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Diversity and cellulolytic activity of cellulase-producing bacteria isolated from the soils of two mangrove forests in Eastern Thailand

TANAPAT BAMRUNGPANICHTAVORN[1,2], SUNISA UNGWIWATKUL[1], PARIMA BOONTANOM[1], AIYA CHANTARASIRI[1,♥]

[1]Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok. Rayong Campus, 19 Moo 11 Nong Lalok, Ban Khai 21120, Rayong, Thailand. Tel./fax.: +66-38-627000 ext. 5446, ♥email: aiya.c@sciee.kmutnb.ac.th
[2]Chanthaburi College of Dramatic Arts. Wat Mai, Mueang Chanthaburi 22000, Chanthaburi, Thailand

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Abstract. *Bamrungpanichtavorn T, Ungwiwatkul S, Boontanom P, Chantarasiri A. 2023. Diversity and cellulolytic activity of cellulase-producing bacteria isolated from the soils of two mangrove forests in Eastern Thailand. Biodiversitas 24: 3891-3902.* The Southeast Asian countries hold the largest proportion of the world's mangrove area. Mangrove forests are a potential source for the isolation of economic microbial enzymes. Cellulases are a widely used microbial enzyme for cellulose degradation in various industries. Therefore, this study aimed to isolate, genetically identify, and enzymatically characterize cellulase-producing bacteria from the soils of two mangrove forests in Eastern Thailand. Twenty-six cellulase-producing bacteria were isolated and subsequently genotyped by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the 16S rRNA genes. Thirteen different RFLP patterns were obtained and genetically analyzed into six bacterial genera comprising *Aeromonas*, *Bacillus*, *Chryseobacterium*, *Lysinibacillus*, *Pseudomonas*, and *Vibrio*. The *Bacillus* species were the predominant cellulase-producing bacteria in the study sites. Moreover, the cellulase-producing *Chryseobacterium* and *Lysinibacillus* had hardly ever been reported. The *Bacillus* sp. strain RY08B was the most active cellulase-producing bacterium with 1.510±0.060 U/mL of CMCase activity. The optimum temperature and pH for the CMCase activity were determined to be 50°C at a pH of 7.0 with a thermal stability range of 25-50°C at a pH of 7.0. This bacterium could be applied in several environmentally friendly industries requiring mild conditions for their production processes.

Keywords: Cellulase, CMCase, mangrove soil, PCR-RFLP, Thailand

INTRODUCTION

Mangrove forests are coastal intertidal wetlands on tropical and subtropical coasts composed of halophytic trees and shrubs (Friess 2016; Friess et al. 2019). Mangrove forests are also significantly important coastal resources, which are vital to ecological importance and socio-economic development (Kathiresan 2012; Biswas et al. 2020). They provide important ecosystem services such as coastal protection, fish, fuelwood, timber, pollution control, and cultural values (Friess et al. 2019). Moreover, mangrove forests are a potential source of effective bacteria that produce active metabolites and industrial enzymes (Pramono et al. 2021). Many valuable industrial bacteria have been isolated from the mangrove environments in the past decade, identified, and characterized (Saravanakumar et al. 2016). Some mangrove-associated bacteria possess valuable enzymes such as alkaline proteases, asparaginases, proteases, amylases, lipases, chitinases, carrageenases, and agarases (Saravanakumar et al. 2016). The cellulolytic bacteria naturally dwell in mangrove soils and produce cellulase enzymes (Thatoi et al. 2013; Thompson et al. 2013; Chantarasiri 2015; Kurniawan et al. 2019; Nursyirwani et al. 2020; Pramono et al. 2021). These bacteria mainly contribute to the decomposition of cellulose-based materials in the mangrove soils, such as mangrove litter leaves and rotting wood (Dewiyanti et al. 2022a). The mangrove cellulase-producing bacteria recently reported consist of *Bacillus altitudinis*, *B. cereus*, *B. safensis*, *B. toyonensis*, *Citrobacter freundii*, *Fictibacillus nanhaiensis*, *Priestia megaterium* (formerly known as *Bacillus megaterium*), and *Vibrio alginolyticus* (Chantarasiri 2015; Kurniawan et al. 2019; Nursyirwani et al. 2020; Pramono et al., 2021; Dewiyanti et al. 2022b). One study reported that the most significant proportion of mangrove forests is in the Southeast Asian region (Friess 2016). However, detailed studies would still be required to assess the diversity of the cellulase-producing bacteria from various mangrove ecosystems in this region.

Cellulases are synergistic enzymes, which comprise endoglucanase (endo-1,4-β-D-glucanase, EG, and EC 3.2.1.4), cellobiohydrolase or exoglucanase (exo-1,4-β-D-glucanase, CBH, and EC 3.2.1.91), and β-glucosidase (1,4-β-D-glucosidase, BG, and EC 3.2.1.21) (Imran et al. 2016; Narkthewan and Makkapan 2019; Chantarasiri 2020). These cellulolytic bacteria can hydrolyze cellulose complexes into simpler sugars, such as oligosaccharides and glucose molecules (Batubara et al. 2021). Cellulases account for a significant share of the global industrial enzyme market nowadays (Zhang and Zhang 2013). Cellulases also have biotechnological potential in various industries, including food, animal feed, brewery and winery, textile and laundry, pulp and paper, agriculture, biofuel, and waste management (Behera et al. 2017).

However, the major limitation of using cellulases in the industry is the high cost of producing the enzymes (Sulyman et al. 2020), even though numerous enzymes obtained from microorganisms are more virulent and stable than those derived from animals and plants (Saravanakumar et al. 2016). It has been reported that the cellulases from cellulolytic bacteria can effectively hydrolyze different cellulosic substrates (Sharada et al. 2013). The cellulolytic bacteria have been isolated from various sources such as compost, rumen ruminants, and soil (Dewiyanti et al. 2022a). Nevertheless, the isolation of cellulolytic bacteria and the characterization of the cellulase components have been provided for more than 50 years (Kuhad et al. 2016). In the last two years, only nine novel bacteria were isolated from various environments and officially described as cellulase-producing bacteria (Menendez et al. 2015).

Therefore, to improve knowledge about the diversity of cellulase-producing bacteria isolated from mangrove soils in the Southeast Asian region, more studies should be conducted. This study aimed to isolate and screen cellulase-producing bacteria from the mangrove soils in Rayong and Chanthaburi Provinces, Thailand. Molecular genetic and phylogenetic analyses of the 16S rRNA genes were used to identify the isolated cellulase-producing bacteria. The most active cellulase-producing bacterium in this study was determined for the endoglucanase (CMCase) activity. The purpose was to review the diversity of the cellulase-producing bacteria from the mangrove ecosystems and determine the potential of the active bacteria for possible use in biotechnological applications.

MATERIALS AND METHODS

Study areas

The study areas were the Phra Chedi Klang Nam Mangrove Forest in Rayong Province (12°39'55.5"N, 101° 14' 50.1" E) and the Kung Krabaen Bay Mangrove Forest in Chanthaburi Province (12°34'21.6" N, 101°53'57.6" E). These provinces are located on the eastern coast of Thailand. The Phra Chedi Klang Nam Mangrove Forest is an estuarine system of the Rayong River, which connects to the Gulf of Thailand. In a previous study by Chantarasiri (2015), the same area reported the isolation, identification, and characterization of an effective cellulase-producing *Bacillus* bacterium isolated from mangrove sediment samples. The Kung Krabaen Bay Mangrove Forest is a small semi-enclosed estuarine system connected to the Gulf of Thailand by a channel in the southeastern corner of the bay (Kunsook et al. 2014). The location of the study areas is shown in Figure 1.

