

HOSTED BY



ELSEVIER

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology



Research Article

Production, purification and characterization of an ionic liquid tolerant cellulase from *Bacillus* sp. isolated from rice paddy field soilMalinee Sriariyanun^{a,*}, Prapakorn Tantayotai^b, Patchanee Yasurin^c,
Peerapong Pornwongthong^d, Kraipat Cheenkachorn^e^a Department of Mechanical and Process Engineering, TGGS, King Mongkut's University of Technology, North Bangkok, Bangsue, Bangkok 10800, Thailand^b Department of Biology, Faculty of Science, Srinakharinwirot University, Bangkok 10110, Thailand^c Food Biotechnology Program, Faculty of Biotechnology, Assumption University, Bangkok 12040, Thailand^d Department of Agro-Industrial, Food and Environmental Technology, Faculty of Applied Science, King Mongkut's University of Technology, North Bangkok, Bangsue, Bangkok 10800, Thailand^e Department of Chemical Engineering, Faculty of Engineering, King Mongkut's University of Technology, North Bangkok, Bangsue, Bangkok 10800, Thailand

ARTICLE INFO

Article history:

Received 31 August 2015

Accepted 17 November 2015

Available online xxxx

Keywords:

Biorefinery

Cellulase

Ionic liquid

Lignocellulosic biomass

ABSTRACT

Background: Lignocellulosic biomass is a renewable, abundant, and inexpensive resource for biorefining process to produce biofuel and valuable chemicals. To make the process become feasible, it requires the use of both efficient pretreatment and hydrolysis enzymes to generate fermentable sugars. Ionic liquid (IL) pretreatment has been demonstrated to be a promising method to enhance the saccharification of biomass by cellulase enzyme; however, the remaining IL in the hydrolysis buffer strongly inhibits the function of cellulase. This study aimed to isolate a potential IL-tolerant cellulase producing bacterium to be applied in biorefining process.

Result: One *Bacillus* sp., MSL2 strain, obtained from rice paddy field soil was isolated based on screening of cellulase assay. Its cellulase enzyme was purified and fractionated using a size exclusion chromatography. The molecular weight of purified cellulase was 48 kDa as revealed by SDS-PAGE and zymogram analysis. In the presence of the IL, 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) concentration of 1 M, the cellulase activity retained 77.7% of non-IL condition. In addition, the optimum temperature and pH of the enzyme is 50°C and pH 6.0, respectively. However, this cellulase retained its activity more than 90% at 55°C, and pH 4.0. Kinetic analysis of purified enzyme showed that the K_m and V_{max} were 0.8 mg/mL and 1000 μ M/min, respectively.

Conclusion: The characterization of cellulase produced from MSL2 strain was described here. These properties of cellulase made this bacterial strain become potential to be used in the biorefining process.

© 2015 Pontificia Universidad Católica de Valparaíso. Production and hosting by Elsevier B.V. All rights reserved.

1. Introduction

Lignocellulosic biomass, the most abundant biomass on earth, has a great potential as renewable feedstock for biorefinery processes including biofuel production. It mainly comprises of three components including cellulose, hemicelluloses and lignin [1]. Deconstruction of lignocellulosic biomass to produce smaller molecules of sugars, which are further used in fermentation process, is the key step to determine the feasibility of the biorefinery process. To overcome the recalcitrance of lignocellulosic biomass, it needs the promising pretreatment method to promote enzyme accessibility to substrate and highly efficient hydrolysis to produce fermentable sugars. Also, to industrialize the biorefining process, the susceptibility and

continuity of pretreatment and hydrolysis process should be achieved as much as possible to minimize the cost, resource and power consumption required for the overall process.

Ionic liquid (IL) pretreatment has received the significant attention because it is green solvent to solubilize cellulose, and can be recycled for many rounds of pretreatment [2,3,4]. Many studies demonstrated that IL-pretreatment has advantages over other pretreatment methods, including high monomeric sugar yields over short pretreatment times, high delignification, and low cellulase-inhibitor formation [5,6]. Although, the economical viability of using IL-pretreatment is still controversial, the techno-economic analysis has been conducted and potentially suggested the strategies to reduce the cost for an economical feasibility [7,8]. ILs have excellent properties for pretreatment, one of their disadvantages is the inhibitory effect that impairs or reduces the activity of cellulase or the growth of microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* [9,10]. Therefore, the intensive

* Corresponding author.

E-mail address: macintous@gmail.com (M. Sriariyanun).

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

washing is required to remove the IL residue before subjecting the pretreated biomass to hydrolysis. Several strategies with state of the art have been developed to overcome the challenges of using IL-pretreatment with the high efficient hydrolysis in biorefining process, for example developing the enzyme that are tolerant to ILs [11,12], identifying metabolic pathways or enzyme properties that improve tolerance to ILs [13,14].

In biorefinery processes of lignocellulosic biomass, a major challenge for economical production is the high cost of cellulase enzyme utilization [3]. Cellulase is an enzyme complex composing of three enzymes namely cellobiohydrolase or exoglucanase, endoglucanase or carboxymethylcellulase, cellobiase or β -glucosidase that synergistically hydrolyzes cellulose into small molecules or monomeric sugars [15]. Several types of microorganisms including fungi and bacteria can produce cellulase enzymes during their growth on cellulosic materials. Fungal cellulases are currently the main commercial products in the markets for many applications. However, bacteria are also potential cellulase producers due to their high growth rate, high variation of glycoside hydrolases because of their diversities in different extreme niches [15,16]. In natural, deconstruction process of lignocellulosic biomass or plant biomass is corporative functions of bacterial and fungal species that accelerate the breakdown of substrates [17]. Therefore, searching for cellulase produced from bacteria has possibility to discover interesting enzyme properties that may be applied in biorefinery process. This study focused on the isolation of cellulase producing bacteria from soil sample obtained from the local rice paddy fields in Thailand. The identification and characterization of purified cellulase on different parameters were conducted to understand its properties including enzyme kinetic and the IL-tolerance ability for application in biorefining process.

