Isolation and Identification of a Cellulase-producing *Bacillus* sp. Strain BR0302 from Thai Coastal Wetland Soil

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Abstract
This study proposed the isolation, screening and identification of cellulase-producing bacteria from Thai coastal wetland soil. Eighty seven bacterial strains which demonstrated cellulase-producing bacteria were investigated for cellulolytic properties. The bacterium identified as Bacillus cereus strain BR0302 exhibited the highest hydrolysis activity on carboxymethylcellulose agar plates. Cellulolytic performance for CMCase activity and FPase activity was 0.121 ± 0.006 and 0.057 ± 0.001 U/mL respectively. Cellulolytic characteristics showed that the *B. cereus* strain BR0302 could be used for the hydrolysis of cellulosic biomass for biofuel production.

Keywords: *Bacillus cereus*, Cellulase-producing bacteria, Coastal wetland soil

1 Introduction

Biotechnological conversion of cellulosic biomass is a sustainable approach to create glucose and reducing sugars for the production of bioethanol and other biofuels. Cellulolytic enzymes have currently become focal biocatalysts in biotechnological conversion applications [1]; they are produced by numerous microorganisms for the depolymerization of cellulose in cellulosic materials. These complex enzymes include endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C. 3.2.1.91, E.C. 3.2.1.176) and β-glucosidases (E.C. 3.2.1.21) which work together to hydrolyse the β-1, 4 glycosidic bonds of cellulose [2]. Most cellulolytic enzymes are produced from fungi, such as the genera *Trichoderma* and *Aspergillus*, and bacteria including the genera *Bacillus*, *Clostridium* and *Cellulomonas* [3], [4]. The bacterial cellulolytic enzyme is often a more effective catalyst because it has short fermentation cycle time, low-cost energy utilization and it is easy to genetically engineer [4], [5]. One major impediment for cellulosic biofuels is the lack of microorganisms which can produce sufficient cellulolytic enzymes to breakdown the cellulose [6].

Coastal wetlands are unique ecosystems with complex interactions between terrestrial, marine and aquatic habitats [7], [8]. They are crucial ecosystems for study and research because of their ecological roles, which include the storage of surface runoff, nutrient cycling, sediment accretion, pollution filtration, shoreline erosion control and the habitats of many shoreline animals [7], [9]. The microbial communities in coastal wetland soil and sediments play a vital role in wetland ecological functions [9]. These include nitrogen and phosphorus transformation processes and organic matter degradation [10]. However, studies on microbial communities, including cellulolytic microbes isolated from coastal wetland soil, are very limited because of frequent flooding conditions resulting in low rates of degradation and low microbial activity [11], [12].

For this study, the cellulase-producing bacterium strain BR0302 was isolated from coastal wetland

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soil and identified by morphology and 16S rDNA sequencing analysis. The purpose was to determine a cellulase-producing bacterium for possible use in biotechnological conversion.

2 Materials and Methods

2.1 *Chemicals, culture media and microorganism*

The chemicals used in this study were of analytical grade, purchased from Sigma-Aldrich (MO, USA), Calbiochem (Darmstadt, Germany) and Merck (Darmstadt, Germany). Culture media were purchased from HiMedia (Mumbai, India). *Bacillus methylotrophicus* strain RYC01101 [13] and *Escherichia coli* isolated from bovine faeces were kindly provided by the Faculty of Science, Energy and Environment, King Mongkut's University of Technology North Bangkok. *Salmonella* sp. was obtained from the Department of Microbiology, Faculty of Science, Mahidol University, Bangkok.

