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Characterization and Secretive Expression in *Bacillus subtilis* of Endoglucanase from *Bacillus safensis* Isolated from Freshwater Swamp Forest

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Abstract

Bacillus safensis M3 was newly isolated from freshwater swamp forest soil in western Thailand. The endoglucanase gene of *B. safensis* M3, *cel9A,* had an open reading frame of 1,848 bp encoding a 616 amino acid protein. Initial expression in *Escherichia coli* yielded a low amount of soluble protein in the cytosolic and secreted fractions. Cel9A was successfully expressed by recombinant *B. subtilis* with a 4-fold greater total enzyme activity than from recombinant *E. coli*. By SDS-PAGE analysis, the molecular weight of Cel9A was estimated to be 70 kDa. The optimal temperature of Cel9A was 55 °C and the optimal pH was 5 - 8. Cel9A had the highest activity in the pH range from 5 - 8, and the highest stability in pH range 4 to 10, which is useful for industrial applications. Notably, Cel9A was able to hydrolyze both mixed linkage glucan (lichenan) and hemicellulose (konjac glucomannan and oat spelt xylan) better than carboxymethylcellulose. Cel9A also showed a tolerance to metal ions and surfactants. In addition, recombinant *B. subtilis* with endoglucanase activity has potential for biotechnological applications and benefits in the optimization of large scale enzyme production with minimal medium using agriculture wastes and other inexpensive feedstock materials.

Keywords: *Bacillus safensis*, cellulases, endoglucanase, recombinant expression, freshwater swamp forest

Introduction

Cellulolytic enzymes can degrade cellulose through the synergistic actions of 3 major classes of enzymes: endoglucanases that cleave the amorphous region of cellulose, exoglucanases that hydrolyze the products of endoglucanases to short chain oligosaccharide and cellobiose, and β-glucosidases or cellobiases [1]. Endoglucanases (EC 3.2.1.4) with desirable properties, i.e., high pH stability and tolerance to metal ions and chelating agents, are in increasing demand for various industrial applications, including the treatment of textiles and animal feeds and use in detergents and sustainable green technology [2,3]. Microbial enzymes have strong potential in commercial enzyme production due to the ease of manipulation of their genes, their great diversity, and the low cost of production, which improves economic feasibility [4]. Hence, several cellulase-producing bacteria have been discovered in a wide variety of environments and investigated in order to realize this potential [5].

Freshwater swamp forest in Kanchanaburi, western Thailand, is a wetland located among limestone mountains and is known as an important ecosystem with vast biodiversity. It is a natural habitat for unique, endangered and novel species of plants and animals. Little work has been done with respect to the bacterial community in this wetland, which may serve as a source of unexplored microorganisms [6].

Accordingly, attempts were made to discover interesting cellulolytic bacteria and examine their enzyme characteristics.

Many approaches are available for the development of recombinant cellulases in the name of productivity, stability, and cost-effectiveness. Optimization of the expression system is one empowering method. Several hosts, including bacteria, yeast, plants, and fungi, have been developed [1]. Among these, *Bacillus subtilis* has advantages in industrial safety, secretory capacity, cost of medium, etc. [7]. Many studies have described efficient recombinant cellulolytic *B. subtilis* [3,8,9]. However, development in the overexpression of convenient secretory cellulases is still necessary to achieve economic and convenient enzyme production [3].

Here we report the identification of a cellulolytic *B. safensis* strain M3 which is newly isolated from freshwater swamp forest soil and the cloning of its endoglucanase gene by PCR methods. The enzyme was overexpressed in *E. coli* and *B. subtilis*, and the *B. subtilis* host facilitated production of a high amount in the secreted form compared to *E. coli*. The recombinant enzyme characteristics were shown to be appropriate for biotechnological applications.

Materials and methods

Bacterial strains, plasmids and growth conditions

E. coli strain DH5α was used as a cloning host for pGEM®-T Easy vector and pET-28a expression vector. *E. coli* strain BL21(DE3) was used as expression host for pET-28a expression vector. *B. subtilis* ISW1214 (derivative of ISW1012, *hsrMl, leuA8, metB5*, tetracycline resistance) was used as an expression host. The shuttle vector, pBCX, a derivative of pBC16 containing a 2.56 kb fragment of pBluescript II, was used for expression in *B. subtilis* [10].

Isolation and identification of soil bacteria for cellulolytic enzymes production

Bacterial isolation was carried out on soil samples from Kanchanaburi province, Thailand. To screen the cellulolytic bacteria on CMC-amido black plates, sample dilutions were spread on tryptic soy agar (TSA) containing 0.5 % (w/v) carboxymethylcellulose (CMC) and 0.05 % (w/v) amido black, and detected by noticing a yellow zone against the dark blue medium. The CMCase or endoglucanase activity was confirmed by streaking on M9 minimal media (composition per liter: 12.8 g $Na_2HPO_4.7H_20$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.024 g $MgSO_4$, 0.011 g $CaCl_2$), supplemented with 0.5 % (w/v) CMC and detected by Congo red indicator [11]. The agar medium was flooded with Congo red (0.1 %, w/v) for 15 min and then washed with 1M NaCl. Cellulolytic colonies were surrounded by a yellow halo against a red background. Colonies with a high activity index, the ratio of the cellulolytic zone radius to the bacteria colony radius, were chosen for further study. Phenotypic and genotypic characterizations of isolates were determined based on biochemical reaction and 16S rRNA gene analysis. The 16S rRNA gene was amplified using the eubacterial universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGCTACCTTGTTACGACTT-3') [2]. The evolutionary history was inferred using the Neighbor-Joining method in MEGA6.