Procedures

Sampling of the mangrove soils

The mangrove soil samples were collected from the study areas during September and October 2016. Fifteen samples were randomly collected from each sampling site. Soil samples beneath the composted plant litter at 0-15 cm depth were sampled to obtain aerobic cellulase-producing bacteria preferentially (Chantarasiri 2015).

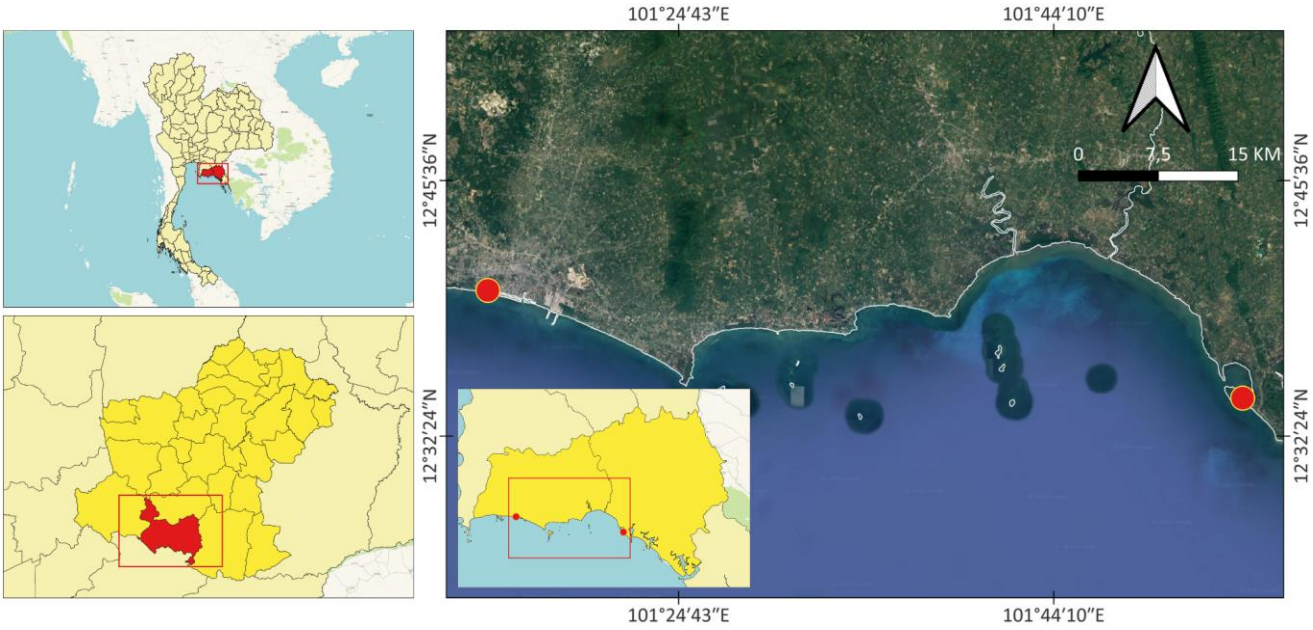

Figure 1. Location of the Phra Chedi Klang Nam Mangrove Forest in Rayong Province and the Kung Krabaen Bay Mangrove Forest in Chanthaburi Province, Thailand

The soil temperatures and pH values were immediately measured at the sampling sites. The temperatures were determined by a needle probe thermometer (Extech Instruments, USA), and the pH values were determined from 1 g of samples suspended in 10 mL of distilled water by a ST20 portable pH meter (Ohaus, USA). All the soil samples were kept in sterilized plastic bags at a temperature of 4°C and taken to the laboratory for further experiments within 24 h of collection.

Isolation and screening of the cellulase-producing bacteria from the mangrove soil samples

One gram of each soil sample was suspended in 10 mL of distilled water and serially 10-fold diluted to obtain 1:100,000 dilutions. Next, 100 µL of the diluted sample was spread-plated on tryptone soya agar (HiMedia, India). The pH value of the tryptone soya agar was adjusted to 6.18 (the average pH of the collected samples). All agar plates were incubated in a KB720 incubator (Binder, Germany) at 28°C (the average soil temperature) for 24 h. The bacterial isolates were selected based on different colony morphology and then colony purified by streak plated on tryptone soya agar. Each pure isolate was named with the code of RY for the isolated bacteria from the Phra Chedi Klang Nam Mangrove Forest in Rayong Province and the code of CH for the isolated bacteria from the Kung Krabaen Bay Mangrove Forest in Chanthaburi Province.

Cellulase-producing bacteria were screened from the previous methodology of Chantarasiri (2015) using carboxymethylcellulose (CMC) agar and an iodine solution staining method. The bacterial isolates were cultured in 1 mL of tryptone soya broth (HiMedia, India) at 28°C for 24 h. Next, 5 µL of each bacterial culture was spot plated on CMC agar to screen the cellulase-producing bacteria. As aforementioned, the pH value of the tryptone soya broth and CMC agar were adjusted to 6.18. All screening plates were incubated in a KB720 incubator at 28°C for 48 h. The incubated plates were flooded with iodine solution for 10 min. The cellulase-producing candidates could produce the cellulolytic zone around their colonies after being flooded by the iodine solution. The cellulolytic performance of the bacterial candidates was evaluated by the Hydrolysis Capacity (HC) value, which was calculated by measuring the ratio of the cellulolytic zone diameter with the bacterial colony diameter (Hidayat 2021). All experiments were performed in triplicate.

The cellulase-producing ability of the isolated bacteria was confirmed by inoculating each bacterium in a basal salt medium containing Whatman filter paper No. 1 strips (Whatman, USA), which was conducted from the methodology of Gupta et al. (2012). These cultures were incubated in a KB720 incubator at 28°C for 28 days. The number of the "+" symbol represented the level of the filter paper degradation observed on the 28th day of incubation. All experiments were performed in triplicate.

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of the 16S rRNA genes

The procedures of the PCR-RFLP analysis of the 16S rRNA genes were conducted by Chantarasiri (2020). The genomic DNA of the isolated cellulase-producing bacteria was extracted and purified by a genomic DNA isolation kit (Bio-Helix, Taiwan) according to the standard protocol described by the company. The extracted genomic DNA was used as the DNA template for the PCR amplification of the 16S rRNA genes. The amplification was conducted using the OnePCR reaction mixture (Bio-Helix, Taiwan) with universal 27F and 1492R primers. The PCR reaction mixtures were amplified in an Eppendorf Mastercycler nexus gradient (Eppendorf, Germany) for 35 amplification cycles. In brief, the amplification conditions involved an initialization at 94°C for 4 min, a denaturation at 94°C for 40 s, an annealing at 55°C for 1 min, an extension at 72°C for 1 min for 10 s, and a final extension at 72°C for 10 min.

The RFLP of the PCR products was performed by the double digestion reaction of *Msp*I and *Alu*I restriction enzymes (New England Biolabs, UK) in a CutSmart buffer (New England Biolabs, UK) following the previous protocol described by Chantarasiri (2020). The reaction mixture was incubated for complete digestion at 37°C for 12 h then the reaction was terminated by heat at 80°C for 15 min. The resulting DNA fragments were electrophoresed on 3% (w/v) of OmniPur agarose gel (Calbiochem, Germany) and visualized by Novel Juice staining (Bio-Helix, Taiwan). The PCR marker used in this experiment was a OneMark 100 RTU DNA ladder (Bio-Helix, Taiwan).

