTG) and antibiotics (ampicillin and kanamycin) were purchased from Sigma (USA) and were used for the selection of recombinant clones. All other chemical reagents were purchased from Merck (Germany), EMD Chemicals (USA) and Thermo Scientific (USA).

2.2. DNA extraction and preparation

B. subtilis UMC7 was cultured overnight in NB at 37°C and 150 rpm in a MAXQ 4000 shaking incubator (Thermo Scientific, USA). The overnight culture was used for genomic DNA extraction using NucleoSpin® Tissue (Macherey-Nagel, Germany) according to the manufacturer's instructions. The presence of DNA was confirmed by gel electrophoresis containing 0.8% agarose. A NanoDrop 2000c spectrophotometer (Thermo Scientific, USA) was used to quantify the purity and concentration of DNA. The extracted genomic DNA was stored at -20°C until further use.

2.3. Amplification of EG1 gene and DNA sequence analysis

Degenerated primers specific for the *B. subtilis* endoglucanase were designed based on the multiple sequence alignment of conserved sequences retrieved from the UniProt BLAST database [14]. The designed PCR primers (without restriction enzyme sites) were confirmed using an *in silico* PCR web server [15]. The forward degenerated primer, EG1-KF which contained a KpnI restriction site (underlined) (AACTGGTACCATGATGCGAAGGAGGAAAAG) and the degenerated reverse primer, EG1-BR, which contained a BamHI restriction site (ACGTGGATCCCTAATTTGGTT CTGTTCCCC), were ordered from BioSune (China). PCR of the EG1 gene was performed using the EconoTaq master mix (Lucigen, USA) according to the manufacturer's instruction, and the amplification procedure consisted of an initial incubation for 2 min at 94°C followed by 30 cycles of incubation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 90 s at 72°C. The reaction was then maintained at 72°C for 10 min. The PCR product was cloned into the pGEM-T easy vector (Promega, USA) and transformed into *E. coli* JM109 cells according to the manufacturer's instruction. Plasmids containing the insert (pGEM-EG1) were screened, extracted using NucleoSpin® Plasmid (Macherey-Nagel, Germany) and sequenced by Genomics BioScience and Technology (Taiwan) using an Applied Biosystems 3730xl DNA

analyzer. The DNA sequences were analyzed, and a BLAST search was performed using NCBI portal [16]. The EG1 gene sequence was deposited in the NCBI GenBank database with accession number KC477685. A BLASTx search was performed to retrieve the homologous proteins, and the translated amino acid sequences were computed using the EXPASY MW tool to obtain the theoretical molecular weight [17]. The phylogenetic tree was drawn using the neighbor-joining (NJ) method with Kimura 2 parameter distances using MEGA 5.0 with 1000 bootstrap replications [18].

2.4. Cloning and expression of EG1 gene in *E. coli*

The EG1 gene was sub-cloned into the pET-30a (+) expression vector and transformed into *E. coli* BL21 (DE3). Plasmids containing the insert were screened and sequenced. The recombinant clone, pET-EG1, was cultured in LB medium containing 30 μ g/mL kanamycin for 16–18 h at 37°C in a shaking incubator (150 rpm). Once the measured cell density (600 nm) of the culture reached an absorbance reading of 0.4, 1 mM IPTG was added to induce protein expression.

2.5. Screening of recombinant endoglucanase activity

The screening of recombinant endoglucanase activity was conducted in three different fractions: extracellular (media), intracellular (supernatant of lysis cells) and cell-bound (cell pellet). The induced bacteria cells were centrifuged at 10,000 g for 15 min, and the media was used as the extracellular fraction. The centrifuged pellet was washed three times with sodium phosphate buffer (50 mM,

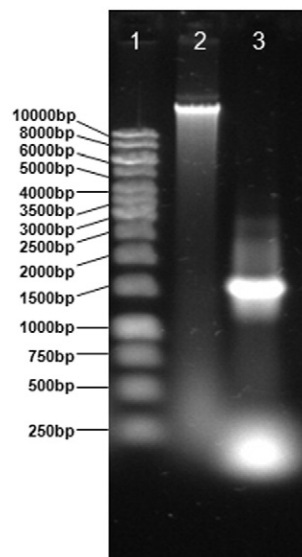


Fig. 1. Amplification of *B. subtilis* UMC7 endoglucanase gene. Lane 1: GeneRuler 1-kb DNA ladder (Thermo Scientific); Lane 2: *B. subtilis* UMC7 genomic DNA; Lane 3: PCR product of *B. subtilis* UMC7 endoglucanase gene.