Cloning of endoglucanase gene

To screen the endoglucanase encoding gene, PCR was performed using genomic DNA as a template, and primers, GH9F1 (5'-AACTATGYMGAGGTKY-3') and GH9R1 (5'-CGCCAGGMGTATA-3'), were designed from consensus sequences found in *Bacillus pumilus* glycoside hydrolase family 9 (GH9) cellulases. To amplify the complete gene encoding mature protein, primers, GH9F2 (5'-GCGGATCCTTGGAATTATTT-3') and GH9R2 (5'-GAGAATTCTTTGTCTGGAAG-3'), were designed based on the gene sequence in the NCBI database. The PCR product was double-digested with *Bam*HI and *Eco*RI and ligated with the pET-28a(+), then the recombinant plasmid was introduced into *E. coli* BL21(DE3). To overexpress the target protein, recombinant *E. coli* was grown to OD600 0.4 - 0.6 and induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG). The induction time and temperature were tested at 30 °C for 5 h and 37 °C for 3 h. The recombinant protein in secreted, soluble, and insoluble forms were collected and tested for endoglucanase activity.

Expression and partial purification of cellulase in *B. subtilis*

To enhance the recombinant endoglucanase productivity, the endoglucanase encoding gene was subcloned into *B. subtilis* ISW1214 with the shuttle vector pBCX. The *cel9A* gene was first ligated into pBluescript II SK (+) to create the restriction sites compatible with pBCX, *Bam*HI, and *Kpn*I. Then, the *cel9A* gene was ligated with pBCX and transformed into *B. subtilis*. *B. subtilis* harboring the recombinant plasmid was grown in LB medium containing 15 mg tetracycline/ml at 37 °C overnight. The secreted protein was harvested by centrifugation at 10,000g for 20 min, and the supernatant was used as crude enzyme. The supernatant was precipitated with 80 % (w/v) ammonium sulfate and dialyzed against 1 mM EDTA to obtain the partially purified protein, which was then concentrated in an ULTRAFREE –MC 300000 NMWL centrifugal filter.

Molecular analyses of the cellulase gene

The nucleotide sequences were obtained by an automated DNA sequencer based on fluorescent dye-terminator sequencing (1st BASE, Malaysia). The sequencing results were analyzed for ORFs using the NCBI ORF Finder Tool. The deduced amino acid and the predicted molecular mass were predicted with ExPASy tools (http://www.expasy.org). Homologous sequences were identified by searches using the BLAST algorithms. The final nucleotide sequence, accession No. KM924440, was deposited in the GenBank database.

Electrophoresis methods for studying the endoglucanase and zymogram analysis

SDS-PAGE was performed with a 10 % separating gel. Native PAGE was conducted similar to SDS-PAGE in the absence of SDS and β-mercaptoethanol, and without heating the sample. To characterize the enzyme activities, duplicate samples were separated on the separating gel into which CMC was incorporated. Zymogram analysis was performed in native PAGE or SDS-PAGE. The SDS-PAGE gel was washed twice with 50 mM citrate buffer, pH 5.0, containing 25 % (v/v) isopropanol for 30 min to remove SDS, then incubated for 30 min at 50 °C and stained with Congo red as described above.

Enzyme activity assay and protein determination

Cell-free extract or culture supernatant was used as crude enzyme to determine the cellulase activity. The cell pellet was resuspended, sonicated, and used as a total cell protein sample. Cellulase activity was measured by the 3,5-dinitrosalicyli acid (DNS) method, through a microplate-based assay [12]. The standard assay mixture contained 30 µl of crude enzyme and 30 µl of 50 mM citrate buffer containing 2 % (w/v) CMC in a PCR microplate. This mixture was incubated for 30 min at 50 °C before 60 µl of DNS solution was added. The treated samples were heated to 98 °C in the PCR machine for 5 min prior to cool down. The OD540 was read against a reagent blank by a microplate reader. One unit (U) of enzyme activity was defined as the amount of enzyme releasing reducing sugar, with a response equivalent to 1 µmol D-glucose in 1 min in this reaction.

The protein concentration of enzyme preparation was performed according to the method described by Bradford, with bovine serum albumin as the standard protein.

Enzyme characterization

The optimal pH was determined at pH values ranging from 3 - 10 in various buffers. The pH stability was determined by incubating the crude enzyme for 2 h at 4 °C in the buffers ranging from pH 3 - 10. The optimal temperature was determined in the range from 20 - 80 °C by conducting the standard assay described above at that temperature. Thermostability was evaluated by pre-incubating the crude enzyme for 1 h at temperatures ranging from 20 - 80 °C. Relative activity at different pH values and temperatures was expressed as a percentage of the maximal activity at the optimum.

The effects of different reagents on the crude enzyme were tested by incubating crude enzyme with metal ions, salt, chelating agent, and surfactant in 50 mM citrate buffer containing 2 % (w/v) CMC at 50 °C for 30 min.

To determine substrate specificity, several insoluble and soluble cellulose forms, β-glucosides, and polysaccharides were tested with crude enzyme, and the enzyme activity determined. Kinetic parameters, K_m and V_{max}, were determined by plotting the Lineweaver-Burk graph using 0.25 - 20 mg/ml CMC.