2.2 *Site description, sample collection and isolation of bacteria*

Sample collection methods and isolation of bacteria were modified from Chantarasiri *et al* [13]. Soil samples were collected from Bueng Samnak Yai (Nong Chamrung) coastal wetland (12°39'39"N, 101°32'09"E) in Rayong Botanical Garden, Rayong Province, Thailand. Bueng Samnak Yai coastal wetland consists of five shoals: Koh-Kok, Koh-Taeo Yai, Koh-Cha Muang, Koh-Taeo Lek and Koh-Mai Nham (Figure 1). The main plants included *Melaleuca quinquenervia* (Thai name: Samed Khao), *Garcinia cowa* (Cha Muang), *Cratoxylum formosum* (Taeo) and *Lepironia articulata* (Kok Krajood). Bueng Samnak Yai coastal wetland was designated as a National Wetland Reserve in 2009. Soil samples were collected during the period from July 2014 to August 2014 (rainy season). The soil samples were collected twice, one in late July and another in late August. Soil samples from soil surface and at a depth of 15 cm were taken in steriled zip-lock polyethylene bags using steriled spatula. The soil samples were kept at 4°C and serially diluted with steriled ultrapure water within 24 h of collection to obtain 1:1,000 dilutions. One hundred microlitres of each diluted sample was cultured in nutrient agar and incubated at 29.2°C, the average soil temperature of the collecting sites, for 48 h. Morphologically dissimilar colonies of bacteria were picked and streak plated on nutrient agar to obtain pure colonies.

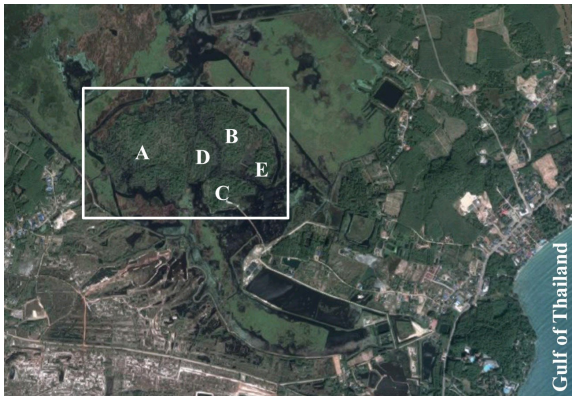

Figure 1: Map of five shoals in Bueng Samnak Yai coastal wetland: (A) Koh-Kok, (B) Koh-Taeo Yai, (C) Koh-Cha Muang, (D) Koh-Taeo Lek and (E) Koh-Mai Nham, covering an area of 130 hectares.

2.3 *Screening of cellulase-producing bacteria*

Screening of the cellulase-producing bacteria was done on carboxymethylcellulose (CMC) agar plates, following the method of Kasana *et al* [14]. Five microlitres of overnight growth culture in the nutrient broth of each bacterial isolate was spot plated on 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). The plates were incubated at 29.2°C for 48 h and then flooded with Gram's iodine solution (0.67% KI and 0.33% I_2) for 5 min. The cellulase-producing isolates were detected by the cellulolytic zone around the colonies after Gram's iodine staining. The hydrolysis capacity (HC) was calculated from the ratio between the diameter of the cellulolytic zone and the diameter of the bacterial colony [13]. The cellulase-producing isolates were further screened by measuring the amount of reducing sugars hydrolyzed from the substrates in the culture medium. The isolates were grown in 10 mL of CMC broth at 29.2°C under aeration condition. After 48 h of incubation, 1 mL of culture medium was collected for reducing sugar measurement by the DNS method [15]. The cell-free culture medium was added by 3.0 mL of 3,5-dinitrosalicylic acid reagent and then

immediately boiled for 5 min. Cooled the solution thoroughly and measured the optical density of the solution at 540 nm. The amount of reducing sugars was calculated using glucose standard curve. The positive control for screening was the cellulolytic bacteria, *B. methylotrophicus* strain RYC01101 and the negative control was *E. coli*.