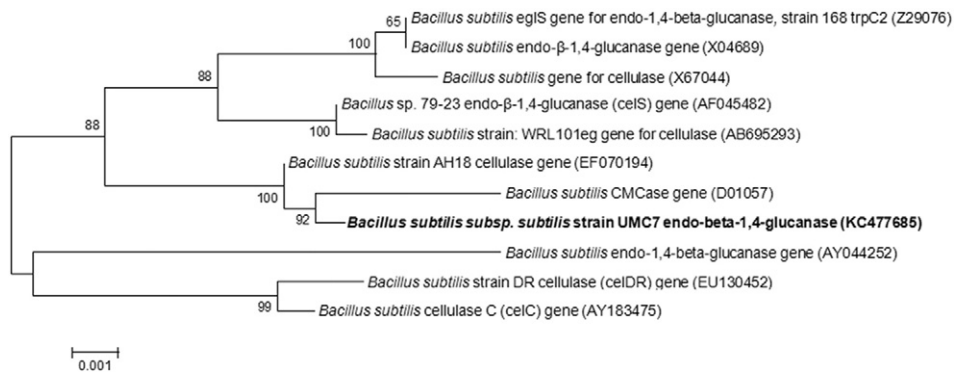


Fig. 2. Phylogenetic relationships of *B. subtilis* subsp. *subtilis* strain UMC7 endo- β -1,4 glucanase gene (KC477685) derived from endoglucanase gene sequence homology. The phylogenetic tree was drawn based on the NJ method with Kimura 2 parameter distances using the MEGA 5.0 software. The bars represent the distance values calculated by MEGA 5.0, and the values at the nodes represent the percentages of 1000 bootstrap replicates.

pH 6.0) and lysed using the B-PER bacterial protein extraction reagent (Thermo Scientific, USA) for 15 min at room temperature. The cell lysate was again centrifuged at 10,000 g for 15 min. The supernatant

obtained was used as the intracellular fraction, and the pellet was re-suspended in sodium phosphate buffer to obtain the cell-bound fraction. The extracellular, intracellular, and cell-bound endoglucanase