Nucleotide sequencing and phylogenetic analysis of the amplified 16S rRNA genes

As aforementioned, the 16S rRNA genes of each cellulase-producing bacterium were amplified by the PCR conditions with universal 27F and 1492R primers. The PCR products were purified by a PCR clean-up and gel extraction kit (PureDireX, Taiwan) according to the standard protocol described by the company. The purified PCR products were nucleotide sequenced for the 16S rRNA genes by Macrogen Inc. (South Korea) services.

The nucleotide sequences of the 16S rRNA genes were aligned for the identity percentage by the BlastN program based on the nucleotide collection (nr/nt) database and a megablast algorithm from the National Center for Biotechnology Information (NCBI). The phylogenetic tree of the isolated cellulase-producing bacteria was analyzed by SeaView software version 5.0.1 (Gouy 2010) and FigTree software version 1.4.4 with the BIONJ algorithm for 100,000 bootstrap replications. All the resulting nucleotide sequences of the cellulase-producing bacteria were deposited in the GenBank database of the NCBI under the accession numbers OR001963 to OR001968, OR001970, OR001971 to OR001975, and OR004586.

Preparation of the crude cellulases from the isolated cellulase-producing bacteria

The crude cellulases were prepared from the five most active cellulase-producing bacteria. The *Chryseobacterium* sp. strain RY03D, and *Vibrio* sp. strain CH06E were preferred as the active bacterial strains based on the HC values. The *Bacillus* sp. strain RY01D, *Bacillus* sp. strain RY08B, and *B. tequilensis* strain CH02H were preferred as the active bacterial strains based on the performance of the

filter paper degradation. All the cellulase-producing bacteria were cultured to prepare the crude cellulases in a CMC broth (Chantarasiri 2015) at 28℃ for 48 h. The pH value of the broth was adjusted to 6.18. All the bacterial cultures were agitated in an LSI-3016R shaking incubator at 150 rpm (Daihan Labtech, South Korea). The crude cellulases were harvested from each culture as the cell-free supernatant by Digicen 21 R centrifugation (Ortoalresa, Spain) at 4,500 ×g for 30 min. The crude enzymes were stored at 4℃ until used.

Cellulase activity assays of the crude cellulases

The bacteria could degrade the cellulose biomass due to their characteristics of producing endoglucanase cellulase or CMCase (Wang et al. 2022). Therefore, several studies had investigated the CMCase activity of aerobic bacteria (Liang et al. 2014). The activity assays were conducted from a previously described study (Chantarasiri 2015). Next, 0.5 mL of crude cellulases were incubated with 0.5 mL of 2% (w/v) of CMC powder in 50 mM of sodium phosphate buffer (pH 7.0) at 50°C for 30 minutes. The reducing sugars released from the CMCase reaction were spectrophotometrically determined by a 3,5-dinitrosalicylic acid (DNS) method at 540 nm (Miller 1959). A standard glucose curve determined the cellulolytic activity value of the CMCase. One unit (U) of the CMCase was defined as the amount of enzyme required to release 1 μmol of the reducing sugars as the glucose equivalents per minute under the assay conditions. All experiments were performed in triplicate.

Enzymatic characterization of the crude cellulases from the most active cellulase-producing bacterium

The study of the enzymatic characterization was determined on the crude cellulases produced from the most active CMCase-producing bacterium; *Bacillus* sp. strain RY08B. The characterization study examined the temperature and pH parameters that affected its CMCase activity. The methodology of the experiments was performed accordingly, as mentioned in Chantarasiri (2020). All experiments were performed in triplicate.

The optimum temperature of the CMCase activity was determined at temperatures ranging from 25°C to 80°C in an assay buffer. The thermal stability was measured by pre-incubating the crude cellulases at temperatures ranging from 25°C to 80°C for 24 h in an assay buffer, and the residual activity of the CMCase was monitored afterward. The assay buffer was a 50 mM sodium phosphate buffer at a pH of 7.0. The optimum pH of the CMCase activity was determined in the pH-varied buffers at 50°C. The assay buffers were 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 measured by pre-incubating the crude cellulases in the buffer mentioned above at 50°C for 24 h, and the residual activity of the CMCase was monitored afterward.

Data analysis

The statistical analyses of the data in this study were performed using R software version 4.2.1 (R Foundation for Statistical Computing, Austria). The multiple comparison analyses were determined by one-way ANOVA followed by Tukey's test with a 95% confidence interval ($p < 0.05$).

RESULTS AND DISCUSSION

Description of the collected mangrove soil samples

Fifteen soil samples were collected from the Phra Chedi Klang Nam Mangrove Forest, and another 15 samples were collected from the Kung Krabaen Bay Mangrove Forest. The soil temperature measured at the sampling sites by a needle probe thermometer ranged from 26.2°C to 37.5°C and the average soil temperature was 28.37±2.61°C. The pH of the suspended soil samples ranged from 4.64 to 7.32, and the average pH of the suspended soil samples was 6.18±0.68. The average temperature and pH values were used as the growth conditions of all the isolated bacteria in this study.

Isolation and screening of the cellulase-producing bacteria from the mangrove soil samples

Sixty-three bacterial isolates were isolated and streak plate-purified from the mangrove soil samples. The screening of the cellulase-producing bacteria by the CMC agar method showed 26 bacterial isolates that were defined as active cellulase-producing bacteria. Furthermore, 15 bacterial isolates (named with the code of RY) were screened from the samples collected from the Phra Chedi Klang Nam Mangrove Forest. Their HC values ranged from 1.045±0.493 (cellulase-producing bacterium isolate RY05B) to 3.550±0.098 (cellulase-producing bacterium isolate RY03D). The other 11 bacterial isolates (named with the code of CH) were screened from the Kung Krabaen Bay Mangrove Forest samples. Their HC values ranged from 1.097±0.048 (cellulase-producing bacterium isolate CH02G) to 3.090±0.181 (cellulase-producing bacterium isolate CH06E). The cellulolytic zone around the bacterial colonies after being flooded by the iodine solution is shown in Figures 2 and 3. The HC values of the cellulase-producing bacteria are shown in Table 1.

The cellulase-producing ability of all the isolated bacteria was confirmed by the qualitative determination of the degradation of Whatman filter paper No. 1 strips. The filter paper degradation results showed three cellulase-producing bacteria that were evaluated as outstanding cellulase-producing bacteria after 28 days of incubation. These were the isolates RY01D, RY08B, and CH02H. Therefore, they were represented by the "+++" level of the filter paper degradation. Interestingly, the HC values of the isolates RY01D, RY08B, and CH02H measured from the previous experiment were 1.343±0.095, 1.367±0.356, and 1.153±0.068, respectively. For the most active cellulase-producing bacteria defined by the CMC agar method, isolates RY03D and CH06E exhibited only a "+" level of filter paper degradation. The qualitative determination of the degradation of Whatman filter paper No. 1 strips is shown in Figure 4. The level of the filter paper degradation of the 26 cellulase-producing bacteria is shown in Table 2.