2.4 *Estimation of cellulase activity*

To determine the cellulase activity, the selected isolate was grown in 100 mL of CMC broth at 29.2°C for 48 h under aeration condition. The crude extracellular cellulase produced in the broth was collected by centrifugation at 4,500 × g for 30 min at 4°C. The cell-free supernatant obtained after centrifugation served as crude cellulase source. The cellulase activity was assayed by incubating the crude cellulase solution with the substrate and estimating the amount of reducing sugars released from the substrate. One unit (U) is defined as the quantity of cellulase activity required to release 1 μmol of reducing sugars as glucose equivalent under standard assay conditions [13]. Endoglucanase activity (CMCase activity) [16] was determined by incubating 0.5 mL of crude cellulase solution with 0.5 mL of 2% CMC sodium salt in 0.05 M sodium citrate buffer (pH 4.8) at 50°C for 30 min. The enzyme reaction was terminated by adding 3.0 mL of 3,5-dinitrosalicylic acid reagent and then immediately boiled for 5 min. The quantity of reducing sugars liberated from the CMC was determined by the DNS method [15]. Total cellulase activity (FPase activity) [17] was measured by incubating 0.5 mL of crude cellulase solution, 1.0 mL of 0.05 M sodium citrate buffer (pH 4.8) and 50 mg of Whatman No.1 filter paper strip at 50°C for 1 h. After incubation, the enzyme reaction was stopped and the amount of reducing sugars liberated from the filter paper strip was measured by the DNS method [15].

2.5 *Identification of cellulase-producing bacteria*

The identification of the selected cellulase-producing isolate was performed by molecular genetics analysis and standard identifications. The PCR amplification and 16S rDNA sequence analysis of the selected isolate were performed by the Thailand Institute of Scientific and Technological Research (Pathum Thani, Thailand). The isolate was further characterized by standard identifications such as Gram staining, endospore staining, motility test, catalase test [18] and oxidase test [19]. Identification of facultative anaerobic bacteria was determined as previously described [13]. The isolate was cultured on anaerobic agar at 29.2°C for 48 h under anaerobic condition in an AnaeroJar (Oxiod, England) assembled with an AnaeroGen sachet (Oxiod). Growth under the saline condition was determined by supplementing nutrient agar with 0–30% (w/v) of NaCl and incubated at 29.2°C for 48 h. For identification of closely related bacteria in the *Bacillus* genus, microscopic methods and antimicrobial activity were further determined. Antimicrobial activity of the cellulolytic isolates was assayed by the disc diffusion method against Gram-negative bacterial pathogens including *E. coli* and *Salmonella* sp. on nutrient agar at 37°C for 48 h.

2.6 *Application on the biotechnological conversion*

To produce reducing sugars using the biotechnological conversion process, the cellulosic biomass was hydrolysed by a selected cellulase-producing bacterium. Rice straw, rubber tree leaves and paper waste, agro-residues and municipal waste in Rayong Province, were used as the carbon source of bacterial culture. The selected cellulase-producing bacterium was cultured in a basal medium (0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl and 0.02% peptone), supplemented with 1% cellulosic biomass powder at 29.2°C under aeration condition for 48 h. The culture medium was collected for reducing sugars determination by the DNS method [15].

3 Results and Discussion

Forty-two soil samples were collected from five shoals in Bueng Samnak Yai coastal wetland. All samples were diluted, spread plated on nutrient agar and incubated under experimental conditions. One hundred and forty-five morphologically dissimilar colonies were isolated and streak plated onto nutrient agar for single colony purification. Eighty-seven isolates exhibited the cellulolytic zone around their colonies after Gram's iodine staining. Hydrolysis capacity values were calculated and a white-pigmented bacterial isolate, strain BR0302, obtained from the Koh-Kok shoal showed a maximum hydrolysis

capacity of 4.15 ± 0.18, greater than the positive control (*B. methylotrophicus* strain RYC01101) by a factor of 1.34. In CMC broth, the bacterium strain BR0302 produced the maximum amount of reducing sugars of 0.158 ± 0.034 mg/mL after 48 h of incubation (Table 1 and Figure 2).