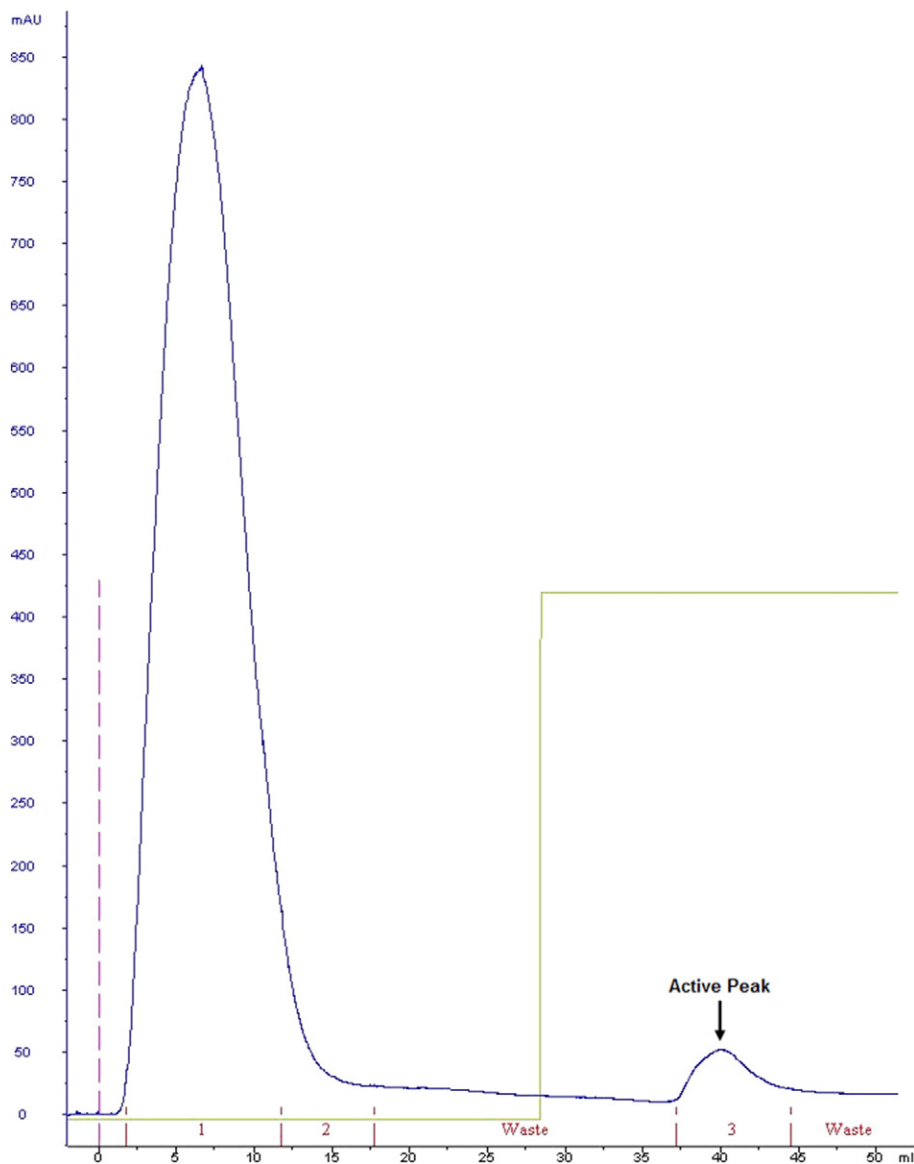


Fig. 3. Chromatography of AKTA FPLC for pET-EG1 protein purification.

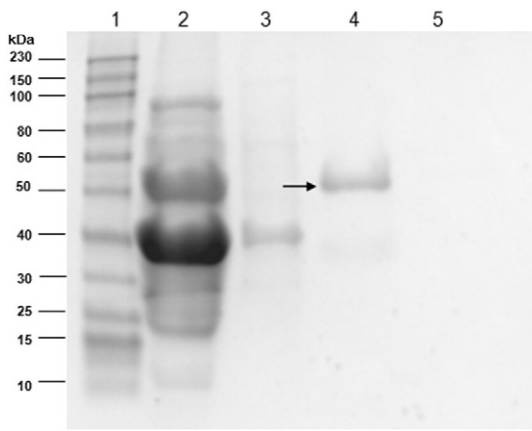


Fig. 4. Protein purification of pET-EG1 using a HiTrap CM FF cation-exchange chromatography column in FPLC. Lane 1: protein ladder (ColorPlus Prestained, New England Biolabs); Lane 2: pET-EG1 intracellular crude enzyme; Lane 3: void; Lane 4: sample after elution (0.5 M NaCl); Lane 5: sample after washing (1 M NaCl).

fractions were assayed to determine their enzyme activities. The amount of reducing sugars released from carboxyl-methylcellulose (CMC) was determined using the 3,5-dinitrosalicylic acid (DNS) assay with glucose as a standard [19]. The reaction mixtures containing 1% (w/v) high-viscosity CMC (EMD Chemicals, USA) in sodium

phosphate buffer were incubated at 60°C for 30 min. The reaction was terminated by the addition of 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent, boiling for 5 min and cooling in ice water. The amount of reducing sugars released was measured at 540 nm using a UV/Vis spectrophotometer (Thermo Scientific, Gensys 20). The amount of protein was determined using the DC™ protein assay (BioRad Laboratories, USA) according to the manufacturer's instructions.

2.6. SDS-PAGE of crude recombinant endoglucanase

All extracellular, intracellular and cell-bound fractions of recombinant clones, pET-EG1 and pET-30a (+) were analyzed by SDS-polyacrylamide gel electrophoresis with 12% (w/v) polyacrylamide gel according to Laemmli [20].