Table 1. HC values of 26 cellulase-producing bacteria isolated from the soil samples of the mangrove forests in Eastern Thailand

HC value	Bacterial isolates	
	Phra Chedi Klang Nam Mangrove Forest (Rayong Province)	Kung Krabaen Bay Mangrove Forest (Chanthaburi Province)
1.00-1.99	RY01D, RY02B, RY02D, RY02E, RY03A, RY03E, RY04E, RY05A, RY05B, RY08A, RY08B, RY09C, RY09D	CH01E, CH02G, CH02H, CH03E, CH05B, CH06K
2.00-2.99	RY09A	CH02I, CH02J, CH05A, CH07B
> 3.00	RY03D	CH06E

Table 2. Level of the filter paper degradation of 26 cellulase-producing bacteria isolated from the soil samples of mangrove forests in Eastern Thailand

Level of the filter paper degradation	Bacterial isolates	
	Phra Chedi Klang Nam Mangrove Forest (Rayong Province)	Kung Krabaen Bay Mangrove Forest (Chanthaburi Province)
+	RY02B, RY02D, RY02E, RY03A, RY03D, RY05A, RY05B, RY08A, RY09C, RY09D	CH06E, CH02G, CH03E, CH05B, CH06K
++	RY03E, RY04E, RY09A	CH01E, CH02I, CH02J, CH05A, CH07B
+++	RY01D, RY08B	CH02H

Table 3. Different RFLP patterns and numbers of the 26 cellulase-producing bacteria isolated from the soil samples of the mangrove forests in Eastern Thailand

RFLP patterns	Bacterial isolates	Total number of bacterial isolates
RFLP-RY01	RY01D, RY02B, RY02E, RY03E, RY08B, RY09A, RY09C	7
RFLP-RY02	RY02D	1
RFLP-RY03	RY03A	1
RFLP-RY04	RY03D	1
RFLP-RY05	RY04E	1
RFLP-RY06	RY05A	1
RFLP-RY07	RY05B	1
RFLP-RY08	RY08A, RY09D	2
RFLP-CH01	CH01E, CH05A, CH06K	3
RFLP-CH02	CH02G	1
RFLP-CH03	CH02H, CH03E, CH05B, CH07B	4
RFLP-CH04	CH02I, CH02J	2
RFLP-CH05	CH06E	1

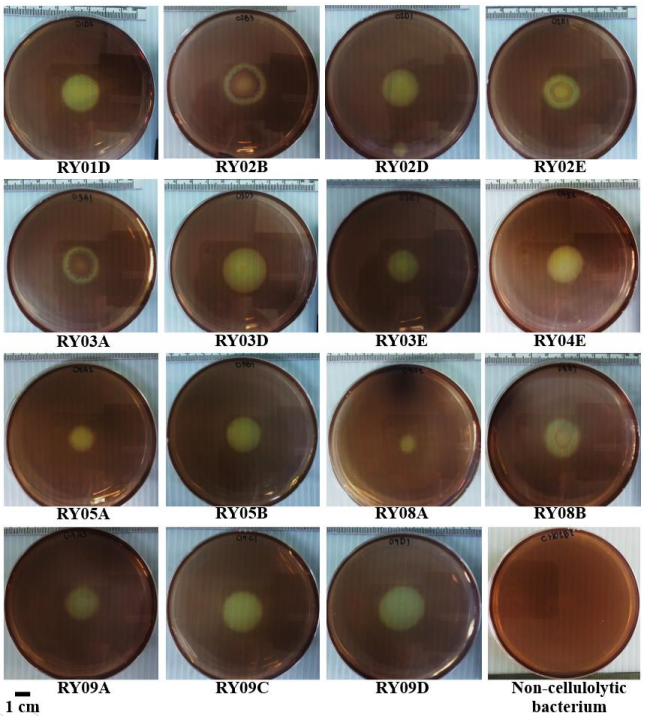

Figure 2. After iodine staining, the cellulolytic zone of 15 cellulase-producing bacteria isolates from the Phra Chedi Klang Nam Mangrove Forest on the CMC agar plates. The negative control was a non-cellulolytic bacterium inoculated on the CMC agar

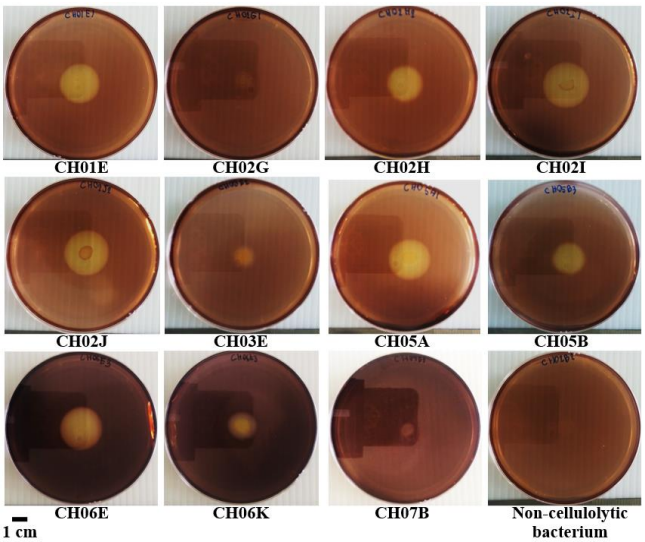

Figure 3. After iodine staining, the cellulolytic zone of 11 cellulase-producing bacteria isolates from the Kung Krabaen Bay Mangrove Forest on the CMC agar plates. The negative control was a non-cellulolytic bacterium inoculated on the CMC agar

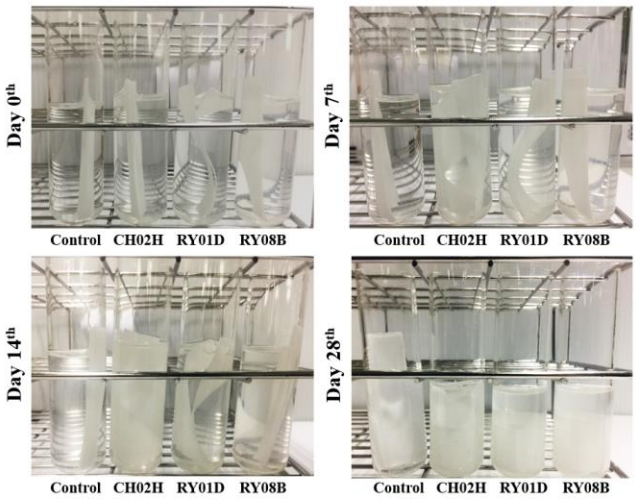

Figure 4. Degradation of the filter paper strips by the cellulase-producing bacteria isolates RY01D, RY08B, and CH02H for 28 days of incubation. The negative control was a basal salt medium containing filter paper strips without bacterial inoculation

PCR-RFLP analysis of the 16S rRNA genes amplified from the cellulase-producing bacteria

The 16S rRNA genes of the isolated 26 cellulase-producing bacteria were amplified, enzymatically digested, and electrophorized by the PCR-RFLP analysis. The resulting RFLP profiles of all the bacteria electrophorized on agarose gel are shown in Figures 5 and 6. There were eight different RFLP patterns obtained from the 15 cellulase-producing bacteria isolated from the Phra Chedi Klang Nam Mangrove Forest and five different RFLP patterns obtained from the 11 cellulase-producing bacteria isolated from the Kung Krabaen Bay Mangrove Forest. The different patterns of the RFLP profiles are summarized in Table 3. The RFLP-RY01 and RFLP-CH03 patterns were the most found in the RFLP profiles of the Phra Chedi Klang Nam Mangrove Forest and the Kung Krabaen Bay Mangrove Forest, respectively. The RFLP-CH01 and RFLP-CH04 were the second and third most patterns of the Kung Krabaen Bay Mangrove Forest. All RFLP patterns were explicit arrangements and practicable for the bacterial categorization in this study.