2. Materials and methods

2.1. Screening of cellulase producing bacterial strains

Soil samples were collected from the local rice paddy fields in Ayutthaya province, Thailand. One gram of each soil samples was resuspended and serially diluted in sterile distilled water. Then, 100 μ L of diluted sample was spreaded on carboxymethylcellulose (CMC, Sigma-Aldrich) agar plates (containing 0.5% CMC, 0.1% NaNO_3 , 0.1% K_2HPO_4 , 0.1% KCl , 0.05% MgSO_4 , 0.05% yeast extract, 1.5% agar) [18] and then incubated at 50°C for 48 h. A total of 200 bacterial colonies grown on agar plates were selected based on the difference in morphology, size and color of the colonies. To screen for cellulase producing bacterial isolates, 5 μ L of each isolate was dropped onto a CMC agar plate and was incubated at 50°C for 48 h. To primarily observe the cellulase activity, culture plates were flooded with 0.1% Congo red solution for 15 min and washed with 0.1 M NaCl solution [18]. The diameter of the clear zone around colony on CMC agar was measured with three replicates. Isolates which had potential to degrade cellulose were selected based on calculating hydrolysis capacity (HC), that is the ratio of diameter of clearing zone and colony [19].

For secondary screening, five bacterial isolates that have larger clear zone than positive control, *Cellulomonas* sp. (Thailand Institute of Scientific and Technological Research (TISTR)) culture collection No. 368, Thailand were selected to test for ability to degrade CMC substrate in solution. 100 μ L of each selected isolate was transferred to 10 mL of fresh CMC broth media, and cultures were incubated at 50°C for 48 h in a rotary shaker. The crude cellulase enzyme from supernatant fractions was collected by centrifugation at 8000 rpm for 10 min. Two types of cellulose substrates, filter paper (Whatman No. 1) and CMC, were added into crude cellulase enzyme (2% w/v) and the mixtures were incubated at 50°C for 2 h, then the amount of reducing sugar was measured by using dinitrosalicylic (DNS) assay [20]. Enzyme activity of cellulase was defined as the amount

of enzyme that released 1.0 μ mol of reducing sugars (measured as glucose) per mL per minute at experimental condition [21]. Protein concentration was measured by using Bio-Rad protein assay kit (Bio-Rad Laboratories Ltd.). All experiments were performed with three replicates.

2.2. Molecular taxonomic identification and sequence analysis

Bacterial isolate, MSL2, was grown in nutrient broth at 50°C for 24 h in a shaking incubator at 200 rpm. Bacterial cells were harvested by centrifugation at 8000 rpm for 5 min and DNA was isolated from these bacterial cells as described in our previous study [22]. The genomic DNA was used as a template in a PCR reaction to amplify a fragment of the 16S rDNA gene. The Universal primers (27 F (forward): 5' AGAGTTTGATCM TGGCTCAG 3' and 1429R (reverse): 5' GGTTACCTTGTTACGACTT 3') were used [23]. The purified PCR products were submitted for DNA sequencing. The sequencing results were individually aligned into the nucleotide BLAST tool via the National Center for Biotechnology Information (NCBI) database to identify the possible genera of the isolates.

2.3. Enzyme production, purification, and identification

MSL2 isolate was cultured in 1 L of CMC broth and incubated at 50°C for 48 h in a shaker incubator. The culture was centrifuged at 8000 rpm for 20 min at 4°C and supernatants were collected as crude cellulase enzyme. Ammonium sulfate salt was added into crude cellulase enzyme to achieve 80% saturation. The mixture was left overnight at 4°C. Pellet was collected by centrifuged at 10,000 rpm for 20 min at 4°C, and dissolved in 10 mL of 50 mM sodium phosphate buffer (pH 5.0) for dialysis using Float-A-Lyzer dialysis membrane (Spectrum Lab) with 10 kDa MWCO. Dissolved pellet was dialyzed against 50 mM sodium phosphate buffer (pH 5.0) at 4°C with three changes of buffer. The dialyzed protein was concentrated by using Vivaspin-500 column (GE Healthcare Life Science). The concentrated protein was applied to a Sephacryl S-100 column (16 \times 600 mm; HiPrep 16/60 Sephacryl S-100 HR, GE Healthcare Life Science) equilibrated with 50 mM sodium phosphate buffer (pH 5.0) at a flow rate of 0.2 mL/min. Each eluted protein fraction was collected separately and subjected to test for enzyme activity with different substrates. The amounts of reducing sugars that are the products of enzyme hydrolysis were measured using DNS assay or Glucose Oxidase assay kit (Megazyme). The active cellulase fractions were pooled together and concentrated again by the Vivaspin-500 column.