Results and discussion

Screening cellulolytic soil bacteria

By a simple screening method, several cellulolytic bacteria were discovered, and the bacteria with the highest activity was identified and selected for further study. In this study, a newly isolated *Bacillus* sp. from freshwater swamp forest soil showed its potential cellulose degrader by demonstrating several cellulases. The isolated bacteria were dominant, with the highest activity index of 18 on M9 agar containing CMC, and showed the activity of 3 kinds of cellulases: β-glucosidase, exoglucanase, and endoglucanase. Bacillus cellulolytic enzymes have been studied for practical biomass digestion in second generation biofuel technology by using cellulosic plant biomass as material [13]. Cellulases with desirable qualities of high thermostability and/or wide pH active have been isolated from *Bacillus* strains. [14] The biochemical tests revealed that the bacterium is *B. safensis* (**Table 1**). The 16S rRNA gene sequence (GenBank accession no. KM924441) showed that the strain was most closely related with *B. safensis*, exhibiting 99 % identity. A phylogenetic tree analysis based on 16S rRNA gene showed the close relationship of strain M3 with *B. safensis* (**Figure 1**). Therefore, this cellulolytic bacterium was identified as *B. safensis* M3. *B. safensis* has been reported as an important industrial producer of enzymes such as endoinulinase, keratinase, and β-galactosidase [15-17]. However, only a few studies on cellulases from this species have been reported. For further study, the endoglucanase gene of *B. safensis* M3 was cloned, identified, and expressed to produce the recombinant enzyme.

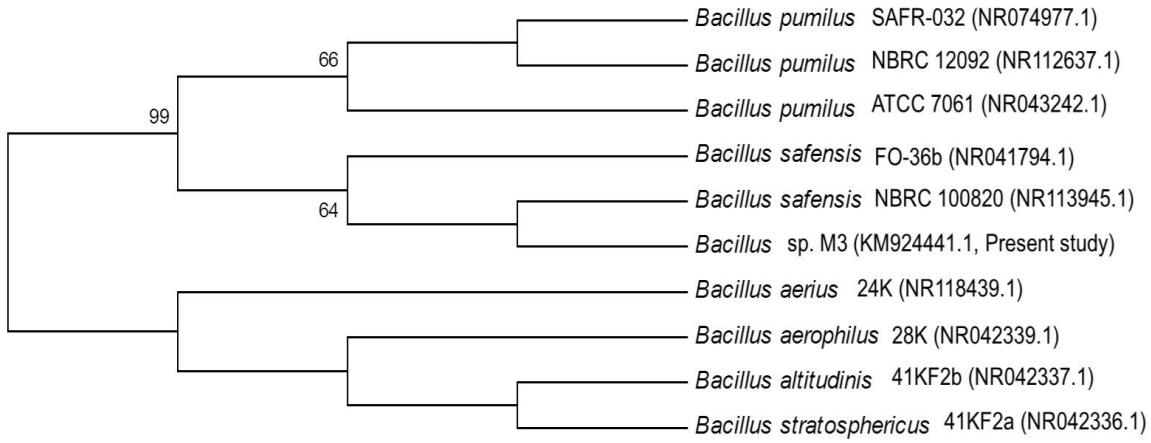

Figure 1 Phylogenetic tree analysis of *Bacillus* sp. M3 based on the 16s rRNA gene sequences with closely related strains. As a result, *Bacillus* sp. M3 was most closely related to *Bacillus safensis*.

Table 1 Phenotypic and biochemical characteristics of strain M3 compared with type strains of *B. pumilus* [18] and *B. safensis* [19].

Characteristics	***B. pumilus* ATCC 7061**	***B. safensis* FO-36b**	**Strain M3**
Colony morphology	Variable; colonies may be wrinkled and irregular; they are unpigmented and most are smooth and opaque.	Round, undulated, dull white, non-luminescent, irregular margin colony	Circular-flat colony, diameter about 3 mm, entire margin, semi-mucoid
Biochemical properties			
Gram stain	Gram-positive or Gram-variable, occurring singly and in pairs, and forming spores.	Gram positive, spore-forming rods	Gram positive (early stage of growth), rod shape, endospore-forming
Spore shape	Cylindrical to ellipsoidal	Ellipsoidal	Ellipsoidal
Spore position	Centrally, paracentrally, and subterminally in unswollen sporangia	Central through paracentral and subterminal	Centrally, paracentrally, and subterminally
Oxygen consumption	Aerobic	Aerobic	Obligate aerobe
Starch hydrolysis test	-	-	-
Casein digestion test	+	+/-	+
Glucose fermentation	+	+	+
Catalase test	+	+	+
Citrate test	+	+	+
6.5 % NaCl growth test	+	+	+
Voges-Proskauer (VP) test	+	+	+
Motility test	+	+	+
Acid from saliacin	+	+	+
Acid from esculin	+	+	+
Acid from insitol	-	+	-
Casein hydrolysis in litmus milk	+	-	+

