DIVERSITY OF CELLULOLYTIC BACTERIA ISOLATED FROM A FRESHWATER WETLAND RESERVE IN THAILAND AND THEIR CELLULOLYTIC ACTIVITY

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Abstract. Freshwater wetlands are unique aquatic ecosystems, which are a tremendous source of organic carbon. A bacterial community plays a significant role in the carbon cycle of organic matter through its cellulolytic enzymes; namely, cellulases. For this study, culturable bacteria were isolated from a freshwater wetland reserve situated in Thailand and screened for their cellulase production. Seventy-six cellulolytic bacteria were grouped by a PCR-RFLP of 16S rDNA technique and identified by a nucleotide sequencing analysis. A total of 17 different RFLP patterns were obtained, belonging to nine bacterial genera including *Acinetobacter*, *Aeromonas*, *Bacillus*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Herbaspirillum*, *Paenibacillus* and *Vibrio*. The predominant genera of the isolated cellulolytic bacteria were *Bacillus*, *Chromobacterium* and *Herbaspirillum*. The cellulolytic bacterium isolated from the moist peat samples designated as *B. megaterium* strain S0702 could produce three types of cellulases and showed the highest CMCase activity at 4.48 ± 0.08 U/mL. The optimum pH and temperature for the CMCase activity were determined to be 45 - 50°C at a pH of 7.0 with a stability range of 25 - 60°C and pH 5.0 - 8.0. The CMCase activity was greatly enhanced by Mn^{2+} and considerably inhibited by EDTA and ethyl-acetate. This enzyme could possibly be used in various biotechnological applications.
Keywords: *aquatic ecosystem, B. megaterium, cellulase, CMCase activity, PCR-RFLP*

Introduction

Wetlands are natural or artificial, permanent or temporary areas with static or flowing water, where the depth at low tide does not exceed six meters; these include, fens, marshes, peatlands, freshwater areas and marine water areas (Bassi et al., 2014; Kalita et al., 2019). Wetlands serve as a source of biogeochemical cycles, bioremediation of contaminants, flood alleviation and production of food (Leff, 2009). Freshwater wetland is a type of saturated land, the nature of which varies based on the hydrological and plant communities. This unique nature affects the microbial communities dwelling in the wetland. Wetland soils and sediments are also a tremendous source of terrestrial carbon including decayed plants and woody organic matter (Gorham, 1991; An et al., 2019). Microbial communities also play a critical role in the detritus decomposition resulting in dissolved organic carbon and related organic compounds which maintain the nutrient cycle and wetland stableness. Cellulolytic microbes mainly provide the decomposition of cellulose-based plant litter through their cellulolytic enzymes producing the simple sugar derivatives in the sediment (Soares-Júnior et al., 2013). Microbial cellulolytic enzymes, generally called cellulase, are complex substances that comprise endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C. 3.2.1.91, and E.C. 3.2.1.176) and β-glucosidases (E.C. 3.2.1.21), which synergistically work to hydrolyze the β-1,4 glycosidic linkages of cellulose polymer (Chantarasiri, 2015). Cellulases account for 20% of the world enzyme market and have biotechnological promise in various industries; such as, agriculture, animal feed, biofuel, breweries, food, laundry, paper and pulp, pharmaceuticals, textiles

and waste management (Juturu and Wu, 2014; Behera et al., 2017). Furthermore, microbial cellulases have been reported in aerobic and anaerobic, mesophilic and thermophilic bacteria and fungi (Sharma and Yazdani, 2016). Cellulolytic microbes have been commonly isolated from soil, decaying organic matter, animal digestive tracts and herbivore dung (Juturu and Wu, 2014); such as, *Aspergillus* (Nwodo-Chinedu et al., 2005; Gao et al., 2008), *Bacillus* (Anand et al., 2010; Chantarasiri, 2014, 2015; Sriariyanun et al., 2016), *Cellulomonas* (Sangkharak et al., 2011), *Clostridium* (Reddy et al., 2010), *Fusarium* (Qin et al., 2010; Nwodo-Chinedu et al., 2005), *Geobacillus* (Ibrahim and El-diwany, 2007; Baharuddin et al., 2010), *Gluconacetobacter* (Wee et al., 2011) and *Penicillium* (Nwodo-Chinedu et al., 2005). Currently, most commercial cellulases in the global enzyme market have been produced by *Trichoderma reesei* and *Aspergillus* sp. (Zhang et al., 2006). Moreover, a few cellulolytic microbes have been isolated from freshwater wetlands and related environments due to their complexity and inaccessibility. The isolated cellulolytic microbes from freshwater wetlands were identified as bacteria belonging to the genera *Bacillus* (Chantarasiri et al., 2015), *Brucella* (Behera et al., 2016), *Nocardia* (Benhadj et al., 2019), *Micromonospora* (Benhadj et al., 2019) and fungi belonging to the genera *Fusarium*, *Peziza*, and *Zygomycete* (Wu et al., 2015). The search for cellulolytic microbes still has much interest, and microbial species and environmental sources of microbial isolation have been reported. However, only a few microbes can produce high cellulolytic activity, and only a few can produce all three cellulase enzymes (Sharma and Yazdani, 2016). To improve the knowledge of cellulolytic microbes and their cellulolytic performance, more research has been conducted. Isolation and screening of cellulase-producing microbes from nature is one of the important ways to obtain novel and effective cellulases.

However, sufficient data of cellulolytic microbes isolated from freshwater wetland ecosystem are required. This research consequently aimed to isolate and screen cellulolytic bacteria from Bueng Samnak Yai, a wetland reserve of Thailand. The molecular genetic methods, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of 16S rDNA and nucleotide sequencing analysis, were used to describe the diversity of a cellulolytic bacterial community. All representative bacteria of each RFLP pattern were determined for their cellulolytic performance. Finally, the most effective bacterium, *B. megaterium* strain S0702, was cellulolytic characterized to evaluate its biotechnological potential.