Concentrated cellulase fraction was analyzed by 12% gel SDS-PAGE to determine the molecular weight. Concentrated cellulase fractions were loaded to 12% gel SDS-PAGE (containing 1% CMC) for zymogram analysis. Then, SDS was removed by soaking the gel in wash buffer (containing 30 mM sodium phosphate buffer and 40% isopropanol (pH 7.2)) for 1 h. The gel was soaked in equilibrated buffer (containing 30 mM sodium phosphate buffer (pH 7.2)) for 1 h, and transferred into renaturing buffer (containing 30 mM sodium phosphate buffer, 5 mM β -mercaptoethanol and 1 mM EDTA (pH 7.2)) at 4°C overnight. The renatured gel was stained with 1% congo red solution for 15 min, and washed with 1 M NaCl solution. The band of active cellulase was seen as a clear zone against background and was cut to be analyzed by LC-MS/MS analysis at Biomedical Laboratory Center, Khon Kaen University, Thailand. Proteins in the selected bands were in-gel digested by Trypsin (Promega). Digested peptides were analyzed with a nano-liquid chromatography system (EASY-nLC II, Bruker) coupled to an ion trap mass spectrometer (Amazon Speed ETD, Bruker) equipped with an ESI nano-sprayer. Peptide sample was loaded onto an C18-A2 EASY-Column (10 cm \times 75 μ m \times 3 μ m) (Thermo Scientific) using a flow rate of 300 nL/min and linear gradient from Solution A (0.1% formic acid) to 40% of Solution B (0.1% formic acid in acetonitrile) in

90 min. Bruker Daltonics software packages, HyStar v.3.2 was used to control the ion trap device. LC-MS/MS spectra were analyzed using Compass Data Analysis v.4.0. Protein identification was performed by searching against the protein database from Bacteria (Eubacteria) using MASCOT program.

2.4. Characterization of cellulase and observation of IL-tolerant property

The optimum temperature and pH of the purified cellulase of MSL2 isolate were determined, by varying reaction temperatures from 30 to 70°C, and pH from 2 to 10. To test the effect of pH, each reaction was performed in appropriate buffer (containing 2% w/v of CMC) that adjusted pH to tested condition (2.0–6.0, sodium acetate; 6.0–8.0 sodium phosphate; 8.0–10.0 Tris-HCl). The reactions were set up at 50°C. To test the effect of temperature, each reaction was performed in 30 mM sodium phosphate buffer containing 2% w/v of CMC at pH of 6.0. After 2 h incubation, each reaction was stopped by heating at 100°C for 5 min; amounts of reducing sugars were measured by using DNS assay.

Enzyme kinetic parameters (K_m and V_{max}) of the purified cellulase were calculated by determining initial reaction velocity of the enzyme at various concentrations of substrates (0.562–18 mg/mL, with 10 min reaction time) and then plotting $1/[S]$ versus $1/[V]$ in Lineweaver-Burk plot. To investigate the IL-tolerant properties of purified cellulase, 0.5 M and 1.0 M of 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) (Sigma-Aldrich) were added into each reactions. The reactions were performed in 50 mM phosphate buffer (pH 6.0) at 50°C for 1 h. The same volume of purified cellulase of MSL2 (11.75 CMCase-U/mL) and CelluClast 1.5 L (72 FPU/mL) were mixed with various concentrations of CMC substrate. The amounts of reducing sugars were quantitated to determine the reduction of enzyme activity by comparing with no-IL reaction. All experiments were conducted in triplicates.

3. Results

3.1. Screening and identification of cellulase producing bacterial strains

Among the total of 200 isolates, five isolates (MSL2, MSL8, MSL141, MSL142, MSC2) that generated the largest clear zone and showed the highest hydrolytic capacity (HC) value on CMC agar plate than *Cellulomonas* sp. were selected for the secondary screening of cellulase enzyme activity (Table 1). The HC values of these 5 isolates ranged from 1.90 to 2.50, and MSL2 isolate had the biggest radius of clear zone and highest of the HC value. The crude cellulase enzymes collected from supernatant fractions of 5 bacterial isolates were mixed with 2 types of cellulose substrates, filter paper (FP) and CMC. The specific enzyme assay for cellulase activity ranged from 0.158 to 0.264 U/mg-protein for FPase and 0.285 to 0.572 U/mg-protein for CMCCase. MSL2 isolate showed the highest FPase of 0.264 U/mg-protein, while MSL141 revealed the highest CMCCase of 0.572 U/mg-protein.

Table 1
Enzymatic activities in selected bacterial isolates from the primary and the secondary screening.

Isolate number	Maximum radius of clear zone (mm)	Average HC value	Crude enzyme specific activity (U/mg-protein)	
			FPase	CMCase
MSL2	12	2.50 ± 0.11	0.264	0.566
MSL8	9	1.90 ± 0.23	0.208	0.285
MSL141	9	2.05 ± 0.14	0.220	0.572
MSL142	10	2.33 ± 0.27	0.158	0.402
MSC2	10	1.74 ± 0.22	0.162	0.303
<i>Cellulomonas</i> sp.	8	1.72 ± 0.15	N.D. ^a	N.D.

^a N.D. is not determined.

Based on the results of primary screening and secondary screening, MSL2 isolate was selected for further study because it had the highest cellulase activity. To identify the MSL2, a fragment of 16S rDNA was generated and sequenced. The sequencing result was aligned online in the nucleotide BLAST tool through the (NCBI) database to identify the possible genera of the isolates base on homology. From BLAST search results, MSL2 isolate has 97% homology to 16S rDNA fragment of *Bacillus* sp. CMJ2-5 (Accession number KC119109.1). Then, 16S rDNA sequences of MSL2 were deposited in NCBI database with an accession number KR732274.1.