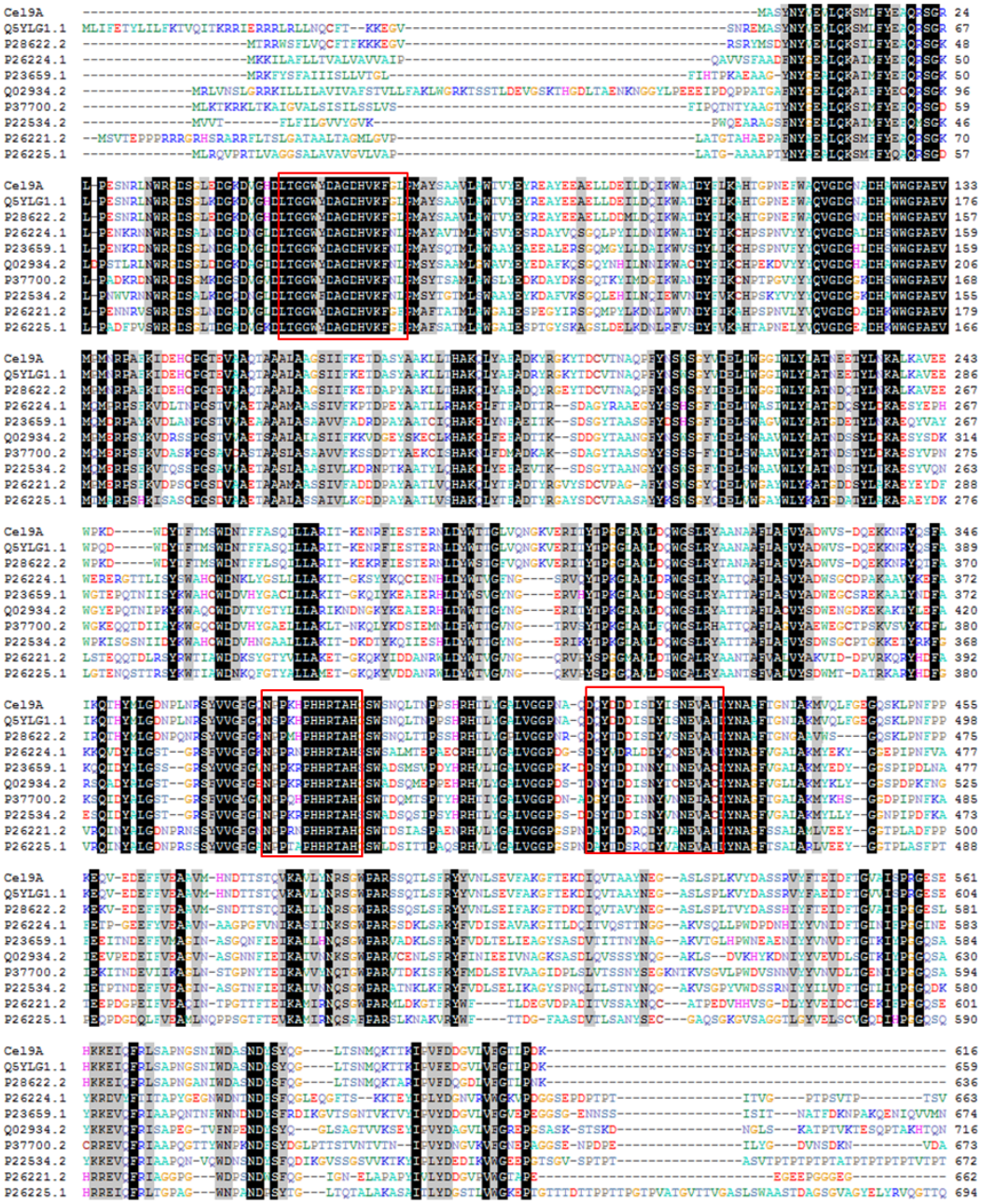

Figure 2 Multiple sequence alignment of Cel9A and related endoglucanase. Three conserved regions are shown in the box. Accession is on the right of the sequence: Endoglucanase A of *B. pumilus* (Q5YLG1.1), Endoglucanase 4 of *Bacillus* sp. KSM-522 (P28622.2), Endoglucanase F of *Ruminiclostridium thermocellum* ATCC 27405 (P26224.1), Endoglucanase Z of *Clostridium stercorarium* (P23659.1), Endoglucanase 1 of *R. thermocellum* ATCC 27405 (Q02934.2), Endoglucanase G of *C. cellulolyticum* H10 (P37700.2), Endoglucanase A of *Caldicellulosiruptor saccharolyticus* (P22534.2), Endoglucanase E-4 of *Thermobifida fusca* (P26221.2), and Endoglucanase B of *Cellulomonas fimi* (P26225.1). Cel9A has revealed those three conserved region of GH9 subgroup E2 endoglucanase.

Cloning endoglucanase gene using degenerate primer and its molecular characterization

A PCR based method using degenerate primer was used to archive the endoglucanase gene. A PCR product of 905 bp was produced with the degenerate primers, GH9F1 and GH9R1. This partial endoglucanase gene had a 99 % similarity to the *B. pumilus* endoglucanase A precursor (*EglA*) gene (AY339624.1). The primers, GH9F2 and GH9R2, were designed from the nucleotide sequence of the closely related *B. pumilus EglA*, and used to amplify the full-length gene, resulting in a 2,196 bp amplicon, named *cel9A*. *cel9A* had an open reading frame of 1,848 bp encoding 616 deduced amino acids. Based on the translated protein sequence, Cel9A had a 90 % similarity to the deduced endoglucanase from *B. pumilus* Z14, EglA from *B. pumilus*, and BglC from *B. pumilus* BY-1. A homology search on NCBI and PROSITE database revealed the conserved patterns of GH9 and CBM3 in Cel9A. Based on domain architecture, GH9 enzymes are categorized in 4 themes: A, B, C, and D. In the present study, Cel9A was placed in 'Theme B1' which contains a single CBM3c in an intermediate downstream of catalytic module [20,21]. Similar domain architectures of *Bacillus* GH9 enzymes was found in EG-IV from *Bacillus* sp. KSM-522 [22], EglA from *B. pumilus* [23], Cel9A from *B. licheniformis* GXN151 [24], and Ba-EGA from *Bacillus* sp AC-1 [25]. The multiple sequence alignment of Cel9A and other GH9 cellulase in **Figure 2** revealed regions of consensus of the GH9 subgroup E2 [26]. GH9 had 3 conserved region where the active residues were detected in Cel9A as Asp^{55} and Asp^{58} in conserved region I, His^{370} in conserved region II, and Asp^{412} and Glu^{430} in conserved region III. The theoretical pI and calculated molecular weight were 5.08 and 69.6 kDa, respectively. Endoglucanases from *Bacillus* sp. have molecular weights in the range of 30 - 97 kDa [2,5]. The size is correlated to the architecture of the endoglucanase, i.e., whether it is a monomeric or a modular enzyme. Similar to the endoglucanases from *Bacillus* AC-1 [27] and endophytic *B. pumilus* strains [23], and EG-IV from *Bacillus* sp. KSM-522 [22], Cel9A shared the same modular structure, and had a similar protein size of about 70 kDa. Additionally, enzymes with modular organizations of GH9-CBM3 always have some properties in common, such as being active in a broad range of pH, a high optimum temperature, and thermostability [22,23,25,27].