2.7. Purification and characterization of recombinant endoglucanase

The recombinant endoglucanase was purified by the AKTA purifier FPLC system (GE Healthcare, Sweden) using a HiTrap CM FF cation exchange chromatography column with a flow rate of 1 mL/min. Sodium phosphate buffer (50 mM, pH 6.0) with a gradient ranging from 0.5 to 1.0 M NaCl was used as the mobile phase. The samples were subjected to SDS-PAGE containing 12% (w/v) polyacrylamide and stained with Coomassie Brilliant Blue R-250. After de-staining, the resulting single band was excised from the gel and was digested with trypsin. The peptide was extracted through standard techniques [21].

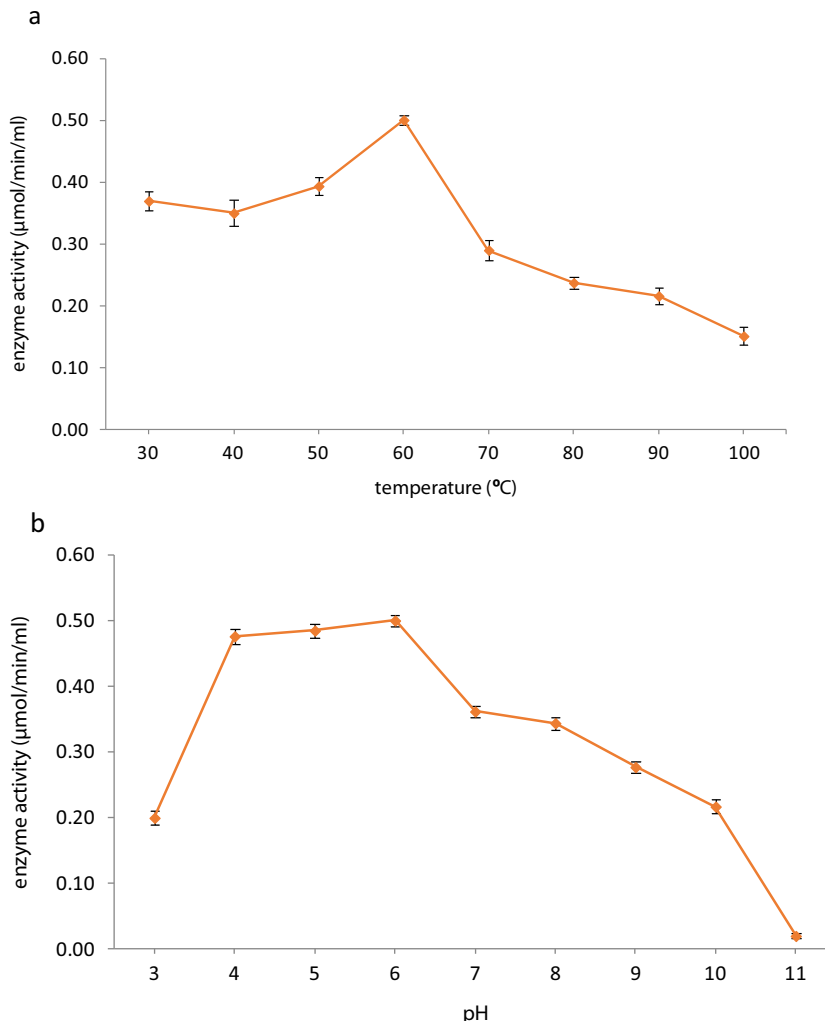


Fig. 5. Screening of optimal physicochemical parameters for endoglucanase activity. (a) Temperature. (b) pH.

The digested peptides were analyzed with a MALDI-TOF/TOF mass spectrometer using a 5800 Proteomics Analyzer (AB Sciex). The spectra were analyzed to identify the protein of interest using the Mascot sequence matching software (version 2.2.03, Matrix Science) with the Ludwig NR Database.