3.2. Enzyme production, purification, and identification

Crude cellulase enzyme of MSL2 was fractionated by using size-exclusion chromatography. Each eluted fraction was separately tested for cellulase activity by mixing each fraction with different types of substrates including CMC, Avicel and cellobiose (2% w/v) (Fig. 1). Enzyme fractions with positive-cellulase activity were collected for subsequent experiments. The positive fractions numbers were 10–14, 5 and 8 from the hydrolysis of CMC, Avicel and cellobiose, respectively. For the fraction 10–14, they were pooled together. To determine the cellulase activity to specific substrates, each selected enzyme fraction was mixed with specific substrate

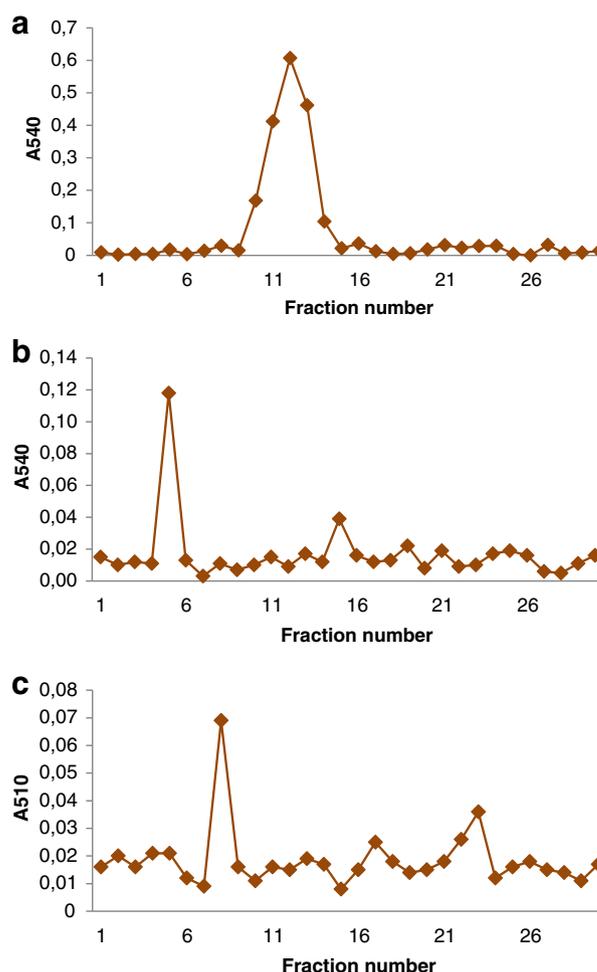


Fig. 1. Chromatogram of cellulase from MSL2 isolate on size exclusion chromatography (HiPrep 16/60 Sephacryl S-100 HR, equilibrated with 50 mM sodium phosphate buffer (pH 5.0) at a flow rate of 0.2 mL/min). Hydrolysis products of three types of substrates were measured, (a) CMC substrate by using DNS assay with absorbance at 540 nm (A_{540}), (b) Avicel substrate by using DNS assay with absorbance at 540 nm (A_{540}) and (c) cellobiose substrate by using glucose oxidase assay with absorbance at 510 nm (A_{510}).

(Pooled fraction Nos. 10–14 was mixed with CMC, fraction No. 5 with Avicel and fraction No. 8 with cellobiose). After 2 h reaction, reducing sugar and glucose contents in each reaction was quantitated and calculated for cellulase activities. Here, the specific activity of CMCase, Avicelase, cellobiase activities was 5.413, 4.765 and 1.151 U/mg-protein, respectively. However, it was also observed that, in 1 L culture, MSL2 produced larger portion of cellulase as CMCase enzyme (20 mL of fraction Nos. 10–14 with total CMCase activity 1.624 U/L-culture). On the other hand, the Avicelase and cellobiase were produced in much less proportion (4 mL each for fraction No. 5 and No. 8 with 0.032 and 0.0087 U/L-culture, respectively). Therefore, the CMCase was selected for subsequent studies including enzyme characterization and IL-tolerance property.

To identify the CMCase enzyme in purified sample, pooled CMCase fraction (Nos. 10–14) was concentrated, then analyzed by SDS-PAGE and CMC-zymogram to estimate protein molecular size (Fig. 2). In SDS-PAGE analysis, there are 3 major bands (with ~65, 48 and 40 kDa size) visualized in the gel. Likewise, CMC-zymogram analysis revealed that a 48 kDa-sized protein band showed clear zone indicating the CMCase activity. Based on SDS-PAGE and CMC-zymogram analysis, the fractionated CMCase enzyme produced from MSL2 was demonstrated to have molecular weight about 48 kDa. Then, this 48 kDa-sized band was excised from SDS-PAGE gel and subjected to trypsin digestion and the peptides were sequenced by LC-MS/MS analysis. The results of MASCOT search indicated that these partial amino acid sequences were 21% homologous (Score: 595 Matches: 24(6) Sequences: 8(4) emPAI: 0.37) to the endoglucanase of *Bacillus amyloliquefaciens* (Accession: gi:696282038) (Fig. 2).

3.3. Effect of pH and temperature on cellulase hydrolysis efficiency and kinetic analysis of purified cellulase

In this section, the optimum pH and temperature for the working condition of purified CMCase which allow to obtain the highest amount of reducing sugars from hydrolysis reaction were studied. To find the optimum pH of purified CMCase, the reactions (containing 2% w/v of CMC) were set up at 50°C. The optimum pH of purified cellulase of MSL2 was pH 6.0 and the enzymatic hydrolysis efficiency retained more than 80% over the range of pH 4.0–7.0 (Fig. 3). The enzyme hydrolysis efficiency greatly declined when pH was less than pH of 4.0, however it retained more than 50% activity at pH of 3.0. When pH of reaction increased to be more than pH 7.0, the efficiency was gradually declined. The optimum temperature was 50°C at pH of 6.0. More than 80% of the maximum hydrolysis efficiency was observed between the range of 45–55°C. However, at 60°C the hydrolysis efficiency still retained at 72% (Fig. 3).