Subcloning of endoglucanase encoding gene in *E. coli* and *B. subtilis*

The *cel9A* gene was cloned into expression vectors in order to express the recombinant endoglucanase, Cel9A. Initially, *E. coli* was evaluated for Cel9A overexpression by subcloning *cel9A* in pET-28a. Unfortunately, Cel9A was expressed as inclusion bodies. To enhance the soluble active form of Cel9A in *E. coli*, induction at the lower temperature was conducted, and it was shown that the most active cellulase fraction was intracellular soluble protein. In the optimized condition (1 mM IPTG at 30 °C for 5 h), the total activities of the soluble and insoluble enzyme were 0.227 and 0.004 U/mg, respectively. **Figure 3a** (Lane 2) indicated that the most active endoglucanase activity was observed in the intracellular soluble fraction. Native PAGE zymogram analysis showed the very clear activity, while SDS-PAGE zymogram analysis required the sample to be concentrated to reveal the 70 kDa activity band (**Figure 3b**). Hence, Cel9A is not secreted in a high amount and does not show activity, so a purification step is necessary, which may affect the cost of enzyme production. To enhance the production to be convenient and efficient for the downstream processing, *cel9A* was cloned into a shuttle vector, pBCX, in order to switch the host from *E. coli* to *B. subtilis*.

Expression of recombinant endoglucanase in *B. subtilis*

In attempting to improve the production of endoglucanase, *cel9A* was subcloned and expressed in *B. subtilis*. *B. subtilis* has been considered as a potential platform for secretory expression of endoglucanase [3,7,9,28]. Previously in our laboratory, pBCX could enhance the expression of the chitinase gene in *B. thuringiensis*, compared with the commercial shuttle vector, pHY300PLK [10]. In this study, recombinant *B. subtilis*, designated as *B. subtilis*/pBCX:*cel9A*, was cultured in LB medium containing 15 mg tetracycline/ml overnight, without any further induction process. *B. subtilis*/pBCX:*cel9A* successfully secreted the recombinant Cel9A into the culture medium for the specific activity of 1.956 U/mg. **Figure 4** shows the comparison of the endoglucanase activity from wild-type *B. safensis* M3, *B. subtilis*, recombinant *E. coli*, and recombinant *B. subtilis*. The total activity of Cel9A in *B. subtilis* was 4-fold greater than in *E. coli* (**Table 2**). This result suggested the potential of *Bacillus* expression system to

enhance the overexpression of cellulase. SDS-PAGE and zymogram analysis of Cel9A detected the active band at around 70 kDa (**Figure 5**). Moreover, *B. subtilis*/pBCX:*cel9A* was able to grow on minimal medium and produced a clear zone greater than recombinant *E. coli*. This preliminary study has provided insight for the further study of the ability of *B. subtilis*/pBCX:*cel9A* to grow on others plant biomasses or agricultural by-products for the low-cost production of biocommodities.

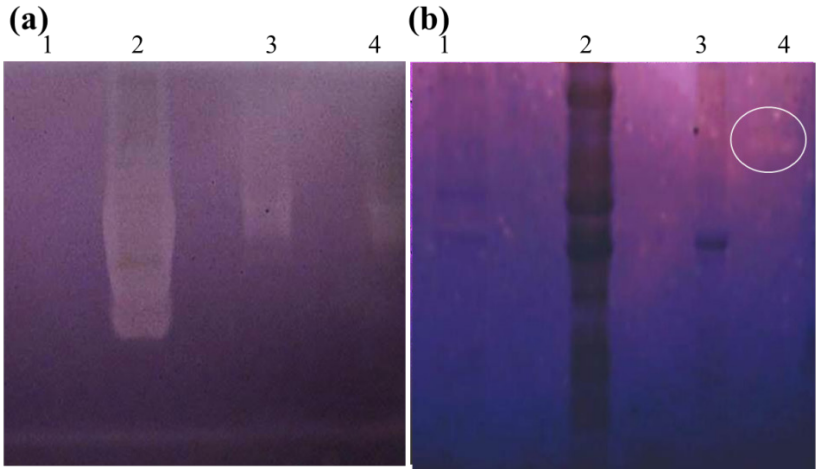

Figure 3 Zymogram analysis of endoglucanase expressed in *E. coli* BL21(DE3): (a) Native PAGE and (b) SDS-PAGE. Lane 1: *E. coli* BL21 harboring pET-28a; Lane 2: soluble protein; Lane 3: insoluble protein; Lane 4: cell-free supernatant in Panel (a) and cell-free supernatant precipitated with acetone in Panel (b). As a result, *E. coli*/pET*:cel9A* was mainly expressed in the intracellular fraction and the recombinant endoglucanase was 70 kDa.