Materials and Methods

Description of the sampling site

The study area in this research was Bueng Samnak Yai (12° 39′ N, 101° 32′ E) in Rayong province, Thailand. Bueng Samnak Yai has been designated as a wetland reserve, which uniquely combines coastal (brackish) wetland and freshwater wetland. The coastal wetland area is grown over by many characteristic plants (Chantarasiri et al., 2017) which those species are similar to those of a freshwater wetland area. Water and moist peat (partially decayed plants) samples were randomly collected from the grass islands floating in the freshwater wetland area. Twenty water samples and 30 moist peat samples were taken at a depth of 0 - 5 cm from the sampling grass islands. The sampling site covered an area of 190 ha with an average depth of water of one meter. The site comprised more than 100 floating and flowing grass islands, which their dimensions ranged from a table-sized to a soccer field-sized island. The grass islands were strongly formed by stem

and rhizome networks of three dominant grass species consisting of *Lepironia articulate* (Retz.) Domin, *Imperata cylindrica* (L.) Beauv. and *Carex baccans* Nees. All samples were collected during September 2017. The samples were kept at 4°C in sterilized plastic bags and taken for bacterial isolation within 24 hours of collection. The locations of the sampling site are shown in *Figure 1*.
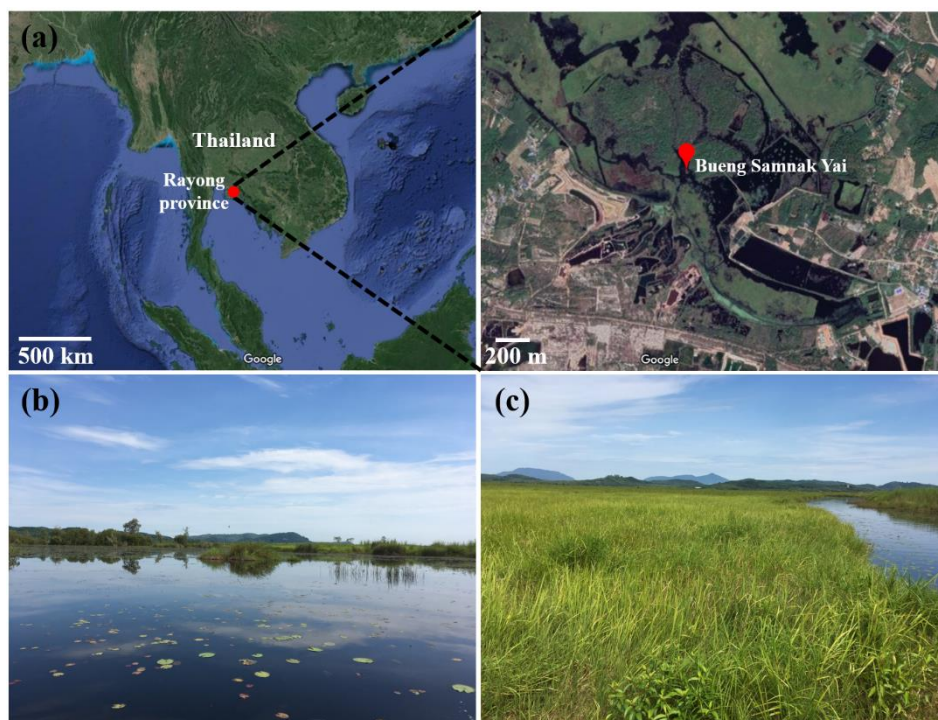

Figure 1. Bueng Samnak Yai: (a) Map of Bueng Samnak Yai (12°39′N, 101°32′E) situated in Rayong province, Thailand (Source: Google Maps). (b) Freshwater wetland area and a few table-sized floating grass islands. (c) A scene of a soccer field-sized floating grass island

Isolation and purification of bacteria from the freshwater wetland samples

Water and moist peat samples were serially diluted with sterilized 0.85% NaCl solution supplemented with 0.1% buffered peptone to obtain 1:10,000 dilutions (Merck, India). One hundred microliters of diluted samples were spread plated on Tryptone soya agar (HiMedia, India) and incubated at $28.5 \pm 0.1°C$ (the average temperature of the sampling site) for 24 hours. The bacterial strains were selected based on the colony's morphology and subsequently the colony was purified by being streak plated on Tryptone soya agar.

Screening of cellulolytic bacteria

The screening of cellulolytic bacteria from an aquatic environment was conducted from that previously described (Chantarasiri, 2015). One drop (five microliters) of overnight growth culture in the Tryptone soya broth (HiMedia, India) of each isolated bacterium was spot plated on carboxymethyl cellulose (CMC) agar (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC sodium salt, 0.02% peptone and 1.7% agar). All culture plates were incubated at $28.5 \pm 0.1°C$ for 48 hours and then flooded

with iodine solution (0.33% I_2 and 0.67% KI) for 10 minutes. The bacterial isolates could produce the cellulolytic zone around the colonies after Gram's iodine staining indicated the synthesis of the extracellular cellulases by the cellulolytic candidates. The cellulolytic performance was evaluated by the hydrolysis capacity (HC) value that was calculated from the ratio between the diameter of the cellulolytic zone and the diameter of the bacterial colony. All experiments were performed in triplicate.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of 16S rDNA

Genomic DNA of each cellulolytic bacteria was extracted by a heat treatment method (Dashti et al., 2009). Polymerase chain reaction (PCR) amplification of the 16S rRNA genes was performed using the OnePCR™ reaction mixture (Bio-Helix, Taiwan). The primer set used for the amplification of the target 16S rRNA genes included the universal forward primer 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and the universal reverse primer 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Sigma-Aldrich, Singapore). The PCR conditions involved a preheating step at 94°C for four minutes, denaturation step at 94°C for 40 seconds, annealing step at 55°C for one minute, extension step at 72°C for 1 minute 10 seconds, and final extension step at 72°C for 10 minutes. PCR was performed for 35 amplification cycles in a Mastercycler® Nexus (Eppendorf, Germany). The 16S rDNA fragments resulting from the PCR processes were approximately 1,500 bp. The restriction fragment length polymorphism (RFLP) analysis was performed by two restriction enzymes of *Msp*I and *Alu*I (New England Biolabs, UK) in a CutSmart® buffer (New England Biolabs, UK). The 16S rDNA fragments were digested with *Msp*I and *Alu*I at 37°C for 12 hours then the digestion reaction was terminated by heating the reaction mixtures at 80°C for 15 minutes following the protocol described by New England Biolabs with minor modifications. The resulting restriction fragments were analyzed using 3% (w/v) OmniPur® agarose gel (Calbiochem, Germany) and visualized by Novel Juice (Bio-Helix, Taiwan). The PCR marker used in this study was a 100 bp DNA ladder RTU (Bio-Helix, Taiwan).

16S rDNA sequencing and phylogenetic analysis

The 16S rDNA PCR products were purified and sequenced by the nucleotide sequencing service of Macrogen Inc. (Seoul, Korea). The sequence similarity analysis of the resulting 16S rDNA was aligned using the BLASTn suite (National Center for Biotechnology Information: NCBI). The phylogenetic tree was analyzed and visualized by the SeaView software version 4.6.4 (Gouy et al., 2010) and FigTree software version 1.4.3 (Institute of Evolutionary Biology, University of Edinburgh, UK). The phylogenetic tree was generated by the neighbor-joining (NJ) method with 100,000 bootstrap replications. All the resulting nucleotide sequences of the identified cellulolytic bacteria from this study were deposited in the GenBank database of NCBI under the accession numbers MN993647, MN993849, MN993893, MN993916, MN994046, MN994069, MN994075, MN994076, MN994079 to MN994082, MN994084 and MN994270 to MN994273.