The kinetic parameters K_m and V_{max} of the purified CMCase enzyme were determined from Lineweaver–Burk double reciprocal plots at 50°C and pH 6.0, using various concentrations of CMC as substrate (0.562–18 mg/mL) (Fig. 4). The CMCase showed a

maximum velocity, V_{max} , of 1000 $\mu\text{M}/\text{min}$ and a Michaelis–Menten constant, K_m , of 0.8 mg/mL CMC.

3.4. Effect of [C2mim][OAc] on purified cellulase of MSL2

Previously, the negative effect of IL, including [C2mim][OAc], to the cellulase activity was demonstrated [5]. In this work, the effect of [C2mim][OAc] on purified cellulase of MSL2 was observed with comparison to commercial cellulase enzyme, CelluClast 1.5 L (Novozymes) (Fig. 5). At high concentration of CMC substrates (0.1–0.2 μM), cellulase activities of MSL2 and CelluClast 1.5 L were reduced in the present of [C2mim][OAc]. The more of [C2mim][OAc] concentration, the less of remaining cellulase activity. At 0.2 μM of CMC concentration, the cellulase activities in the reactions, which contained 0.5 M and 1 M of [C2mim][OAc], retained 88.2% and 77.7% activity of control reaction (No-IL), respectively. While the activity of CelluClast 1.5 L reduced to 85.7% and 74.1% in the presence of 0.5 M and 1 M of [C2mim][OAc], respectively. This result suggested that purified cellulase of MSL2 had little higher tolerance to [C2mim][OAc] compared to the CelluClast 1.5 L. Interestingly, at lower concentration of CMC substrates (0.0125–0.04 μM), the [C2mim][OAc] insignificantly showed negative effect on the cellulase activity in both MSL2 and CelluClast 1.5 L. Basically, the enzyme activity depends on the enzyme–medium–substrate interactions. Many factors could affect the enzyme activity in IL medium, for example anions of IL, the alkyl chain composition, IL polarity, viscosity, and hydrophobicity [24]. In the case of hydrophilic IL, including [C2mim][OAc], more water in the medium is needed because hydrophilic IL tends to remove the water molecule from the enzyme causing enzyme deactivation [25]. Additionally, CMC also requires surrounding water molecules to let itself to dissolve. Therefore, it is possible that, at low concentration of CMC substrate, there is less competition between CMC and enzyme molecules to grab water molecule. On the other hand, at high concentration of CMC substrate, there are high competition between CMC and enzyme to attract water molecules, and leads to the loss of enzyme activity.

4. Discussion

Cellulase produced from MSL2 was purified and fractionated by using size exclusion liquid chromatography and ultrafiltration. Each fraction was tested for hydrolysis activities on three different types of substrates including CMC, Avicel, and cellobiose. Previously, these three types of substrates were studied and suggested to be used as indicators for different types of cellulase enzymes. CMC, Avicel and cellobiose are general substrates for endoglucanase, exoglucanase, and cellobiase, respectively [15]. From the size exclusion liquid chromatography, active fractions of endoglucanase, exoglucanase, and cellobiase were eluted with different retention time or fraction numbers indicating that MSL2 produced these enzymes with different

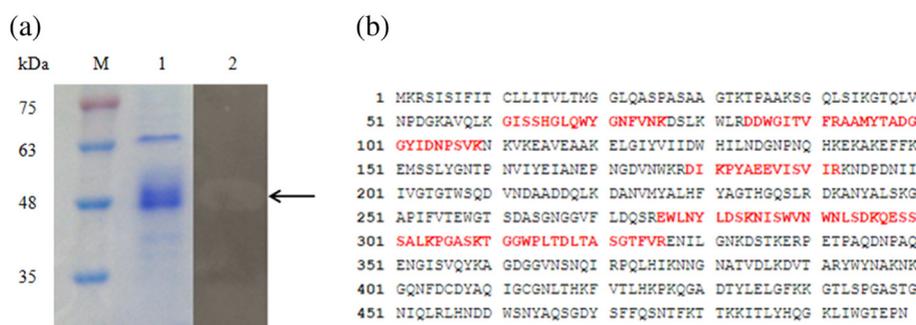


Fig. 2. (a) SDS-PAGE analysis and CMC-zymogram analysis of purified CMCase. Lane-M molecular mass markers (BLUeye Prestained Protein Ladder, GeneDirex). Lane 1 purified CMCase. Lane 2 purified CMCase in CMC-zymogram gel. (b) MASCOT search analysis of partial amino acid sequences of 48 kDa-sized band (arrow pointed band). The red color sequences are matched amino acids to the endoglucanase (Accession: gi: 696282038).