2.8. Screening of optimal temperature and pH

The purified recombinant endoglucanase was mixed with 1% (w/v) CMC in sodium phosphate buffer and incubated at temperatures ranging from 30 to 100°C at 10°C intervals to screen for optimal endoglucanase activity. To study the effect of pH, the CMC assay described above was repeated with citrate-phosphate buffer (pH 3.0 to 5.0), sodium phosphate buffer (pH 6.0 to 7.0) and glycine-HCl buffer (pH 8.0 to 11.0).

2.9. Screening of the effect of metal ions

The effect of individual ions, namely Mg^{2+} , Ca^{2+} , Ni^{2+} , Cu^{2+} , Mn^{2+} , Fe^{3+} , Zn^{2+} , Cd^{2+} and Cr^{2+} , at a concentration of 1 mM on the activity of the purified endoglucanase was determined using the CMC assay described above.

2.10. Screening of substrate specificity

Carbohydrate substrates consisting of 1% (w/v) CMC, cotton linter, avicel, filter paper, oat spelt xylan, starch, chitin and pectin in sodium phosphate buffer were incubated at 60°C for 30 min to determine the substrate specificity of the purified endoglucanase. The reactions were performed in 500 μ L of 50 mM sodium phosphate buffer (pH 6.0) containing the respective substrate and 500 μ L of the enzyme solution. One unit of endoglucanase activity was defined as the amount of enzyme that released 1 μ mol of glucose/min under the assay conditions [19]. One unit of xylanase activity was defined as the amount of enzyme that released 1 μ mol of xylose/min under the assay conditions [22]. One unit of amylase activity was defined as the amount of enzyme that released 1 μ mol of glucose/min under the assay conditions [23]. One unit of chitinase activity was defined as the amount of enzyme that released 1 μ mol of N-acetylglucosamine/min under the assay conditions [24]. One unit of pectinase activity was defined as the amount of enzyme that released 1 μ mol of galacturonic acid/min under the assay conditions [25]. The β -glucosidase activity was measured using p -nitrophenyl- β -D-glucopyranoside (pNPG) as the substrate. The β -glucosidase assay reaction mixture containing 0.5 mL of 1 mg/mL pNPG and 0.5 mL of enzyme solution was incubated at 60°C for 30 min. The reaction was stopped by the addition of 2 mL of 1 M sodium carbonate, and the absorbance was measured at 400 nm. One unit of β -glucosidase activity was defined as the amount of enzyme that released 1 μ mol of para-nitrophenol/min under the assay conditions [26].

Table 2

Enzyme activity in the presence of various types of metal ions.

Metal ions	Concentration (mM)	Enzyme activity (μ mol/min/mL)
Fe^{3+}	1.00	0.05 \pm 0.01
Ni^{2+}	1.00	0.14 \pm 0.01
Cu^{2+}	1.00	0.27 \pm 0.02
Mn^{2+}	1.00	0.29 \pm 0.01
Zn^{2+}	1.00	0.29 \pm 0.01
Mg^{2+}	1.00	0.36 \pm 0.02
Cd^{2+}	1.00	0.39 \pm 0.01
Cr^{2+}	1.00	0.46 \pm 0.01
Ca^{2+}	1.00	0.53 \pm 0.02
Control	–	0.50 \pm 0.01

All of the reactions were performed at pH 6.0 and 60°C.

Table 3

Enzyme activity in the presence of various types of substrates.

Substrates	Enzyme activity (μ mol/min/mL)
CMC (low viscosity)	0.73 \pm 0.02
CMC (high viscosity)	0.49 \pm 0.02
Cotton linter	0.49 \pm 0.02
Filter paper	0.12 \pm 0.01
Avicel	0.11 \pm 0.02
Starch	n.d.
Xylan	n.d.
Chitin	n.d.
Pectin	n.d.
4-Nitrophenyl α -D glucopyranoside	n.d.

n.d. = no enzyme activity was detected. All of the reactions were performed at pH 6.0 and 60°C.