Preparation of the crude cellulases

The cellulolytic bacteria were cultured in a CMC liquid medium. All bacterial cultures were shaken under an aeration condition in a baffled flask (Schott-Duran, Germany) at

150 rpm, 28.5 ± 0.1°C, for 48 hours. The bacterial cells were then removed from the liquid medium to obtain the crude cellulases by a centrifugation method at 4,500 ×*g* at 4°C for 30 minutes (Chantarasiri, 2015). The crude cellulases were concentrated by 30-kDa Amicon® ultra centrifugal filter units (Millipore, Ireland).

Cellulolytic activity assays of the crude cellulases

The cellulolytic activity assays of the crude cellulases were conducted from that previously described (Chantarasiri, 2015). The endoglucanase activity (CMCase) was measured by incubating 0.5 mL of crude cellulases with 0.5 mL of 2% CMC in an assay buffer at 50°C for 30 minutes. The exoglucanase activity (Avicelase) was measured by incubating 0.5 mL of crude cellulases with 0.5 mL of 2% Avicel® PH-101 (Sigma-Aldrich, Germany) in an assay buffer at 50°C for one hour. The reducing sugars liberated from the CMCase and Avicelase reactions were spectrophotometrically determined by a 3,5-dinitrosalicylic acid (DNS) method at 540 nm (Miller, 1959). The cellulolytic activity values of CMCase and Avicelase were calculated by a glucose standard curve. One unit (U) of CMCase and Avicelase was defined as the amount of enzyme required to release 1 µmol of the reducing sugars as glucose equivalents per minute under the assay conditions. The β-glucosidase activity was measured by incubating 0.5 mL of crude cellulases with 1 mL of 0.1% *p*-nitrophenyl-β-D-glucopyranoside (Sigma-Aldrich, Germany) in an assay buffer at 50°C for one hour. The reaction was terminated by adding 2 mL of 1 M Na_2CO_3 solution. The reaction mixture was spectrophotometrically measured at 400 nm. The cellulolytic activity values of β-glucosidase were calculated by a *p*-nitrophenol standard curve. One unit (U) of β-glucosidase was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol per minute under the assay conditions. The assay buffer used in this study was 50 mM sodium phosphate buffer at a pH 7.0, which its pH value was conducted from Samira et al. (2011) and Shobharani et al. (2013). All experiments were performed in triplicate.

Characterization of the cellulolytic activity from the most active cellulolytic bacteria

The characterization of the cellulolytic activity was examined on the crude cellulases produced from the most active endoglucanasic bacterium, *B. megaterium* strain S0702. The study on the cellulolytic activity was determined based on its CMCase activity. The enzymatic characterization focused on three parameters, which affected the cellulolytic activity consisting of temperature, pH and some chemical additives. All experiments were performed in triplicate.

Effect of temperature on the cellulolytic activity and thermal stability

The CMCase activity was measured accordingly as mentioned in the section "Cellulolytic activity assays of the crude cellulases" at temperatures ranging from 25°C to 80°C in an assay buffer. Thermal stability was examined by pre-incubating the crude enzyme at temperatures ranging from 25°C to 80°C in an assay buffer for 24 hours, and the relative activity of CMCase was monitored using 2% CMC sodium salt as a substrate under the mentioned CMCase conditions. The assay buffer used in this study was a 50 mM sodium phosphate buffer at a pH 7.0.

Effect of pH on the cellulolytic activity and pH stability

The CMCase activity was measured accordingly as mentioned above in the pH-varied buffers at 50°C. The assay buffer used in this study was a 50 mM citrate buffer (pH 4.0 - 6.0), 50 mM sodium phosphate buffer (pH 6.0 - 8.0) and 50 mM glycine-NaOH buffer (pH 8.0 - 10.0). The pH stability was examined by pre-incubating the crude enzyme in the above-mentioned buffer at 50°C for 24 hours, and the relative activity of CMCase was monitored using 2% CMC sodium salt as a substrate under the mentioned CMCase conditions.

Effect of chemical additives on the cellulolytic activity

The CMCase activity was measured accordingly as mentioned above. Crude cellulases were pre-incubated with metal ions, a chelating agent and organic solvents. Ten metal ions were used comprising Ca^{2+} (as $CaCl_2$), Co^{2+} (as $CoCl_2$), Cu^{2+} (as $CuCl_2$), Fe^{2+} (as $FeCl_2$), Hg^{2+} (as $HgCl_2$), K^+ (as KCl), Mn^{2+} (as $MnCl_2$), Ni^{2+} (as $NiCl_2$), Pb^{2+} (as $PbCl_2$) and Sr^{2+} (as $SrCl_2$). The chelating agent used in this study was ethylene diamine tetra-acetic acid (EDTA) sodium salt. The final concentration of the metal ion and chelating agent solutions was 5 mM following the study of Seo et al. (2013). The relative activity of CMCase was monitored using 2% CMC sodium salt as a substrate after being incubated with the metal ions and chelating agent at 50°C for one hour (Annamalai et al., 2013). There were six organic solvents used comprising acetone, dichloromethane, ethanol, ethyl-acetate, methanol and *n*-hexane with the final concentration of 25% of various organic solvents. The relative activity of CMCase was monitored using 2% CMC sodium salt as a substrate after being incubated with the organic solvents at 50°C for four hours (Annamalai et al., 2013).

Statistical analysis

The statistical analysis in this study was analyzed by one-way ANOVA followed by Tukey's test with a 95% confidence interval using R software version 3.6.2 (R Core Team, 2017).

Results and Discussion

Description of the freshwater wetland samples

Twenty water samples and 30 moist peat samples were randomly collected from the sampling site, Bueng Samnak Yai. The average temperature of the 50 sampling points was 28.5 ± 0.1°C. All samples were a dark brown color due to the large amount of organic matter and humic substances (Leff, 2009).

Isolation, purification and screening of bacteria from the freshwater wetland samples

Two-hundred and eight bacterial strains were isolated and purified from the freshwater wetland samples. There were 88 bacterial strains isolated from the water samples and 120 bacterial strains isolated from the moist peat samples with a dissimilar morphological colony. Most aquatic bacteria had a yellow pigmentation, circular shape, entire margin and convex elevation, whereas the moist peat bacteria usually had a white pigmentation, circular shape, entire margin and raised elevation. The percentage of the morphology of the isolated bacteria from the freshwater wetland samples is shown in *Table 1*.