Genetic identification and phylogenetic analysis of the cellulase-producing bacteria

The genomic DNA was extracted and purified from 13 representative cellulase-producing bacteria based on the RFLP patterns. The PCR conditions of the 16S rRNA gene amplification amplified each genomic DNA sample. The nucleotide sequencing and alignment results showed that the isolated cellulase-producing bacteria from the mangrove soil samples belonged to six genera consisting of the genus *Aeromonas* of the Phylum Pseudomonadota, genus *Bacillus* of the Phylum Bacillota, genus *Chryseobacterium* of the Phylum Bacteroidota, genus *Lysinibacillus* of the Phylum Bacillota, genus *Pseudomonas* of the Phylum Pseudomonadota, and genus *Vibrio* of the Phylum Pseudomonadota. The cellulase-producing bacteria isolated from the Phra Chedi Klang Nam Mangrove Forest were closely similar to the bacteria in the genera of *Aeromonas*, *Bacillus*, *Chryseobacterium*, *Lysinibacillus*, *Pseudomonas*, and *Vibrio* with a 93-98% identity. The cellulase-producing bacteria isolated from the Kung Krabaen Bay Mangrove Forest were closely similar to the bacteria in the genera of *Aeromonas*, *Bacillus*, *Pseudomonas*, and *Vibrio* with a 97-99% identity. The nucleotide alignment result of the cellulase-producing bacteria by the BlastN program is shown in Table 4.

A circular phylogenetic tree of the cellulase-producing bacteria was established using the BIONJ algorithm for 100,000 bootstrap replications (Figure 7). Six RFLP patterns fell into the phylogenetic cluster of the Phylum Bacillota. The patterns RFLP-RY01, RFLP-RY07, RFLP-RY08, RFLP-CH03, and RFLP-CH04 were formed into the clade of the genus *Bacillus* with a bootstrap value of 71-100, while the pattern RFLP-RY06 was formed into the clade of the genus *Lysinibacillus* with a bootstrap value of 59. The other six RFLP patterns fell into the phylogenetic cluster of the Phylum Pseudomonadota. The patterns RFLP-RY02 and RFLP-CH01 were formed into the clade of the genus *Aeromonas* with a bootstrap value of 78. The patterns RFLP-RY03 and RFLP-CH05 were clustered into the clade of the genus *Vibrio* with a bootstrap value of 99-100. The patterns RFLP-RY05 and RFLP-CH02 were formed into the clade of the genus *Pseudomonas* with a bootstrap value of 52. Lastly, the pattern RFLP-RY04 fell into the genus *Chryseobacterium* of the Phylum Bacteroidota with a bootstrap value of 52, considered the unique phylogenetic clade of this circular phylogenetic tree.

The nucleotide alignment and phylogenetic tree of the 16S rRNA gene sequences evidenced the genetic identification of the cellulase-producing bacteria isolated from the mangrove soil samples. The cellulase-producing bacteria were found to be closely related based on the nucleotide sequence alignment results of the 16S rRNA genes when the identity was more than 99%, and the bootstrap value was more than 70, such as *B. tequilensis* strain CH02H. Apart from the mention, the ones were presented at the genus level, such as *Bacillus* sp. strain RY08B. All the nucleotide sequences of the 16S rRNA genes obtained from this study were deposited in the GenBank database of the NCBI under accession numbers OR001963 to OR001968, OR001970, OR001971 to OR001975, and OR004586. The genetic identification of the 13 cellulase-producing bacteria is shown in Table 5.

Cellulase activity assays of the crude cellulases

Five active cellulase-producing bacteria were preferred for cellulase activity by the CMCase activity assay. The crude cellulases prepared from those bacteria were used to determine the cellulase activity. The activity assays showed they could produce crude cellulases with 0.573±0.050 to 1.510±0.060 U/mL of the CMCase activity. The *Bacillus* sp. strain RY08B was considered the most active CMCase bacterium with significant cellulase activity among this study's five active cellulase-producing bacteria ($p<0.05$). Their cellulase activities are shown in Table 6.

Table 4. Identity percentages of the 16S rRNA gene sequences of the 13 cellulase-producing bacteria with closely related bacteria obtained from the BlastN program

RFLP patterns	Representative bacterial isolate	Closely related bacteria	GenBank accession No. (references)	Query cover (%)	Identity (%)	E value
RFLP-RY01	RY08B	*Bacillus stratosphericus* strain RRD69	KJ534473.1	99	98.00	0.0
RFLP-RY02	RY02D	*Aeromonas hydrophila* strain BJ	EU696781.1	99	97.25	0.0
RFLP-RY03	RY03A	*Vibrio* sp. strain PrVb096	MF948987.1	99	97.58	0.0
RFLP-RY04	RY03D	*Chryseobacterium indologenes* strain VIT-CMJ1	KJ437473.1	99	98.25	0.0
RFLP-RY05	RY04E	*Pseudomonas* sp. strain PrPz072	MF948933.1	99	98.16	0.0
RFLP-RY06	RY05A	*Lysinibacillus fusiformis* strain D4	OP115606.1	99	98.59	0.0
RFLP-RY07	RY05B	*Bacillus toyonensis* strain FS-8	KY649418.1	99	96.14	0.0
RFLP-RY08	RY09D	*Bacillus siamensis* strain SDI-28	KT021508.1	99	93.59	0.0
RFLP-CH01	CH05A	*Aeromonas* sp. AE-51	AY987731.1	99	97.16	0.0
RFLP-CH02	CH02G	*Pseudomonas* sp. strain T34	MT337580.1	99	99.66	0.0
RFLP-CH03	CH02H	*Bacillus tequilensis* strain JAAKPT	MN049471.1	99	99.05	0.0
RFLP-CH04	CH02I	*Bacillus cereus* strain IHB B 6826	KF668460.1	99	97.09	0.0
RFLP-CH05	CH06E	*Vibrio* sp. strain CHZNd-3	MW785575.1	99	97.69	0.0

Note: The identity results were analyzed on 15 May 2023

Table 5. Genetic identification of the 13 cellulase-producing bacteria and their deposited GenBank accession numbers

RFLP patterns	Identified species	GenBank accession no. (deposited)
RFLP-RY01	*Bacillus* sp. strain RY08B	OR001963
RFLP-RY02	*Aeromonas* sp. strain RY02D	OR001964
RFLP-RY03	*Vibrio* sp. strain RY03A	OR001966
RFLP-RY04	*Chryseobacterium* sp. strain RY03D	OR004586
RFLP-RY05	*Pseudomonas* sp. strain RY04E	OR001965
RFLP-RY06	*Lysinibacillus* sp. strain RY05A	OR001968
RFLP-RY07	*Bacillus* sp. strain RY05B	OR001967
RFLP-RY08	*Bacillus* sp. strain RY09D	OR001970
RFLP-CH01	*Aeromonas* sp. strain CH05A	OR001971
RFLP-CH02	*Pseudomonas* sp. strain CH02G	OR001972
RFLP-CH03	*Bacillus tequilensis* strain CH02H	OR001973
RFLP-CH04	*Bacillus* sp. strain CH02I	OR001974
RFLP-CH05	*Vibrio* sp. strain CH06E	OR001975

Note: The nucleotide sequences of the 16S rRNA gene were deposited in the GenBank database of NCBI on 19 May 2023

Table 6. Cellulase activity of the five active cellulase-producing bacteria

Active cellulase-producing bacteria	CMCase activity (U/mL) with a pH of 7.0 and temperature of 50°C
Bacillus sp. strain RY01D	1.098 ± 0.059^{a}
Chryseobacterium sp. strain RY03D	0.813 ± 0.059^{c}
Bacillus sp. strain RY08B	1.510 ± 0.060^{d}
B. tequilensis strain CH02H	1.192 ± 0.013^{a}
Vibrio sp. strain CH06E	0.573 ± 0.050^{b}