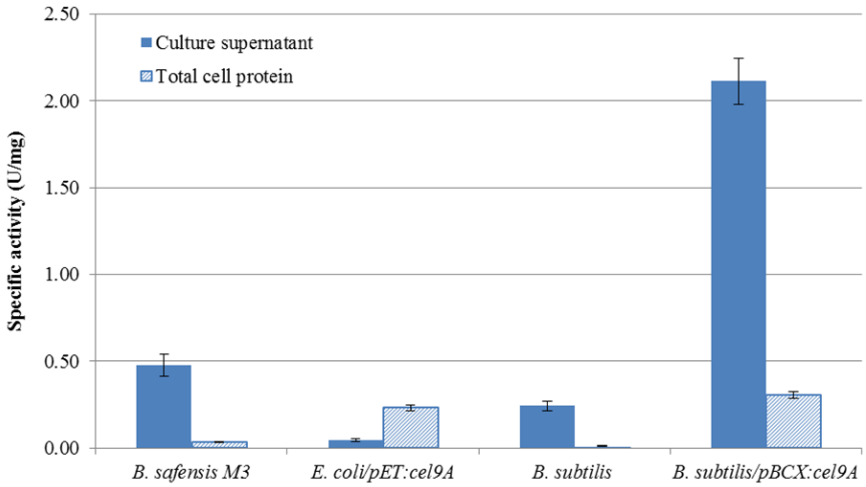

Figure 4 Enhanced expressions of endoglucanase activity in culture supernatant (extracellular fraction, ■) and total cell protein (intracellular fraction, ▨). *B. safensis M3* as the parent strain, *E. coli*/pET*:cel9A* as *E. coli* BL21(DE3) harboring plasmid pET-28a backbone with *cel9A* gene, *B. subtilis* ISW1214 as the controlled host strain, *B. subtilis*/pBCX:*cel9A* as *B. subtilis* ISW1214 harboring pBCX backbone with *cel9A* gene. *B. subtilis*/pBCX:*cel9A* had the highest activity compared with the parent strain and recombinant *E. coli*/pET*:cel9A*.

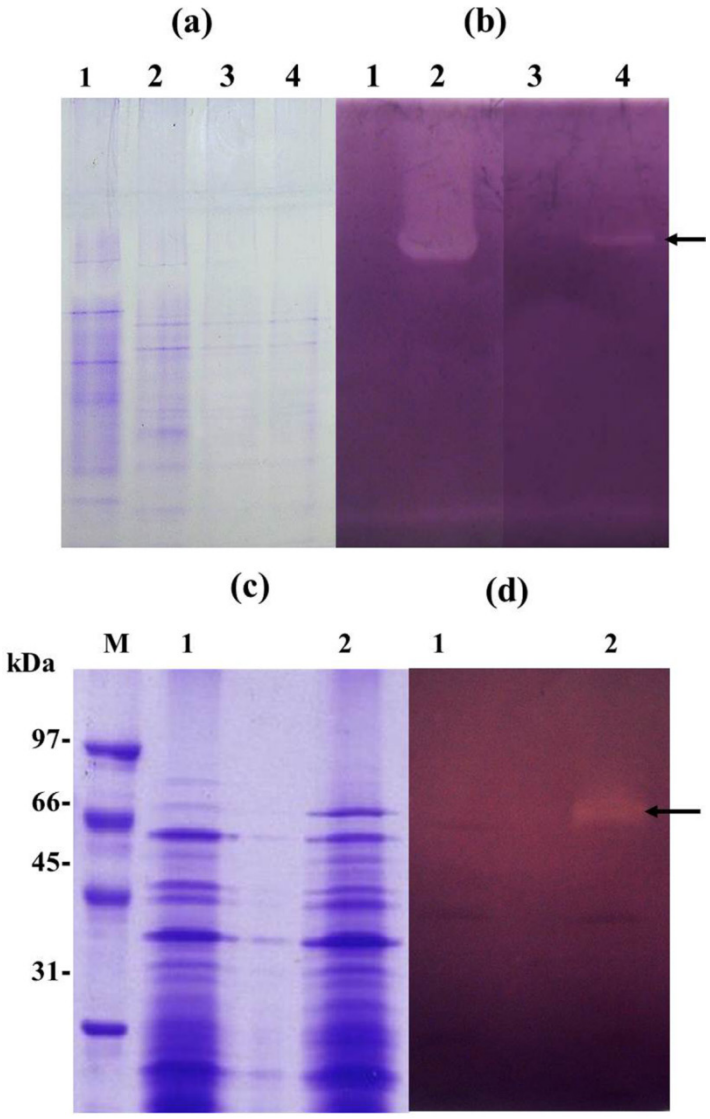

Figure 5 PAGE and Zymogram analysis of endoglucanase activity of Cel9A expressed in *B. subtilis* ISW1214: Panel (a) and (b) were Native-PAGE and its zymogram analysis (Lane 1: total cell protein of *B. subtilis* ISW1214 harboring pBCX; Lane 2: total cell protein of *B. subtilis*/pBCX:*cel9A*; Lane 3: cell-free supernatant of *B. subtilis* ISW1214 harboring pBCX; Lane 4: cell-free supernatant of *B. subtilis*/pBCX:*cel9A*); Panel (c) and (d) were SDS-PAGE and its zymogram analysis (Lane M: marker; Lane 1: supernatant of *B. subtilis* ISW1214 harboring pBCX concentrated by ultrafiltration; Lane 2: supernatant of *B. subtilis*/pBCX:*cel9A* concentrated by ultrafiltration). The activity band was shown at 70 kDa.

Table 2 Comparison of endoglucanase activity and expression level in recombinant *E. coli* and *B. subtilis*.