Table 1. *Percentage of the morphology of the isolated bacteria from the freshwater wetland samples*

Bacteria	Pigmentation (Percentage)		Shape (Percentage)		Margin (Percentage)		Elevation (Percentage)	
Aquatic Bacteria (88 strains)	Violet	11.10	Circular	90.00	Entire	80.00	Convex	34.34
	White	37.38	Irregular	7.78	Erose	4.44	Flat	32.34
	Yellow	44.85	Punctiform	2.22	Lobate	5.56	Raised	30.00
	Translucent	6.67	Filamentous	-	Undulate	10.00	Umbonate	3.32
	Total	100.00	Total	100.00	Total	100.00	Total	100.00
Moist Peat Bacteria (120 strains)	Violet	4.17	Circular	95.83	Entire	97.50	Convex	35.00
	White	68.33	Irregular	2.50	Erose	-	Flat	23.33
	Yellow	19.17	Punctiform	1.67	Lobate	1.67	Raised	40.00
	Translucent	8.33	Filamentous	-	Undulate	0.83	Umbonate	1.67
	Total	100.00	Total	100.00	Total	100.00	Total	100.00

The screening of the cellulolytic bacteria using the CMC agar method showed 22 bacterial strains isolated from the water samples (25% of the isolated aquatic bacteria) and 54 bacterial strains isolated from the moist peat samples (45% of the isolated moist peat bacteria) that were defined as active cellulolytic bacteria. The hydrolysis capacity (HC) values of the aquatic bacteria ranged from 1.54 to 3.54, while that of the moist peat bacteria ranged from 1.57 to 3.55. The aquatic bacterium strain W0105 and moist peat bacterium strain S0804 showed a maximum HC of 3.54 ± 0.13 and 3.55 ± 0.33, respectively. The cellulolytic zone around the bacterial colonies on the CMC agar plates after Gram's iodine staining is shown in *Figure 2*. Chantarasiri et al. (2015) reported that there were 87 bacterial strains (60% of the isolated bacteria) isolated from the coastal wetland soils of Bueng Samnak Yai defined as cellulolytic bacteria. The bacterium with the highest HC values of that study was the *Bacillus* sp. strain BR0302 with a HC value of 4.15 ± 0.18. From the hydrolytic performance on the CMC agar plates, it was believed that the cellulolytic bacteria dwelling in the moist peat and related terrestrial samples were more abundant than bacteria dwelling in a water sample.
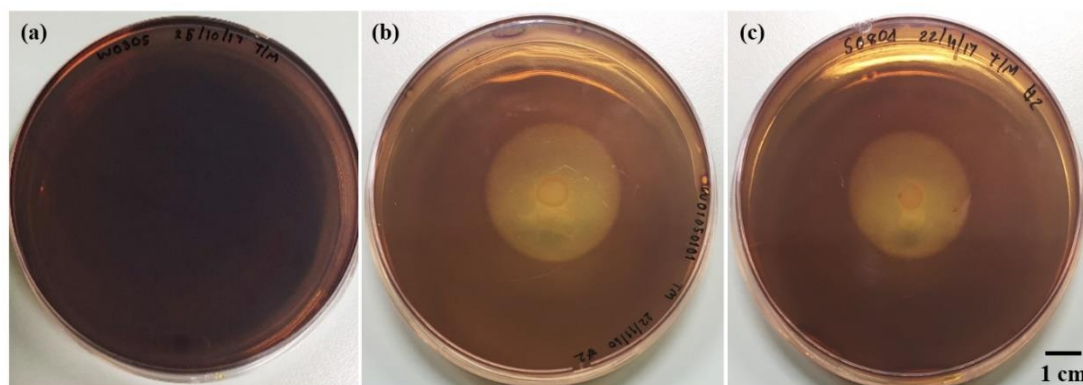

Figure 2. *The cellulolytic zone around the colonies on the CMC agar plates after Gram's iodine staining. (a) Non-cellulolytic bacterium. (b) Aquatic-cellulolytic bacterium strain W0105.(c) Moist peat-cellulolytic bacterium strain S0804*

PCR-RFLP analysis of the 16S rDNA fragments amplified from the cellulolytic bacteria

The 16S rRNA genes of the isolated cellulolytic bacteria were amplified using the PCR method with a set of primers consisting of the 27F-forward primer and 1492R-reverse primer. The 16S rDNA fragments resulting from that amplification were digested by the *Msp*I and *Alu*I restriction enzymes. The resulting RFLP profiles electrophorized on agarose gel are shown in *Figure 3*. There were seven different patterns in the RFLP profile obtained from the 22 strains of cellulolytic bacteria isolated from the water samples and 10 different patterns in the profile obtained from the 54 strains of cellulolytic bacteria isolated from the moist peat samples. The different patterns of the RFLP are summarized in *Tables 2 and 3*. Patterns W1 and W7 were the ones most commonly found in the cellulolytic bacteria isolated from the water samples by 27%. Pattern W6 showed a smear arrangement; however, there was only one bacterial strain (W1401) in this pattern (*Table 2*). Therefore, it did not affect the categorization of the pattern and identification of this bacterium. Pattern S4 was the one most commonly found in the cellulolytic bacteria isolated from the moist peat samples by 37%. All patterns from the moist peat bacteria were explicit arrangements and practicable for bacterial categorization. Interestingly, several patterns of the two RFLP profiles were similar; such as, the pair of patterns W1-S4 (*Figure 3*). They were possibly believed to be the same species of cellulolytic bacteria like a pair of patterns W2-S5 and a pair of patterns W5-S1 (*Figure 3*).
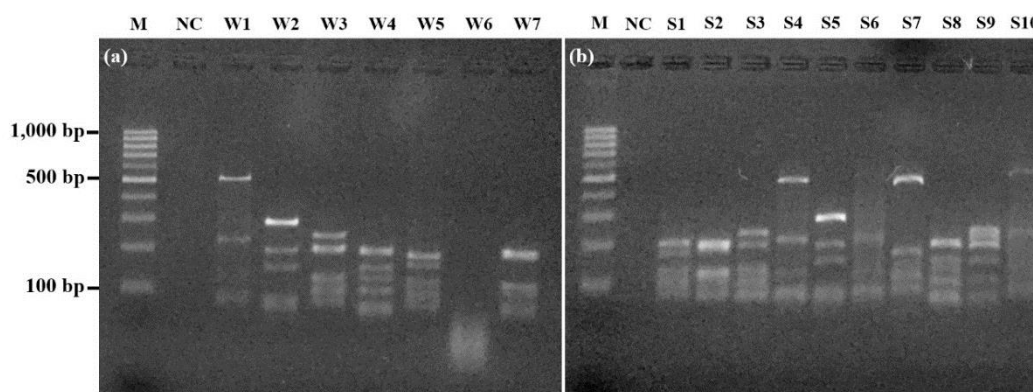