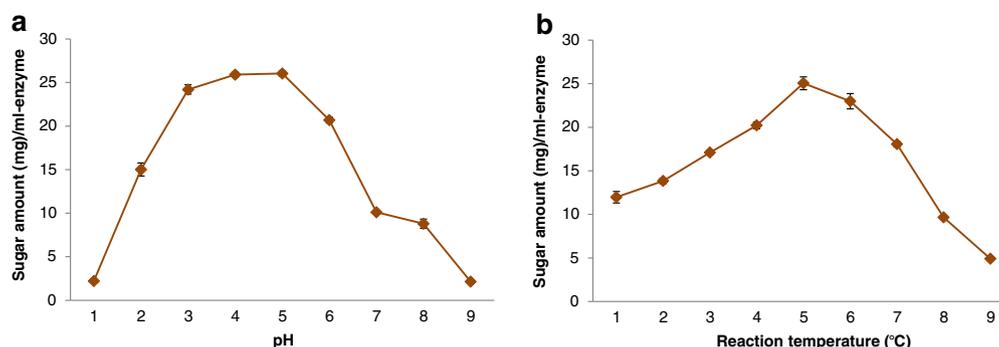


Fig. 3. Effect of reaction pH and temperature on cellulase hydrolysis efficiency of MSL2. Reactions were performed for 2 h at various (a) pH (2.0–10.0) and (b) temperature (30–70°C).

molecular weight (Fig. 1). Here, the retention time of Avicelase is the fastest, then followed with cellobiase and CMCase, respectively. The purified CMCase, Avicelase and cellobiase exhibited a specific activity of 5.413, 4.675 and 1.151 U/mg-protein, respectively. However, in terms of the volume and amount of each type of enzyme produced from MSL2 culture, CMCase was likely to be the largest portion of total cellulase. Therefore, only CMCase characterization was mainly focused in this study.

A SDS-PAGE and CMC-zymogram analysis of purified CMCase presented a protein band with significant activity corresponding to 48 kDa (Fig. 2). This finding is consistent with the review of the investigations on bacterial CMCase (35–57 kDa) [1,16]. The 48 kDa-sized band was subjected to analyzed by LC-MS/MS, and the amino acid sequence of digested peptides were matched to endoglucanase protein of *B. amyloliquefaciens* (gi: 696282038) (Fig. 2). This result confirmed that this purified cellulase active fraction is CMCase (endoglucanase) enzyme.

The temperature optimum of purified CMCase was 50°C at pH of 6.0. The enzymatic hydrolysis efficiency was maintained with more than 80% of the maximum efficiency over the temperature of 45 to 55°C and pH of 4.0 to 7.0 (Fig. 3). With this regard, the influence of pH and/or temperature in the enzymatic hydrolysis within these range is found to be little, so this cellulase may be exploited for industrial usage. The kinetic parameters, K_m and V_{max} , of the purified CMCase enzyme of MSL2 were determined from Lineweaver–Burk double reciprocal plots using CMC as substrate at 50°C, pH 6.0 (Fig. 4). The K_m value of purified CMCase was 0.80 mg/mL and V_{max} was 1000 μ M/min, respectively. This K_m value was lower than those

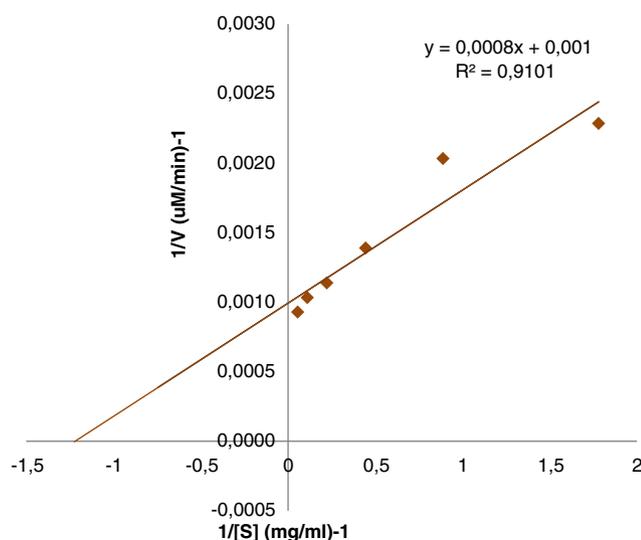


Fig. 4. Lineweaver–Burk plot of purified CMCase of MSL2.

obtained in other endoglucanase isolated from metagenome studies (Umcel5 (K_m of 16 mg/mL) [26], C67-1 (K_m of 37 mg/mL) [27]), and other bacteria (*Pseudomonas fluorescens* (K_m of 3.6 mg/mL) [28], *Actinobacteria anitratus* (K_m of 4.97 mg/mL) [29]). This lower K_m value indicated that the purified CMCase from MSL2 had higher affinity for CMC substrate.

The IL tolerance property of purified CMCase of MSL2 was monitored in comparison with commercial cellulase enzyme, CelluClast 1.5 L. Previously, a few IL stable cellulases derived from metagenome and from culturable microbes such as *Enterobacter lignolyticus*, *Penicillium janthinellum*, *Thermotoga maritime*, *Pyrococcus horikoshii*, and *Fusarium oxysporum* have been identified and reported [11,12]. In this study, CMCase of MSL2 retained activity to 88.2% and 77.7% in 0.5 M and 1 M [C2mim][OAc], which was higher than that of commercial cellulase, CelluClast 1.5 L (Fig. 5). Comparing to other studies, MSL2 has comparable tolerance to IL, for example, CMCase from *Paenibacillus tarimensis* and *Aspergillus terreus* UniMAP AA-6 can retain >80% and >60% activity in 20% concentration (~1.2 M) of [C2mim][OAc] [30, 31], or *Aspergillus fumigatus* has >50% activity in 30% concentration (~1.8 M) of [C2mim][OAc] [12] suggesting that CMCase of MSL2 is a potential IL-tolerance enzyme.