3. Results and discussion

A termite has the ability to degrade lignocellulosic biomass due to its partnership with the community of microorganisms present in its gut, which breaks down plant materials into nutrient sources. An earlier study revealed that *B. subtilis* UMC7 isolated from the gut of the indigenous termite *Macrotermes malaccensis* produces an endoglucanase with an enzymatic activity of 0.12 \pm 0.01 μ mol/min/mL. The ability of an enzyme to enhance cellulase production and its recovery is vital for economically viable industrial applications. *E. coli* is commonly used as a host for heterologous recombinant expression because it is well-characterized, grows rapidly and able to produce products at high yields [27,28]. In this study, a gene encoding the endoglucanase derived from *B. subtilis* UMC7 was successfully cloned in the pET-30a (+) expression vector and expressed in *E. coli* BL21 (DE3). *In silico* PCR and agarose gel electrophoresis of the PCR product revealed that the size of the EG1 gene was 1527 bp, as shown in Fig. 1. The full-length sequence of the recombinant endoglucanase was determined and deposited in the NCBI GenBank database with accession number KC477685.

The gene product was determined to have the same length (1527 bp) as the digested pGEM-EG1 recombinant plasmid and was analyzed by 0.8% agarose gel electrophoresis. The phylogenetic tree computed using the NJ method showed that the isolated *B. subtilis* *subsp. subtilis* strain UMC7 endoglucanase gene was classified in clade with other strains of *B. subtilis* (Fig. 2) and was particularly similar to the *B. subtilis* CMCase gene (D01057). The theoretical molecular weight for the translated endoglucanase DNA sequence (KC477685), which was calculated using the EXPASY MW tool, was found to be 56.452 kDa.

The expression vector pET-30a (+), recombinant cloned plasmid pET-EG1 and the digested pET-EG1 were also analyzed. Endoglucanase activities were detected in all three fractions (extracellular, intracellular and cell-bound) of the recombinant clone, and the highest endoglucanase activity was observed with the intracellular fraction followed by the cell-bound fraction and then the extracellular fraction. The intracellular fraction of crude recombinant endoglucanase showed an enzymatic activity of 0.51 \pm 0.02 μ mol/min/mL, whereas the cell-bound and extracellular fractions showed enzymatic activities of 0.37 \pm 0.02 μ mol/min/mL and 0.33 \pm 0.01 μ mol/min/mL, indicating an approximately three-fold increase in extracellular recombinant endoglucanase activity compared with the extracellular native endoglucanase (*B. subtilis* UMC7), which was found to exhibit an enzymatic activity of 0.12 \pm 0.01 μ mol/min/mL. Koide et al. [29] also reported the distribution of cellulase of the cloned cellulase gene of *B. subtilis* IF03034 in all three fractions of *E. coli*. Our results show similarity to the findings reported by Kim et al. [30], who found higher CMCase activity in the intracellular fraction followed by the cell-bound and extracellular fractions.

It has been reported that a number of factors influence the distribution of enzymes in *E. coli* clones. The gene copy number, regulation or inducibility of a gene, the stability of enzyme and/or a combination of any of these factors will influence the distribution of enzymes in a host [30]. A higher endoglucanase activity was observed in the extracellular fraction of the recombinant clone (pET-EG1) compared with the wild-type endoglucanase from *B. subtilis* UMC7. This result indicates that the *E. coli* system has the capability for higher endoglucanase production due to its inducible recombinant expression. A further analysis of the crude recombinant endoglucanase using SDS-PAGE showed the presence of endoglucanase in the intracellular fraction by the appearance of a clear band with a protein size of 56 kDa. This molecular weight, which was determined by SDS-PAGE, matches the theoretical value of 56.452 kDa calculated using the EXPASY MW tool.

The recombinant endoglucanase was further purified by AKTA Fast Performance Liquid Chromatography (FPLC) using a HiTrap CM FF cation exchange chromatography column (Fig. 3), and the SDS-PAGE result showed a single band at approximately 56 kDa for the recombinant EG1 endoglucanase (Fig. 4). As expected, the MALDI-TOF MS-MS fingerprinting and sequencing results confirmed that the targeted protein sample was *B. subtilis* endoglucanase and matched the theoretical molecular weight of 56.452 kDa calculated for KC477685 using the EXPASY MW tool. The analysis of the protein sample resulted in a protein sequence coverage of 25% with a Mascot score of 471 (>200), indicating extensive homology at a significance threshold of $p < 0.05$. The expressed recombinant endoglucanase has a similar molecular weight compared with other reported recombinant endoglucanases from *B. subtilis* [28,31,32]. The purified recombinant enzyme had an activity of $0.50 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$, as determined by the carboxymethyl cellulose (CMC) assay at 60°C and pH 6. Hence, our endoglucanase activity is 3.2-fold greater than that of the recently studied endoglucanase of *Paenibacillus* sp. MTCC 5639 expressed in *E. coli* BLR (DE3) [33]. At present, the secretion of endoglucanase by *E. coli* into the extracellular medium at a sufficient amount remains a difficult task, and a systematic analysis using a consolidated bioprocessing approach is therefore required [34].