Figure 3. RFLP profiles resulting from the PCR-RFLP analysis of the isolated cellulolytic bacteria. (a) RFLP profile of the cellulolytic bacteria isolated from the water samples (pattern W1 to W7).(b) RFLP profile of the cellulolytic bacteria isolated from the moist peat samples (pattern S1 to S10). M denoted 100 bp DNA ladder RTU. NC denoted negative control of PCR

Table 2. Different RFLP patterns and number of the cellulolytic bacteria isolated from the water samples

RFLP Pattern	Bacterial Strain	Total No. of Bacterial Strains
W1	W0105, W0902, W1307, W1802, W1902, W2002	6
W2	W0203, W0301, W2003	3
W3	W0303	1
W4	W0306, W2004	2
W5	W0205, W1103, W1504	3
W6	W1401	1
W7	W0903, W1104, W1105, W1506, W1507, W1508	6
	Total	22

Table 3. *Different RFLP patterns and number of the cellulolytic bacteria isolated from the moist peat samples*

RFLP Pattern	Bacterial Strain	Total No. of Bacterial Strains
S1	S0104, S0303, S2802	3
S2	S0503	1
S3	S0701, S1202, S2604, S3003	4
S4	S0103, S0201, S0204, S0703, S0804, S0902, S0903, S1103, S1302, S1304, S1306, S1702, S2004, S2303, S2601, S2605, S2803, S2804, S2904, S3004	20
S5	S0402, S0501, S0601, S0702, S0904, S0905, S0906, S0907, S0908, S1601, S1701, S2001, S2902, S2903, S2905, S2906, S2907, S3006	18
S6	S1305, S1401, S1404	3
S7	S1402	1
S8	S1602	1
S9	S2606	1
S10	S3007, S3008	2
	Total	54

Identification of the cellulolytic bacteria by 16S rDNA sequencing and phylogenetic analysis

The genomic DNA was extracted from 17 different bacterial strains based on the RFLP profiles as mentioned above. The 16S rDNA amplification was performed by the universal primers, 27F and 1492R. The 16S rDNA PCR-products were purified, sequenced and aligned. The alignment results of the cellulolytic bacteria belonged to nine genera: *Acinetobacter*, *Aeromonas*, *Bacillus*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Herbaspirillum*, *Paenibacillus* and *Vibrio* (*Table 4*). The cellulolytic bacteria isolated from the water samples were closely similar to the bacteria in the genera of *Bacillus*, *Chromobacterium* and *Enterobacter* with a 94 - 98% identity. The cellulolytic bacteria isolated from the moist peat samples were closely similar to bacteria in the genera of *Acinetobacter*, *Aeromonas*, *Bacillus*, *Chromobacterium*, *Citrobacter*, *Herbaspirillum*, *Paenibacillus* and *Vibrio* with a 93 - 98% identity. The moist peat samples had a greater biodiversity of cellulolytic bacteria than the aquatic environment in the freshwater wetland. This could be related to their amount of organic matter and related carbon sources, which would be essential for bacterial life. The phylogenetic tree of the isolated bacteria with 100,000 bootstrap replications is shown in *Figure 4*.

The cellulolytic bacteria were designated as being closely related based on the alignment results of the 16S rDNA sequence when the identity was more than 98%; such as, *B. wiedmannii* strain W1401. The ones which were lower than a 98% identity were presented at the genus level; such as, *Bacillus* sp. strain W0105 and *Bacillus* sp. strain S0804 (the cellulolytic bacteria with the maximum HC values as previously mentioned). The alignment and phylogenetic tree results confirmed the hypothesized identification of the same RFLP patterns as mentioned above comprising patterns W1-S4, W2-S5 and W5-S1 (*Table 4*).

All the 16S rDNA sequences from this study were deposited in the GenBank database of the NCBI under the accession numbers MN993647, MN993849, MN993893, MN993916, MN994046, MN994069, MN994075, MN994076, MN994079 to MN994082, MN994084 and MN994270 to MN994273, as mentioned above in the Materials and Methods section.

Table 4. *Identity percentage of the 16S rDNA sequences for the isolated cellulolytic bacteria*

RFLP Pattern	Closely Related Bacteria	GenBank Accession No. (Database)	Identity (%)*	GenBank Accession No. (Deposited)
W1	*Bacillus cereus* strain ATCC 14579	NR_074540.1	94.49	MN993849
W2	*Bacillus megaterium* strain ATCC 14581	NR_117473.1	97.98	MN993647
W3	*Chromobacterium piscinae* strain LMG 3947	NR_114953.1	97.20	MN993916
W4	*Enterobacter asburiae* strain JM-458	NR_145647.1	96.74	MN994046
W5	*Chromobacterium violaceum* strain ATCC 12472	NR_074222.1	97.55	MN993893
W6	*Bacillus wiedmannii* strain FSL W8-0169	NR_152692.1	98.83	MN994075
W7	*Chromobacterium amazonense* strain CBMAI 310	NR_136426.1	96.80	MN994069
S1	*Chromobacterium violaceum* strain NBRC 12614	NR_113595.1	98.25	MN994079
S2	*Chromobacterium vaccinia* strain MWU205	NR_109451.1	95.85	MN994081
S3	*Herbaspirillum frisingense* strain NBRC 102522	NR_114140.1	98.61	MN994082
S4	*Bacillus cereus* strain ATCC 14579	NR_074540.1	95.27	MN994076
S5	*Bacillus megaterium* strain ATCC 14581	NR_117473.1	98.54	MN994080
S6	*Aeromonas veronii* strain JCM 7375	NR_112838.1	93.09	MN994084
S7	*Paenibacillus chibensis* strain JCM 9905	NR_040885.1	97.24	MN994270
S8	*Citrobacter koseri* strain CDC-8132-86	NR_104890.1	97.43	MN994271
S9	*Acinetobacter calcoaceticus* strain NCCB 22016	NR_042387.1	97.76	MN994272
S10	*Vibrio fluvialis* strain NBRC 103150	NR_114218.1	95.51	MN994273