5. Conclusion

In this report, MSL2 isolate was screened by using congo red plate assay from a total 200 isolates of bacteria collected from the local rice paddy fields in Thailand. The clear zone observed on the stained plate indicated that MSL2 secreted cellulase to extracellular compartment. From the screening experiment, MSL2 showed the highest global cellulase activity (as the FPU), and the second-highest of CMCase activity, therefore it was selected as the best candidate of cellulase-producer of our collection. Cellulase of MSL2 was purified and characterized to find the specific hydrolysis activities with different types of substrates. CMCase fraction of MSL2 was identified to be endoglucanase by using LC-MS/MS analysis. The optimum conditions and kinetic properties of enzyme hydrolysis were studied which could be later used as information for future studies. Our efforts here to identify IL-tolerant property of MSL2 cellulase for application in one-pot process that incorporates pretreatment and saccharification, lead to the development of new technology for lignocellulosic biorefineries. The enzyme reported in this study can be developed and applied in this technology in the future.

Financial support

The authors would like to thank King Mongkut's University of Technology, North Bangkok (Research University Grant Nos. KMUTNB-GOV-57-41, KMUTNB-GOV-58-22 and KMUTNB-GOV-58-27), and The Institute for the Promotion of Teaching Science and

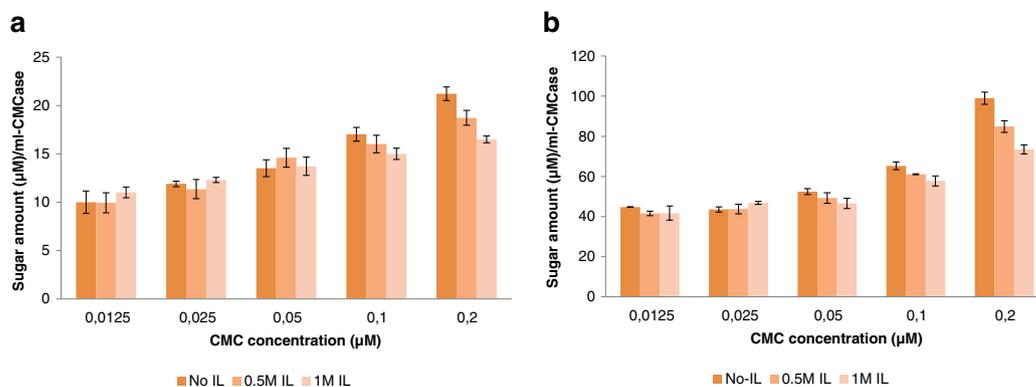


Fig. 5. Effect of [C2mim][OAc] on (a) purified cellulase of MSL2 (11.75 CMCCase-U/mL) and (b) CelluClast 1.5 L (72 FPU/mL) hydrolysis efficiency of CMC substrate (with 0.0125–0.2 µM concentration). White, gray and black bars represented control reaction (No-IL), addition of 0.5 M and 1 M of [C2mim][OAc], respectively.

Technology, Thailand (IPST) (Research Grant No. 022/2555) for financial support of this work.

Conflict of interest

There is no conflict of interest.