Vector/host	Endoglucanase activity[a] (U) in			Expression level (mg protein per milliliter)	Clear zone[b] (mm)
	Extracellular fraction	Intracellular fraction	Total		
pET-28a:*cel9A* /*E. coli* BL21(DE3)	0.035	0.119	0.154	0.002	5.3±0.6
pBCX:*cel9A* /*B. subtilis* ISW1214	0.587	0.013	0.600	0.030	11.3±0.6

[a]*E. coli* and *B. subtilis* recombinant strains were cultivated in 5 ml of LB medium and their endoglucanase activities in extracellular and intracellular fractions were determined, respectively.
[b]Clear zones of recombinant bacteria were determined in triplicate using 5 µl of culture broth equivalent to McFarland 0.5 dropped on M9 containing CMC and incubated at 37 °C for 3 days.

Characterization of recombinant endoglucanase

Although the *cel9A* gene was quite similar to *B. pumilus eglA*, EglA has not been extensively characterized [23]. As shown in **Figure 6a**, Cel9A had 2 pH optima at 5 - 8, and maintained > 80 % optimal activity from pH 5 - 9. Similar observation can be noticed in EglA from *B. pumilus*, which has an optimum pH range of 5 - 8 [23]. However, the major of endoglucanases from other *Bacillus* strains usually have a pH range of 7 - 9 [2,4,22,29]. Notably, Cel9A had stability in a broad range of pH; 46 % of the activity was recovered after incubation in pH 3, and around 75 % was recovered with pre-incubation in the pH range 4 - 10. Chistakopoulos *et al*. also reported the alkali-resistance of *B. pumilus* endoglucanase, and some *Bacillus* alkali-endoglucanases are patented for their potential uses in industrial applications [1,29].

For an optimum temperature, the enzyme showed maximum activity when incubated at 55 °C (**Figure 6b**), in agreement with a previous report, where *B. pumilus* endoglucanase was most active around 50 - 60 °C [5]. Although Cel9A retained over 75 % of its activity when incubated at 50 °C for 1 h, its activity decreased by half when incubated at 60 °C. In agreement with Chistakopoulos *et al*. [29], endoglucanase from *B. pumilus* had an optimum temperature and thermostability at 60 °C. Li *et al*. [27] also reported the rapid decrease in activity of Ba-EGA from *Bacillus* sp. AC-1 when incubated at 60 °C [27].

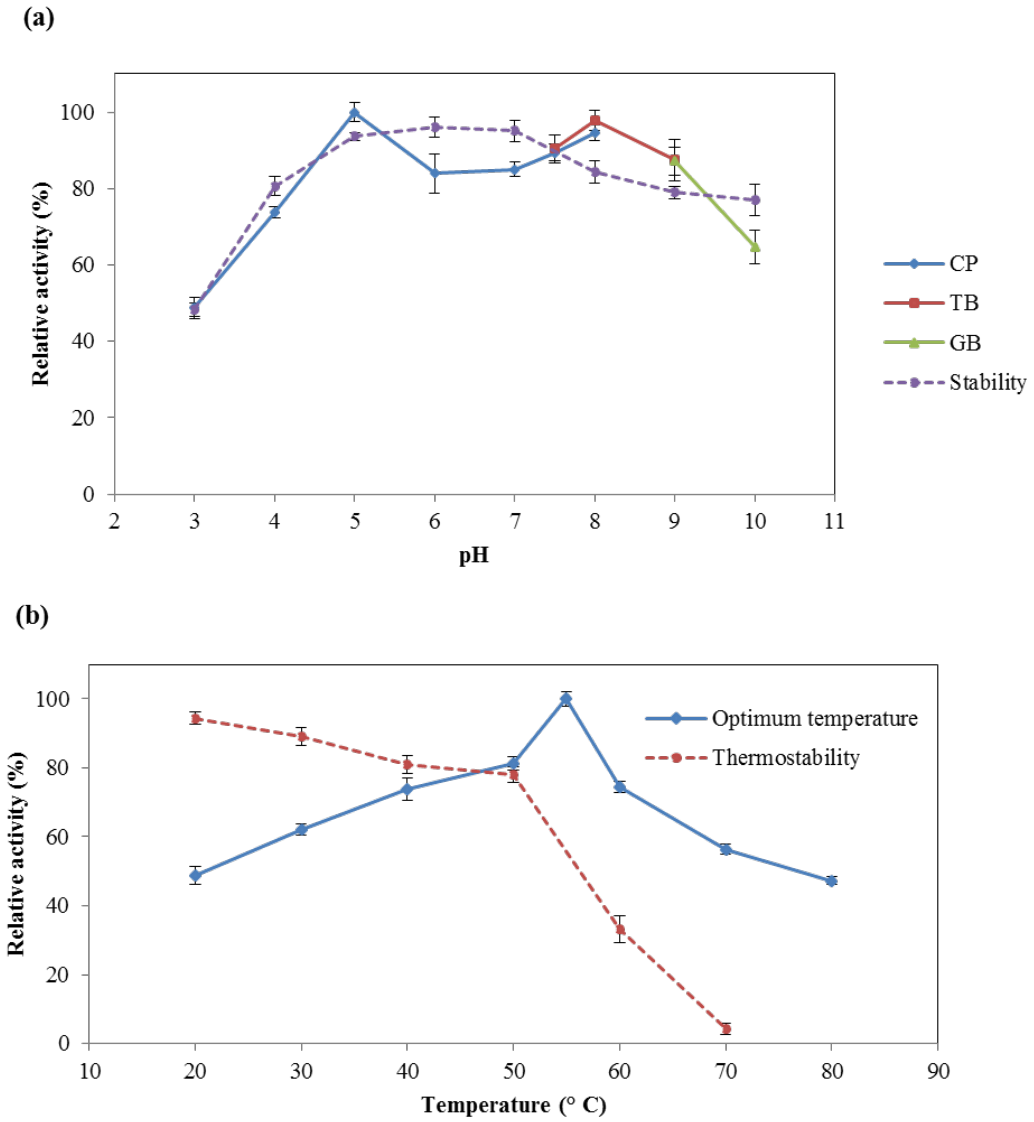

Figure 6 Characterization of the recombinant endoglucanase, Cel9A: (a) optimum pH and stability and (b) optimum temperature and thermostability (CP, citrate phosphate buffer; PB, phosphate buffer; TB, Tris buffer; GB, glycine buffer).