Remark: * The identity results were analyzed on January 28, 2020

The predominant bacterial genera of the isolated cellulolytic bacteria in this study were *Bacillus* of the Firmicutes by 63%, *Chromobacterium* of the Proteobacteria by 14%, and *Herbaspirillum* of the Proteobacteria by 5%. The previous study reported that the bacterial diversity in wetland soils showed predominant bacterial phyla belonging to Proteobacteria, Bacteroidetes, Acidobacteria, Firmicutes and Actinobacteria (Lv et al., 2014). *Bacillus* is a genus of ubiquitous bacteria frequently isolated from various environments including air, dust, soil, and water. The cellulolytic *Bacillus* in this study was isolated from both the water and moist peat samples. It was closely related to *B. cereus*, *B. megaterium* and *B. wiedmannii*. Previous reports showed that many *Bacillus* species were effective cellulolytic bacteria; such as, *B. cereus*, *B. circulans*, *B. licheniformis*, *B. megaterium*, *B. methylotrophicus* and *B. subtilis* (Chantarasiri, 2014, 2015; Azadian et al., 2016; Shahid et al., 2016). Importantly, this study has now confirmed that the *B. wiedmannii* was cellulolytic bacteria. *B. wiedmannii* was firstly isolated from a raw milk sample, named, and described in 2016 (Miller et al., 2016). It

was recently defined as a rice root-associated bacterium and found its cellulase gene by molecular detection in 2020 (Khaskheli et al., 2020). *Chromobacterium* is a genus of saprophytic bacteria, which are generally isolated from soil and freshwater (Soby et al., 2013). The cellulolytic *Chromobacterium* was found in both samples similar to the *Bacillus* species. It was closely related to *C. amazonense*, *C. piscinae*, *C. vaccinia* and *C. violaceum*. *Chromobacterium* species have been previously reported as cellulolytic bacteria (Vazquez-Arista et al., 1997; Sudiana et al., 2001). *Herbaspirillum* is a genus of nitrogen-fixing bacteria associated with the roots of many grasses including rice, maize and sorghum (Kirchhof et al., 2001). *H. frisingense* was the only species of *Herbaspirillum* isolated from the moist peat samples. It has been defined as a cellulolytic species described in a previous study (Fujii et al., 2012). The other isolated genera belonging to *Acinetobacter*, *Aeromonas*, *Citrobacter*, *Enterobacter*, *Paenibacillus* and *Vibrio* had been previously reported as cellulolytic bacteria (Gao et al., 2012; Poomai et al., 2014; Pawar et al., 2015; Islam and Roy, 2018; Waghmare et al., 2018).
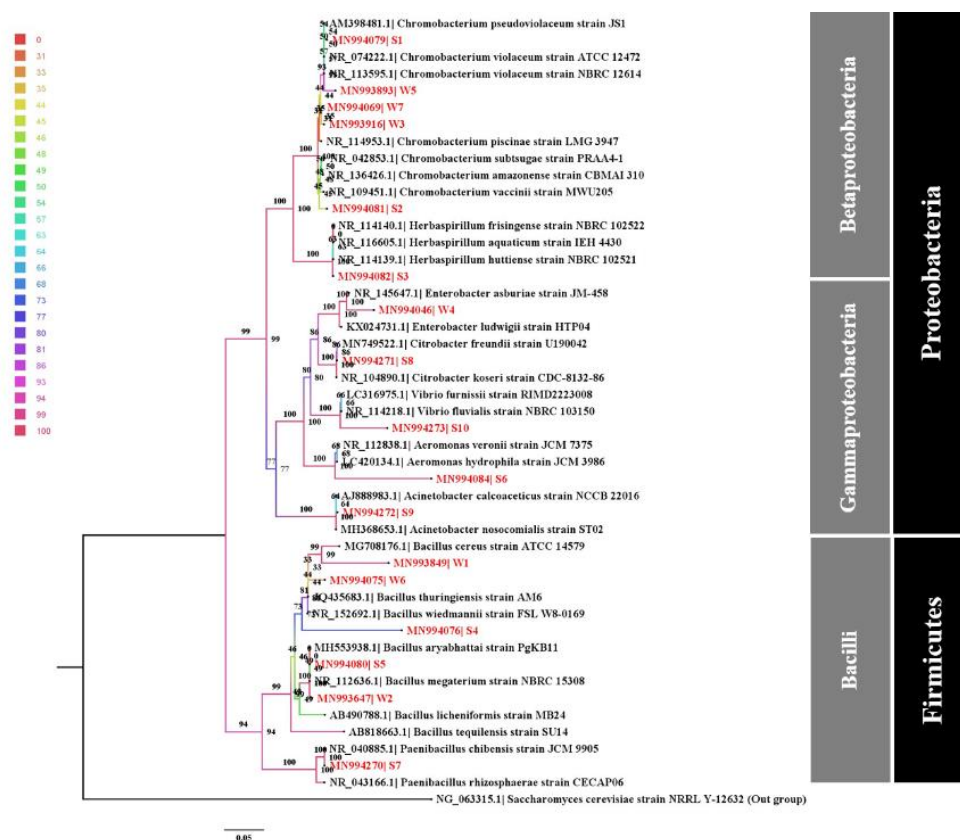

Figure 4. *Phylogenetic tree of the 16S rDNA sequences of the cellulolytic bacteria. The neighbor-joining (NJ) method with 100,000 bootstrap replications was used in the infer tree topology. The phylogenetic tree was generated and visualized by the SeaView program version 4.6.4 and FigTree program version 1.4.3. The colour-coding represents bootstrap values*

Cellulolytic activity assays of the crude cellulases

The representative bacterium from each RFLP pattern was examined for the cellulolytic activity assays consisting of endoglucanase (CMCase), exoglucanase (Avicelase) and β-glucosidase activities. The assays showed that they could yield crude

cellulases from 0.57 to 4.48 U/mL of the CMCase activity, 0.06 to 0.44 U/mL of the Avicelase activity and 0.01 to 0.19 U/mL of the β-glucosidase activity (*Table 5*). All representative bacteria satisfactorily produced CMCases; however, they barely produced any Avicelases and β-glucosidases. It could be stated that endoglucanases were the mainly produced enzyme in their cellulolytic system.