Note: The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$) among the active cellulase-producing bacteria

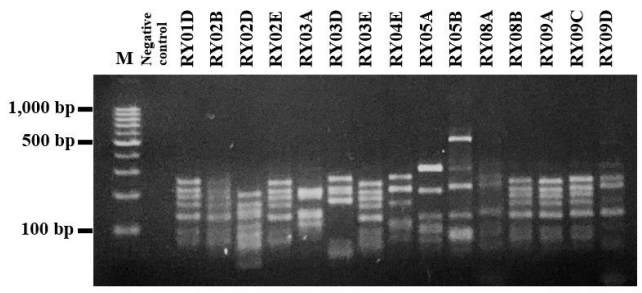

Figure 5. RFLP profiles resulting from the PCR-RFLP analysis of the 15 cellulolytic bacteria isolated from the Phra Chedi Klang Nam Mangrove Forest. M denotes the OneMark 100 RTU DNA ladder

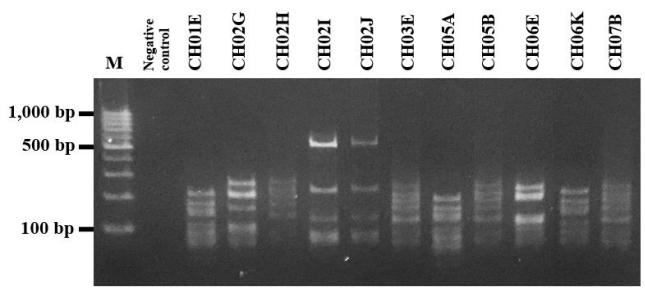

Figure 6. RFLP profiles resulting from the PCR-RFLP analysis of the 11 cellulolytic bacteria isolated from the Kung Krabaen Bay Mangrove Forest. M denotes the OneMark 100 RTU DNA ladder

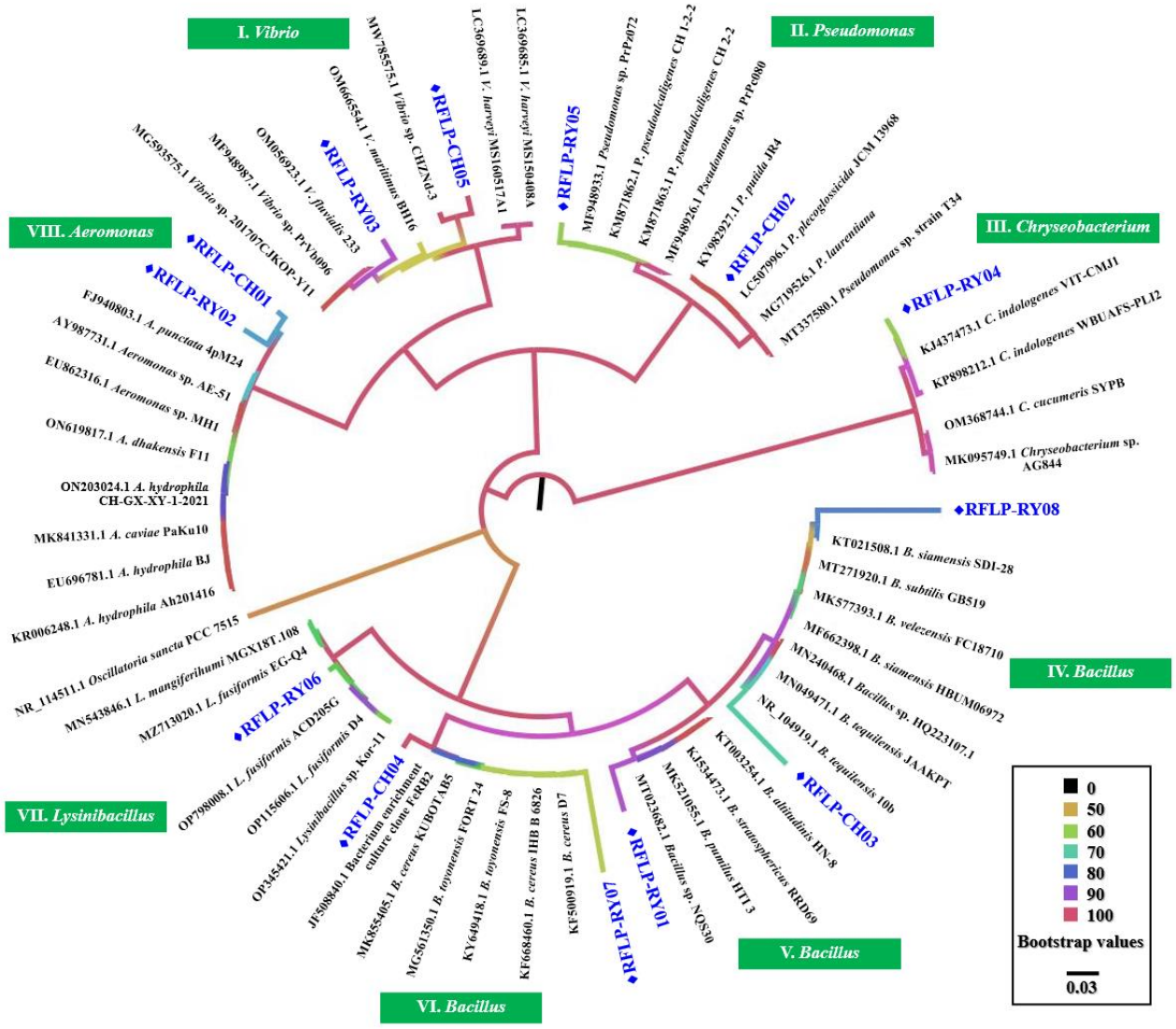

Figure 7. The circular phylogenetic tree of the cellulase-producing bacteria is shown using the BIONJ algorithm for 100,000 bootstrap replications. The phylogenetic tree was analyzed by SeaView software version 5.0.1 and FigTree software version 1.4.4

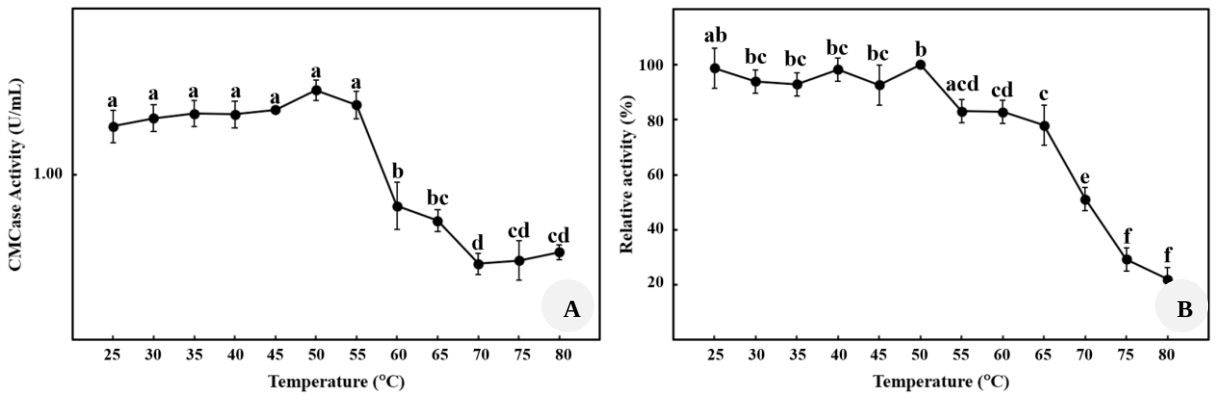

Figure 8. A. Effect of the temperature on the CMCase activity and B. Thermal stability from the cellulases of the *Bacillus* sp. strain RY08B. The error bars represent the standard deviation of the triplicate. The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$). The experiments were performed in triplicate