References

- Juturu V, Wu J. Microbial cellulases: Engineering, production and applications. *Renew Sust Energ Rev* 2014;33:188–203. <http://dx.doi.org/10.1016/j.rser.2014.01.077>.
- Sriariyanun M, Yan Q, Nowik I, Cheenkachorn K, Phusantisampan T, Modigell M. Efficient pretreatment of rice straw by combination of screw-press and ionic liquid to enhance enzymatic hydrolysis. *Kasetsart J* 2015;49:146–54.
- Agbor V, Cicek N, Sparling R, Berlin A, Levin DB. Biomass pretreatment: Fundamentals toward application. *Biotechnol Adv* 2011;29:675–85. <http://dx.doi.org/10.1016/j.biotechadv.2011.05.005>.
- Wang Y, Song H, Peng L, Zhang Q, Yao S. Recent developments in the catalytic conversion of cellulose. *Biotechnol Biotechnol Equip* 2014;28:981–8. <http://dx.doi.org/10.1080/13102818.2014.980049>.
- Engel P, Mladenov R, Wulforth H, Jager G, Spiess AC. Point by point analysis: How ionic liquid affects the enzymatic hydrolysis of native and modified cellulose. *Green Chem* 2010;12:1959–66. <http://dx.doi.org/10.1039/c0gc00135j>.
- Ang T, Ngoh GC, Chua AS, Lee MG. Elucidation of the effect of ionic liquid pretreatment on rice husk via structural analyses. *Biotechnol Biofuels* 2012;5:67. <http://dx.doi.org/10.1186/1754-6834-5-67>.
- Klein-Marcuschamer D, Simmons B, Blanch H. Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. *Biofuels Bioprod Biorefin* 2011;5:562–9. <http://dx.doi.org/10.1002/bbb.303>.
- Konda N, Shi J, Singh S, Blanch H, Simmons BA, Klein-Marcuschamer D. Understanding cost drivers and economic potential of two variants of ionic liquid pretreatment for cellulosic biofuel production. *Biotechnol Biofuels* 2014;7:86. <http://dx.doi.org/10.1186/1754-6834-7-86>.
- Ouellet M, Datta S, Dibble DC, Tamrakar PR, Benke PI, Li C, et al. Impact of ionic liquid pretreated plant biomass on *Saccharomyces cerevisiae* growth and biofuel production. *Green Chem* 2011;13:2743–9. <http://dx.doi.org/10.1039/c1gc15327g>.
- Ghanem OB, Mutalib MIA, El-Harabawi M, Gonfa G, Kait C, Alitheen NB, et al. Effect of imidazolium-based ionic liquids on bacterial growth inhibition investigated via experimental and QSAR modelling studies. *J Hazard Mater* 2015;297:198–206. <http://dx.doi.org/10.1016/j.jhazmat.2015.04.082>.
- Park J, Steen EJ, Burd H, Evans SS, Redding-Johnson AM, Batth T, et al. A thermophilic ionic liquid-tolerant cellulase cocktail for the production of cellulosic biofuels. *PLoS One* 2012;7: e37010. <http://dx.doi.org/10.1371/journal.pone.0037010>.
- Xu J, He B, Wu B, Wang B, Wang C, Hu L. An ionic liquid tolerant cellulase derived from chemically polluted microhabitats and its application in *in situ* saccharification of rice straw. *Bioresour Technol* 2014;157:166–73. <http://dx.doi.org/10.1016/j.biortech.2014.01.102>.
- Jaeger V, Burney P, Pfaendtner J. Comparison of three ionic liquid-tolerant cellulases by molecular dynamics. *Biophys J* 2015;108:880–92. <http://dx.doi.org/10.1016/j.bpj.2014.12.043>.
- Nordwald E, Brunecky R, Himmel ME, Beckham GT, Kaar JL. Charge engineering of cellulases improves ionic liquid tolerance and reduces lignin inhibition. *Biotechnol Bioeng* 2014;111:1541–9. <http://dx.doi.org/10.1002/bit.25216>.
- Lynd LR, Weimer PJ, Van Zyl WH, Pretorius IS. Microbial cellulose utilization: Fundamentals and biotechnology. *Microbiol Mol Biol Rev* 2002;66:506–77. <http://dx.doi.org/10.1128/MMBR.66.3.506-577.2002>.
- Sadhu S, Maiti T. Cellulase production by bacteria: A review. *Br Microbiol Res J* 2013; 3:235–58. <http://dx.doi.org/10.9734/BMRJ/2013/2367>.
- Xu W, Shi L, Chan O, Li J, Casper P, Zou X. Assessing the effect of litter species on the dynamic of bacterial and fungal communities during leaf decomposition in microcosm by molecular techniques. *PLoS One* 2013;8:e84613. <http://dx.doi.org/10.1371/journal.pone.0084613>.
- Kasana R, Salwan R, Dhar H, Dutt S, Gulati A. A rapid and easy method for the detection of microbial cellulases on agar plates using gram's iodine. *Curr Microbiol* 2008;57:503–7. <http://dx.doi.org/10.1007/s00284-008-9276-8>.
- Hendricks CW, Doyle JD, Hugley B. A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Appl Environ Microbiol* 1995;61:2016–9.
- Miller G. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* 1959;31:426–8. <http://dx.doi.org/10.1021/ac60147a030>.
- Ghose TK. Measurement of cellulase activities. *Pure Appl Chem* 1987;59:257–68. <http://dx.doi.org/10.1351/pac198759020257>.
- Sriariyanun M. Response surface methodology for optimization of biodiesel production by *Acinetobacter baylyi*. *KMUTNB Int J Appl Sci Technol* 2014;7:47–52. <http://dx.doi.org/10.14416/j.ijast.2014.09.003>.
- Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. Microbial diversity in water and sediment of Lake Chaka, an Athalassohaline Lake in Northwestern China. *Appl Environ Microbiol* 2006;72:3832–45. <http://dx.doi.org/10.1128/AEM.02869-05>.
- Naushad M, Othman ZAAL, Khan AB, Ali M. Effect of ionic liquid on activity, stability, and structure of enzymes: A review. *Int J Biol Macromol* 2012;51:555–60. <http://dx.doi.org/10.1016/j.ijbiomac.2012.06.020>.
- Noritomi H, Suzuki K, Kikuta M, Kato S. Catalytic activity of a-chymotrypsin in enzymatic peptide synthesis in ionic liquids. *Biochem Eng J* 2009;47:27–30. <http://dx.doi.org/10.1016/j.bej.2009.06.010>.
- Liu L, Feng Y, Duan CJ, Pang H, Tang JL, Feng JX. Isolation of a gene encoding endoglucanase activity from uncultured microorganisms in buffalo rumen. *World J Microbiol Biotechnol* 2009;25:1035–42. <http://dx.doi.org/10.1007/s11274-009-9983-8>.
- Duan C, Xian L, Zhao GC, Feng Y, Pang PH, Bai XL, et al. Isolation and partial characterization of novel genes encoding acidic cellulases from metagenomes of buffalo rumens. *J Appl Microbiol* 2009;107:246–56. <http://dx.doi.org/10.1111/j.1365-2672.2009.04202.x>.
- Bakare M, Adewale IO, Ajayi A, Shonukan OO. Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*. *Afr J Biotechnol* 2005;4:898–904.
- Ekperigin MM. Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. *Afr J Biotechnol* 2007;6:28–33.
- Khudyakov J, D'haeseleer P, Borglin SE, DeAngelis KM, Woo H, Lindquist EA, et al. Global transcriptome response to ionic liquid by a tropical rain forest soil bacterium, *Enterobacter lignolyticus*. *Proc Natl Acad Sci U S A* 2012;109:2173–82. <http://dx.doi.org/10.1073/pnas.1112750109>.
- Gunny A, Arbain D, Gumba R, Jong B, Jamal P. Potential halophilic cellulases for *in situ* enzymatic saccharification of ionic liquids pretreated lignocelluloses. *Bioresour Technol* 2014;155:177–81. <http://dx.doi.org/10.1016/j.biortech.2013.12.101>.