Table 5. *Cellulolytic performances of the representative bacteria*

RFLP Pattern	Bacterial Representative	CMCase Activity (U/mL) with a pH 7.0	Avicelase Activity (U/mL) with a pH 7.0	β-Glucosidase Activity (U/mL) with a pH 7.0
W1	*Bacillus* sp. strain W0105	3.68 ± 0.54^{ef}	0.13 ± 0.03^{bc}	0.06 ± 0.01^{bc}
W2	*Bacillus* sp. strain W0301	3.84 ± 0.27^{fg}	0.44 ± 0.04^{d}	0.06 ± 0.01^{bc}
W3	*Chromobacterium* sp. strain W0303	0.57 ± 0.02^{a}	0.07 ± 0.01^{ab}	0.02 ± 0.00^{a}
W4	*Enterobacter* sp. strain W0306	3.91 ± 0.08^{fg}	0.08 ± 0.03^{ab}	0.06 ± 0.01^{bc}
W5	*Chromobacterium* sp. strain W1103	3.65 ± 0.17^{ef}	0.10 ± 0.03^{ac}	0.06 ± 0.00^{bc}
W6	*B. wiedmannii* strain W1401	3.87 ± 0.32^{fg}	0.08 ± 0.01^{ab}	0.03 ± 0.01^{ab}
W7	*Chromobacterium* sp. strain W0903	1.19 ± 0.24^{ab}	0.06 ± 0.00^{a}	0.02 ± 0.00^{a}
S1	*C. violaceum* strain S2802	2.15 ± 0.17^{cd}	0.15 ± 0.03^{c}	0.06 ± 0.01^{bc}
S2	*Chromobacterium* sp. strain S0503	1.01 ± 0.10^{a}	0.07 ± 0.01^{ab}	0.02 ± 0.00^{a}
S3	*H. frisingense* strain S0701	0.90 ± 0.07^{a}	0.08 ± 0.01^{ab}	0.02 ± 0.00^{a}
S4	*Bacillus* sp. strain S0804	3.17 ± 0.07^{e}	0.12 ± 0.00^{ac}	0.06 ± 0.01^{bc}
S5	*B. megaterium* strain S0702	4.48 ± 0.08^{g}	0.07 ± 0.00^{ab}	0.08 ± 0.02^{c}
S6	*Aeromonas* sp. strain S1401	2.21 ± 0.18^{cd}	0.08 ± 0.03^{ab}	0.06 ± 0.01^{bc}
S7	*Paenibacillus* sp. strain S1402	1.73 ± 0.11^{bc}	0.08 ± 0.03^{ab}	0.07 ± 0.01^{c}
S8	*Citrobacter* sp. strain S1602	2.40 ± 0.29^{d}	0.10 ± 0.03^{ac}	0.08 ± 0.02^{c}
S9	*Acinetobacter* sp. strain S2606	1.08 ± 0.17^{a}	0.07 ± 0.01^{ab}	0.01 ± 0.00^{a}
S10	*Vibrio* sp. strain S3007	3.94 ± 0.07^{fg}	0.07 ± 0.00^{a}	0.19 ± 0.02^{d}

Remark: The mean values in the same row followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the representative bacteria

B. megaterium strain S0702 was the significant endoglucanasic bacteria at 4.48 ± 0.08 U/mL ($p < 0.01$). It was considered as a bacterial model for the following experiments of this study because it was generally considered to be a non-pathogenic and well-known bacterium. Chantarasiri (2015) reported that *B. cereus* strain JD0404 isolated from mangrove swamp soils was an active endoglucanasic bacterium and primary degraded CMC with its endoglucanase activity. Interestingly, *B. megaterium* strain S0702

was not the most active cellulolytic bacterium based on the HC value determination on the CMC agar. It exhibited only 2.27 ± 0.17, which was less than that of *Bacillus* sp. strains W0105 and S0804. This conflicting result may be due to the fluctuations in some experimental parameters which affected the cellulase producing processes described in several previous reports (Ahmad et al., 2013; Chantarasiri et al., 2015). The cellulolytic activity of *B. megaterium* strain S0702 was compared to other bacteria in the *Bacillus* genus isolated from the wetland ecosystems (*Table 6*). *Bacillus* sp. strain W0301 significantly produced the high activity of Avicelase by 0.44 ± 0.04 U/mL ($p < 0.01$); however, it could not indicate being an effective cellulolytic bacterium due to its medium CMCase and low β-glucosidase performances. This enzymatic performance was in agreement with the lack of the complete cellulolytic system of the *Bacillus* genus (Kim et al., 2012). Moreover, *Bacillus* sp. strain W0301 was closely related to *B. cereus* that was generally considered as a pathogenic bacterium. *Vibrio* sp. strain S3007 showed quite a high activity of CMCase and significant β-glucosidase activity of 0.19 ± 0.02 U/mL ($p < 0.01$). The *Vibrio* species could produce β-glucosidase for utilizing the glucans and related compounds (Wang et al., 2015). However, it was not appropriate for further experiments and industrial applications, as it was believed to be a human-pathogenic bacterium.

Table 6. *Cellulolytic performance of B. megaterium strain S0702 and the related bacteria in the Bacillus genus isolated from the wetlands and related environments*

Bacteria	Source of Isolation	CMCase Activity (U/mL)	Avicelase Activity (U/mL)	β-Glucosidase Activity (U/mL)	References
Bacillus sp. strain BR0302	Coastal wetland	0.12	ND	ND	Chantarasiri et al. (2015)
B. cereus strain JD0404	Mangrove swamp	1.78	0.08	0.05	Chantarasiri (2015)
B. licheniformis strain CDB-12	Mangrove in a river delta	98.25*	ND	ND	Behera et al. (2016)
B. subtilis strain A-53	Seawater	92*	ND	ND	Kim et al. (2009)
B. megaterium strain S0702	Freshwater wetland	4.48	0.07	0.08	This current study.

Remark: * = purified cellulases. ND = not determined

Characterization of the cellulolytic activity from B. megaterium strain S0702

Crude cellulases from *B. megaterium* strain S0702 were characterized for the CMCase performance in different experimental conditions; such as, temperature, pH and chemical additives. The optimum temperature and pH for the CMCase activity of the crude cellulases were 45 - 50°C ($p < 0.01$) with a pH 7.0 ($p < 0.01$) (*Figures 5a and 6a*). The enzyme remained stable at up to 60°C ($p < 0.01$) and a pH range of 5.0 - 8.0 ($p < 0.01$) for 24 hours (*Figures 5b and 6b*). The different buffers with the same pH were not significantly affected by the CMCase activity (*Figure 6b*). The cellulases produced from other *B. megaterium* were studied and evaluated for their CMCase activity. It was found that *B. megaterium* strain BM05 had the optimum pH and temperature of 6.5 and 50°C with a stability range of 6.0 - 8.0 and 30 - 40°C (Shahid et al., 2016). The CMCase from *B. megaterium* strain CB-sw1-I was optimally active with a pH 6.0 and temperature of