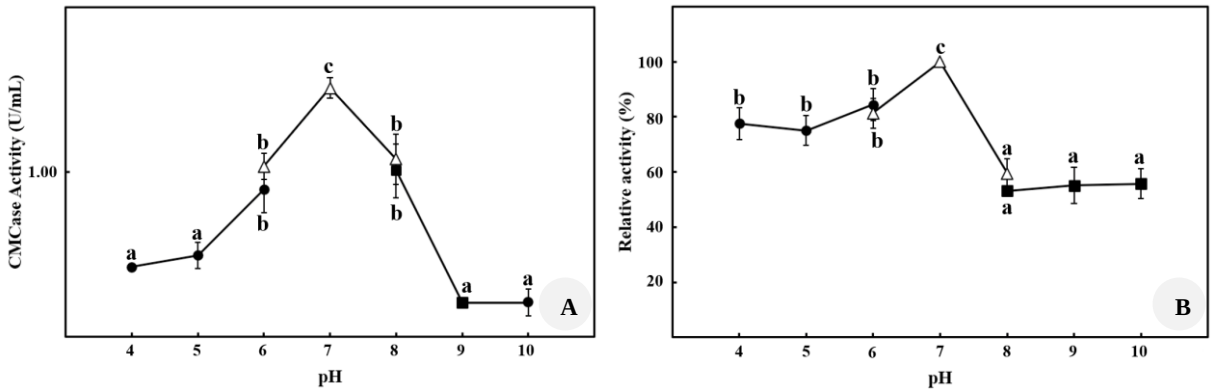

Figure 9. Effect of pH on the CMCase activity and B. pH stability from the cellulases of the *Bacillus* sp. strain RY08B. The activity of the enzyme was determined in a citrate buffer (●), sodium phosphate buffer (Δ), and glycine-NaOH buffer (■). The error bars represent the standard deviation of the triplicate. The mean values followed by the same letter were not significantly different according to Tukey's test ($p < 0.05$). The experiments were performed in triplicate

Enzymatic characterization of the crude cellulases from the *Bacillus* sp. strain RY08B

Crude cellulases from the most active cellulase-producing bacteria, *Bacillus* sp. strain RY08B, were characterized for the CMCase activity at various temperatures and pH conditions. The meso-temperature ranging from 25°C to 55°C was favored for the CMCase activity ($p<0.05$) with the optimum temperature of 50°C. The optimum pH was 7.0 in a sodium phosphate buffer ($p<0.05$). The CMCase activity at the same pH value did not significantly affect the buffer types. The optimum temperature and pH for the CMCase activity are shown in Figures 8.A and 9.A, respectively.

The CMCase activity remained stable up to 50°C ($p < 0.05$), while the enzyme activity was reduced to below 50% of the relative activity after incubation at a temperature above 70°C in a sodium phosphate buffer at a pH of 7.0. The enzyme was stable at a neutral pH of 7.0 ($p < 0.05$) and inactivated at a strong alkaline condition with less than 50% relative activity remaining. The thermal and pH stability results for the CMCase activity are shown in Figures 8.B and 9.B, respectively.

Discussion

Mangrove plant materials and related components must be decomposed by the microbial metabolic processes (Biswas et al. 2020). Cellulase-producing bacteria is one of the diverse groups of microbes dwelling in the mangrove ecosystems that mainly have a vital role in these metabolic processes. This study collected mangrove soil samples from two mangrove ecosystems in Rayong and Chanthaburi Provinces, Thailand, to study cellulase-producing bacteria's diversity and cellulolytic activity. In addition, the collected soil sample's average temperature and pH values were 28.37°C and 6.18, respectively. These characteristics were closely similar to the average temperature and pH values of some mangrove soils collected from Indonesia (Dewiyanti et al. 2021) and Thailand (Klinfoong et al. 2022).

Moreover, 26 bacterial isolates were identified as cellulase-producing bacteria by the CMC agar method, with HC values ranging from 1.05 to 3.55. Bacterial isolates RY03D and CH06E were considered the most active cellulase-producing bacteria based on the HC values of 3.55 and 3.09, respectively. However, this was lower than the researcher's previous report, which found 22 cellulase-producing bacteria isolated from muddy sediments from the mangrove forest in Rayong Province, Thailand, with HC values ranging from 3.10 to 4.47 (Chantarasiri 2015). A recent report from Indonesia showed that the HC values of 22 cellulase-producing bacteria isolated from mangrove soil in Aceh Province ranged from 0.31 to 4.82 (Dewiyanti et al. 2022b). Furthermore, the filter paper degradation method was confirmed for screening the cellulase-producing bacteria. The degradation level exhibited that the bacterial isolates RY01D, RY08B, and CH02H were the most active cellulase-producing bacteria. Interestingly, the bacterial isolates RY03D and CH06E were not the most active cellulase-producing bacteria based on the level of filter paper degradation. These conflicting results could be due to fluctuations in some experimental factors which affected the production and enzymatic performance of the cellulases described in several previous reports (Ahmad et al. 2013; Chantarasiri 2020). Therefore, the HC values should be considered as the presumptive method for screening cellulase-producing bacteria. Another screening method would need to be confirmed for accuracy and precision.

Many microbiological studies in mangrove ecosystems have been reported in recent years (Liu et al. 2019). The study on the diversity and taxonomy of soil bacteria in the mangrove forests of Panama Bay, Panama, showed the three dominant phyla of the found bacteria, which were Pseudomonadota (formerly known as Proteobacteria), Desulfobacterota, and Chloroflexota (formerly known as Chloroflexi) (Quintero et al. 2020). In addition, a recent study aimed to isolate and identify the microbial diversity of Khlong Tub Mangrove Forest in Chon Buri Province, Thailand (Ganjanasiripong et al. 2022). The predominant

bacterial genera were *Acinetobacter*, *Aeromonas*, *Aneurinibacillus*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Staphylococcus*, and *Vibrio*. In this study, the isolated cellulase-producing bacteria were analyzed by the PCR-RFLP analysis of the 16S rRNA genes. PCR-RFLP is a highly reliable, inexpensive, and fast genotyping method for species identification. Thirteen different RFLP patterns were obtained, and the representative cellulase-producing bacteria from each pattern were genetically and phylogenetically identified. The identification revealed that the cellulase-producing bacteria belonged to the three phyla of Pseudomonadota, Bacillota, and Bacteroidota.