The purified recombinant endoglucanase was screened at a temperature range from 30°C to 100°C to determine its optimal temperature. The result indicated that this enzyme has a broad range of activity with optimal activity of $0.50 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$ at 60°C, as shown in Fig. 5a. The enzyme also showed a broad range of activity at pH 3.0 to 11.0, although the optimal endoglucanase activity of $0.50 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$ was found to be at pH 6.0, as shown in Fig. 5b. The recombinant endoglucanase was also screened to determine its enzymatic activity in the presence of different metal ions, namely Mg^{2+} , Ca^{2+} , Ni^{2+} , Cu^{2+} , Mn^{2+} , Fe^{3+} , Zn^{2+} , Cd^{2+} and Cr^{2+} . The recombinant endoglucanase activity was found to be enhanced to $0.53 \pm 0.02 \mu\text{mol}/\text{min}/\text{mL}$ in the presence of Ca^{2+} . However, as shown in Table 2, the enzyme was inhibited by the presence of metal ions in the following order: $\text{Fe}^{3+} > \text{Ni}^{2+} > \text{Cu}^{2+} > \text{Mn}^{2+} = \text{Zn}^{2+} > \text{Mg}^{2+} > \text{Cd}^{2+} > \text{Cr}^{2+}$.

The optimal physicochemical parameters of the purified recombinant endoglucanase for CMC specificity were found to be activated by Ca^{2+} at 60°C and pH 6. The optimal temperature and pH value determined in this study are similar to those reported for other recombinant endoglucanases from *B. subtilis* [32,35,36,37]. The analysis of the effect of metal ions revealed that the addition of Ca^{2+} resulted in elevated endoglucanase activity, whereas the enzyme activity was inhibited by Fe^{3+} . This result is comparable to the results of studies on the endoglucanase from *B. subtilis* YJ1 [35], an anaerobic sulfidogenic bioreactor [38], and *B. subtilis* JS2004 [39]. A previous study also showed that the addition of Ca^{2+} enhances endoglucanase activity [39]. It is hypothesized that the presence of Ca^{2+} ions may enhance the substrate binding affinity of the enzyme by stabilizing the conformation of the catalytic site [40], whereas the addition of Fe^{3+} has a deleterious effect. The inactivation of the recombinant

endoglucanase from *B. subtilis* A8-8 by Fe^{3+} was also observed in a previous study [32].

The results of the substrate specificity assay, as shown in Table 3, revealed that the maximum endoglucanase activity was found to be $0.72 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$ for CMC (low viscosity), followed by CMC (high viscosity) with an enzyme activity of $0.49 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$, cotton linter with an enzyme activity of $0.49 \pm 0.02 \mu\text{mol}/\text{min}/\text{mL}$, filter paper with an enzyme activity of $0.12 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$ and avicel with an enzyme activity of $0.11 \pm 0.01 \mu\text{mol}/\text{min}/\text{mL}$. The ability of the recombinant endoglucanase to hydrolyze substrate-containing cellulose in the major amorphous regions confirms that the purified enzyme is an endoglucanase. The recombinant endoglucanase exhibited its highest activity toward low-viscosity CMC followed by high-viscosity CMC, cotton linter, filter paper and avicel. No enzymatic activity was detected in the presence of xylan, starch, chitin, pectin and p-nitrophenyl- β -glucoside. Further bioprocess optimization and mutagenesis studies are needed to improve and achieve maximal bioconversion of the cellulosic biomass.

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ejbt.2014.12.007>.

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