60°C (Shobharani et al., 2013). The CMCases from many *Bacillus* species were active at a temperature range of 50 - 60°C and a pH range of 4.8 - 11.0 (Sadhu and Maiti, 2013; Chantarasiri, 2015). This *B. megaterium* CMCase was preferred for various industrial applications; such as, bioethanol industries and agricultural industries because the enzyme could be active and hydrolyze the cellulose-based materials under mild conditions with a neutral pH and meso-temperature.
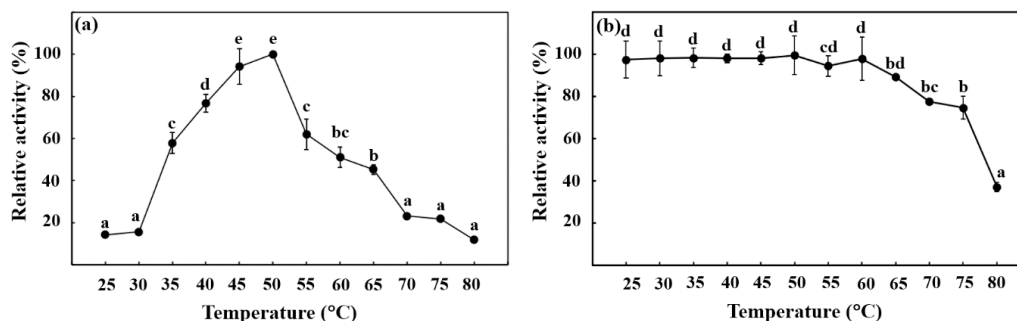

Figure 5. Effect of temperature on the CMCase activity (a) and stability (b) from B. megaterium strain S0702. Error bars represent the standard deviation of the three replicates. The mean values followed by the same letter were not significantly different according to Tukey's test (p < 0.05) among the CMCase activity
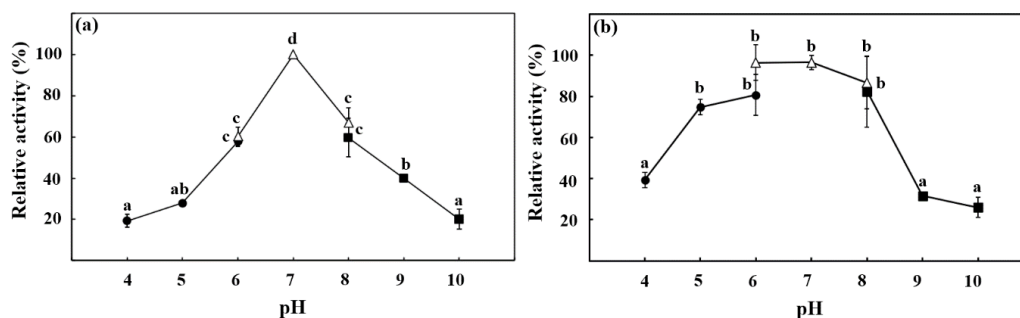

Figure 6. Effect of pH on the CMCase activity (a) and stability (b) from B. megaterium strain S0702. The CMCase activity was measured in a citrate buffer (●), sodium phosphate buffer (△) and glycine-NaOH buffer (■). Error bars represent the standard deviation of the three replicates. The mean values followed by the same letter were not significantly different according to Tukey's test (p < 0.05) among the CMCase activity

The effect of various chemical additives is shown in *Table 7*. The results of the metal ions revealed that the CMCase activity of *B. megaterium* strain S0702 was significantly enhanced by Mn^{2+}, Ca^{2+}, Co^{2+} and Sr^{2+} ($p < 0.01$). Similarly, many previous reports showed that these metal ions could activate the CMCase activity of *Bacillus* cellulases (Shobharani et al., 2013; Chantarasiri, 2015; Shahid et al., 2016). It was believed that they could possibly respond to certain amino acid residues in the active site and promote the favorable conformation of the enzyme to the substrate binding and enzyme activity (Azzeddine et al., 2013; Shahid et al., 2016). Mn^{2+} could be promised as the great activator of the CMCase in further biotechnological applications due to being less toxic than those of other metal ions. Many Mn^{2+} compounds were known; such as, manganese

sulfate ($MnSO_4$) and manganese chloride ($MnCl_2$). Most metal ions and organic solvents could inhibit the CMCase activity of *B. megaterium* strain S0702. The activity was significantly inhibited by EDTA and ethyl-acetate. The reduction of the cellulolytic performance by a chelating agent EDTA revealed that the CMCase from *B. megaterium* strain S0702 could be identified as a metalloenzyme (Annamalai et al., 2013). The results indicated that this CMCase was not remarkably appropriate for any organic solvent related applications.

Table 7. Effect of the various chemical additives on the CMCase activity from B. megaterium strain S0702

Chemical Additives	Relative Activity (%)
Ca^{2+}	241.25 ± 7.43[j]
Co^{2+}	221.01 ± 0.78[i]
Cu^{2+}	77.51 ± 7.79[ef]
Fe^{2+}	68.06 ± 5.45[e]
Hg^{2+}	64.91 ± 6.18[de]
K^+	50.52 ± 7.79[cd]
Mn^{2+}	625.87 ± 0.78[k]
Ni^{2+}	74.81 ± 2.06[ef]
Pb^{2+}	63.56 ± 1.35[de]
Sr^{2+}	199.42 ± 7.43[h]
EDTA	23.98 ± 4.34[ab]
Acetone	38.82 ± 4.43[bc]
Dichloromethane	68.51 ± 7.79[e]
Ethanol	95.05 ± 2.06[g]
Ethyl-acetate	22.63 ± 3.12[a]
Methanol	63.56 ± 4.05[de]
n-Hexane	88.30 ± 0.78[fg]

Remark: The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the CMCase activity

Conclusion

The freshwater wetland ecosystem is a potential source for the isolation of cellulolytic bacteria. There were nine genera of cellulolytic bacteria isolated from Bueng Samnak Yai, a freshwater wetland in Thailand based on the RFLP-PCR of a 16S rDNA and nucleotide sequencing analysis comprising *Acinetobacter*, *Aeromonas*, *Bacillus*, *Chromobacterium*, *Citrobacter*, *Enterobacter*, *Herbaspirillum*, *Paenibacillus* and *Vibrio*. The cellulolytic performance of the representative bacteria from each RFLP pattern was determined. It revealed that *B. megaterium* strain S0702 was the most active CMCase bacterium. Its CMCase was characterized and found that it could possibly be used in various biotechnological applications. This CMCase was not remarkably appropriate for some metal ions and organic solvent related applications. Finally, further study on enzyme purification, enzyme kinetic and applications of CMCase are suggested.

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