Table 3 shows the effects of 5 mM metal ions and chelating agent and 0.5 % detergents on Cel9A activity. Mg^{2+} and Mn^{2+} increased the activity of Cel9A by roughly 20 %, in concordance with a previous report that they had enhanced the activity of EglA from *B. pumilus* [23]. Little or no effect was seen with Zn^{2+}, Li^{+}, Na^{+}, and EDTA, while 70 % inhibition of Cel9A was observed in Cu^{2+}. The activities of endoglucanases from *Bacillus* sp. also were strongly inhibited by Cu^{2+} [5,30]. Moderate activation was noticed in the nonionic surfactants Tween 20 and Triton X-100. Li *et al*. [23] described the activation of enzyme by nonionic surfactant, which is the result of cellulose surface property modification. In contrast, the same concentration of SDS inhibited Cel9A by 35 %, which is similar to its effect on EglA from *B. pumilus*.

Table 3 Effect of different chemical reagents on the activity of recombinant endoglucanase.

Reagent	Concentration	Relative activity (%)
None		100 ± 1.0
NaCl	5 mM	102 ± 2.4
LiCl	5 mM	102 ± 2.3
$MgCl_2$	5 mM	123 ± 3.6
$MnCl_2$	5 mM	117 ± 4.3
$ZnCl_2$	5 mM	89 ± 2.7
$CuCl_2$	5 mM	31 ± 2.0
EDTA	5 mM	90 ± 7.5
Triton X-100	0.5 % (v/v)	122 ± 1.0
SDS	0.5 % (w/v)	65 ± 3.4
Tween 20	0.5 % (v/v)	130 ± 2.9
Glycerol	0.5 % (v/v)	90 ± 5.4

Table 4 Substrate specificity of recombinant endoglucanase.

Substrate	Activity (U/mg)
CMC, carboxymethyl cellulose	1.32 ± 0.04
Avicel, microcrystalline cellulose	0.16 ± 0.04
Cellulose from Spruce	0.22 ± 0.01
Konjac glucomannan	6.79 ± 058
Lichenan	5.16 ± 1.58
Oat spelt xylan	5.82 ± 0.50
Chitin	0.11 ± 0.01
pNPG	0.49 ± 0.12
Cellobiose	0.18 ± 0.01

The relative activities in **Table 4** indicate the substrate specificity of Cel9A. Cel9A had high specific activities against β-1,4-glucans. Interestingly, Cel9A could degrade the mixed linkage substrates (lichenan and konjac glucomannan) better than CMC, which is different from a previous report of *B. pumilus* CMCase [5]. To exclude intrinsic activity from the *B. subtilis* host strain, Cel9A, purified from *E. coli*/pET:*cel9A* using a HiTrap™ Chelating HP column, was used to determine its substrate preference, and similar results were obtained; that is, the purified Cel9A was also highly active against mixed linkage substrate (lichenan, barley β-glucan, and glucomannan). This effect may be due to the flexible active site structure of the enzymes against the equatorial 2-OH (in cellulose) to axial 2-OH (in mannan) [31]. Moreover, crude Cel9A also had activity against oat spelt xylan, unlike alkali-resistant endoglucanase from *B. pumilus* [29]. Cel9A showed a little β-glucosidase activity by hydrolyzing pNPG (p-Nitrophenol-β-D-Glucopyranoside) and cellobiose. Such activities can be found in GH9 cellulases (www.cazy.org/GH9.htm). The ability to grow and produce activity on M9+CMC of recombinant *B. subtilis*, along with the wide substrate specificity of Cel9A, may indicate the possible utilization of a variety of substrates to serve as carbon sources in minimal media for enzyme production. The K_m and V_{max} values of crude enzyme for CMC were 3.8 mg/ml and 0.011 μmol/min/mg, respectively.

Conclusions

In this study, identification of cellulolytic bacteria involved in phenotypic and genomic methods of bacterial strain M3 have revealed that it is *B. safensis*. The endoglucanase from newly isolated cellulolytic bacteria *B. safensis* M3 was successfully cloned and expressed in *B. subtilis*. Compared with expression in recombinant *E. coli*, recombinant *B. subtilis* produced a crude enzyme preparation with higher specific activity and the ability to grow on M9+CMC without an inducer (IPTG) could reduce the enzyme production cost of medium and inducer. The recombinant endoglucanase Cel9A showed nearly optimal activity over the pH range from 5 - 8, and a broad pH range for stability as well, which are important characteristics for industrial utilization. Substrate specificity patterns different from some *B. pumilus* endoglucanases may provide insight into the basis for this selectivity. Moreover, its broad range of substrate utilization could benefit the optimization for large scale production with minimal medium. The optimal conditions of this study will be used for investigating the application of the recombinant endoglucanase to agriculture wastes and other inexpensive feedstock materials.

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