In this study, the cellulase-producing bacteria belonging to Phylum Pseudomonadota were genera *Aeromonas*, *Pseudomonas*, and *Vibrio*. Bacteria in the genus *Aeromonas* naturally inhabit aquatic environments and mangrove soil samples (Ganjanasiripong et al. 2022; Pessoa et al. 2022). The present study revealed that the *Aeromonas* sp. strain RY02D and *Aeromonas* sp. strain CH05A were designated as cellulase-producing bacteria. Many previous studies reported that *Aeromonas* were the producers of cellulases. The effective strains were isolated from various samples, such as animals, moist peat samples, sedimentary water, and sugar industry waste (molasses) (Islam and Roy 2018; Chantarasiri 2020; Chantarasiri 2021b; Canellas and Laport 2022). At present, *Aeromonas* is considered an interesting candidate for many biocatalyst industries. The isolated *Pseudomonas* sp. strain RY04E and *Pseudomonas* sp. strain CH02G were also considered cellulase-producing bacteria. *Pseudomonas* is a versatile bacterial genus widely found in environments such as soils and water (Gonzalez-Pimentel et al. 2022). Furthermore, many studies showed that the *Pseudomonas* species could be retrieved from mangrove soil samples in Thailand (Chantarasiri 2021a; Ganjanasiripong et al. 2022; Klinfoong et al. 2022). Some *Pseudomonas* species were isolated and identified as cellulase-producing bacteria, such as *P. fluorescens* from soil (Sethi et al. 2013), *P. mendocina* from rotten straw (Zhang et al. 2016), *P. psychotolerans* from mangrove sediments (Kurniawan et al. 2019), and *P. stutzeri* from forest soil (Rattanasuk et al. 2020). The last cellulase-producing bacteria of the Phylum Pseudomonadota found in this study were the *Vibrio* sp. strains RY03A and CH06E. *Vibrio* is a genus of heterotrophic bacteria widely distributed in aquatic environments, such as estuarine, freshwater, marine, and mangrove ecosystems (Chantarasiri 2020; Ganjanasiripong et al. 2022; Sampaio et al. 2022). The *Vibrio* species can produce cellulases, such as *V. xiamenensis* and *V. alginolyticus*, which could be isolated from mangrove soil samples (Ming Gao et al. 2012; Kurniawan et al. 2019). In a previous study, a cellulase-producing *Vibrio* sp. was isolated from moist peat samples in Thailand (Chantarasiri 2020).

Bacillus and *Lysinibacillus* of the Phylum Bacillota were isolated and considered cellulase-producing bacteria. These bacterial genera had been reported to be a bacterial community dwelling in mangrove soil (Ganjanasiripong et al. 2022). It is well-known data that the *Bacillus* species are commonly found in soil and water environments. Furthermore, five strains of *Bacillus* comprising RY05B, RY08B, RY09D, CH02H, and CH02I were investigated in this study. Notably, the *Bacillus* sp. strain CH02H was genetically identified as *B. tequilensis* strain CH02H. The variety of *Bacillus*-producing cellulases included strains of *Bacillus* sp., *B. cereus*, *B. licheniformis*, and *B. subtilis* (Kim et al. 2012). They were employed for the prolific production of cellulases, and their cellulases are now reported frequently (Fatani et al. 2021). Moreover, *Bacillus* cellulases facilitate easy extraction and purification processes (Malik and Javed 2021). Many previous studies reported cellulase-producing *Bacillus* species that had been isolated from mangroves and related ecosystems, such as *B. altitudinis*, *B. cereus*, *B. safensis*, *B. subtilis*, and *B. toyonensis* (Chantarasiri 2015; Naresh et al. 2019; Nursyirwani et al. 2020; Dewiyanti et al. 2022b). *B. tequilensis* was also designated as cellulase-producing bacteria, such as *B. tequilensis* strain G9, which was isolated from the gut fluid of a giant African land snail (Dar et al. 2019) and *B. tequilensis* strain UTMSA14 isolated from a geothermal hot spring (Fachrial et al. 2020). Interestingly, the Phra Chedi Klang Nam Mangrove Forest in Rayong Province was the area of a previous study in 2015 (Chantarasiri 2015), but it had no cellulase-producing *B. cereus* found. In addition, *Lysinibacillus* is a new genus of bacteria recently reclassified from the genus *Bacillus*. *Lysinibacillus* species have been reported to have the potential to control pests, remediate heavy metal-contaminated environments, and increase crop yields (Ahsan and Shimizu 2021). This study classified a bacterial isolate of *Lysinibacillus* as a cellulase-producing bacterium. It had the 16S rRNA gene nucleotide sequence closely related to *L. fusiformis* and was finally genetically identified as the *Lysinibacillus* sp. strain RY05A. A previous study reported that *Lysinibacillus* sp. isolated from estuary sediment could secrete cellulases (Mahalik et al. 2018). To the best of the researcher's knowledge, the data on cellulase-producing *Lysinibacillus* have been limited.

Another cellulase-producing bacterium of the Phylum Bacteroidota found in this study was the *Chryseobacterium* sp. strain RY03D. This bacterial strain was isolated from the Phra Chedi Klang Nam Mangrove Forest in Rayong Province and had the nucleotide sequence of the 16S rRNA gene closely related to *C. indologenes*. *Chryseobacterium* species are common pathogens of plants and animals, which normally exist in soil, water, and plants (Lin et al. 2019). To date, some *Chryseobacterium* species have been studied and reported as cellulase producers, such as the *Chryseobacterium* sp. strain HT1 isolated from the dung of straw-fed cattle (Tan et al. 2018), *C. indologenes* strain LA4K isolated from agar-agar industry solid waste (Munifah et al. 2020), and *C. luteola* isolated from the guts of termites (Egwuatu and Appeh 2018). However, the cellulase-producing *Chryseobacterium* isolated from mangrove forests had been scarce.

The most active cellulase-producing bacteria based on the CMCase activity was the *Bacillus* sp. strain RY08B with a significant CMCase activity of 1.510 U/mL. It was slightly less than the previously reported *B. cereus* strain

JD0404 isolated from the same study site, which exhibited 1.778 U/mL of CMCase activity (Chantarasiri 2015). Crude cellulases of the *Bacillus* sp. strain RY08B were enzymatically characterized at various temperature and pH conditions. The CMCase activities of the crude cellulase from the *Bacillus* sp. strain RY08B were active and stable under meso-temperature and neutral pH conditions in a sodium phosphate buffer. This was closely similar to the CMCase activity of other cellulase-producing bacteria isolated from mangrove ecosystems, which were active at a meso-temperature range of 50°C and a pH range of 6.0-7.0 (Chantarasiri 2015; Naresh et al. 2019). These reported mangrove cellulase-producing bacteria were also stable at a meso-temperature range of 40-60°C and a pH range of 6.0-8.0 (Chantarasiri 2015; Naresh et al. 2019). The results suggested that the cellulases of the *Bacillus* sp. strain RY08B could be preferred in biotechnological and industrial applications with mild conditions, such as food, animal feed, and environmentally friendly biofuel industries. However, the industrial cellulase producers should be a non-pathogenic and well-known bacterium. Therefore, the explicit identification of the *Bacillus* sp. strain RY08B should be advanced further.

In conclusion, the Southeast Asian mangrove ecosystems are a potential source for isolating cellulase-producing bacteria. There were six genera of cellulase-producing bacteria isolated from two mangrove forests in Eastern Thailand based on the PCR-RFLP of the 16S rRNA genes and nucleotide sequencing analysis comprising *Aeromonas*, *Bacillus*, *Chryseobacterium*, *Lysinibacillus*, *Pseudomonas*, and *Vibrio*. Cellulase-producing *Bacillus* were the predominant bacteria found in this study. The results revealed that the *Bacillus* sp. strain RY08B was the most active cellulase-producing bacterium based on its CMCase activity. Its crude cellulases were characterized, and it could be used in biotechnological applications with meso-temperature and neutral pH conditions. Further studies should also be recommended on optimizing cellulase production, determining chemical additives on cellulase activity, and enzyme purification.

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