Table 1: Hydrolysis capacity of bacteria after culture on CMC agar and amount of reducing sugars after culture in CMC broth at 29.2°C for 48 h

No.	Bacterial strain	Hydrolysis capacity (HC)	Amount of reducing sugars (mg/mL)
1	BR0102	3.22 ± 0.08	0.132 ± 0.007
2	BR0202	3.24 ± 0.14	0.130 ± 0.002
3	BR0204	3.30 ± 0.05	0.138 ± 0.003
4	BR0302	4.15 ± 0.18	0.158 ± 0.034
5	BR0502	3.60 ± 0.14	0.138 ± 0.002
6	BR0603	3.19 ± 0.20	0.126 ± 0.008
7	BR0701	3.33 ± 0.06	0.138 ± 0.006
8	BR0702	3.42 ± 0.15	0.133 ± 0.017
9	BR0906	3.11 ± 0.19	0.132 ± 0.005
10	BR1101	3.14 ± 0.18	0.132 ± 0.007
11	BR1401	3.60 ± 0.29	0.122 ± 0.005
12	BR1604	3.30 ± 0.14	0.136 ± 0.005
13	BR1901	3.69 ± 0.05	0.134 ± 0.002
14	BR2402	3.16 ± 0.10	0.126 ± 0.002
15	BR2605	3.44 ± 0.20	0.129 ± 0.007
16	BR2901	3.22 ± 0.51	0.130 ± 0.002
17	BR3504	3.26 ± 0.05	0.132 ± 0.001
18	*B. methylotrophicus* strain RYC01101*	3.09 ± 0.39	0.163 ± 0.003

* positive control
Note: The hydrolysis capacity values of any isolates less than the positive control are not shown.

The bacterium strain BR0302 was selected for crude extracellular cellulase production and the cellulase activity was determined. Cellulase activity assays showed that BR0302 yielded 0.121 ± 0.006 U/mL of CMCase activity and 0.057 ± 0.001 U/mL of FPase activity. Surprisingly, BR0302 showed a maximum hydrolysis capacity but enzyme activity was less than the positive control (Table 1 and 2). Ahmad *et al.* [20] suggested that this conflicting result may be due to fluctuations in some experimental parameters which affect the enzyme producing processes such as pH changes in medium, incubation time and incubation temperature. This enzyme activity was compared to other bacteria in the *Bacillus* genus isolated from soil and oil palm meal (Table 2). The comparisons showed that bacterium strain BR0302 had a moderate cellulase activity.

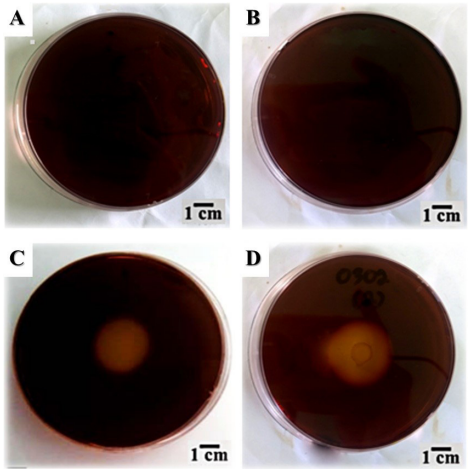

Figure 2: The cellulolytic zone around the colonies on CMC agar plates after Gram's iodine staining. (A) Uninoculated. (B) Inoculated with *E. coli* (negative control). (C) Inoculated with *B. methylotrophicus* strain RYC01101 (positive control). (D) Inoculated with bacterium strain BR0302.

Table 2: Cellulase activity of bacterium strain BR0302 and related species in the *Bacillus* genus

Bacteria	Source	CMCase activity (U/mL)	FPase activity (U/mL)	Ref.
B. velesensis strain P3-1	Soil	0.015	ND	[21]
B. velesensis strain P4-6	Soil	0.015	ND	[21]
B. safensis strain PJ1-24S	Oil palm meal	0.233	ND	[22]
B. altitudinis strain PH27	Oil palm meal	0.170	ND	[22]
B. cereus strain PH4-36	Oil palm meal	0.039	ND	[22]
B. methylotrophicus strain RYC01101*	Bovine faeces	0.230	0.080	[13]
Bacterium strain BR0302	Coastal wetland soil	0.121	0.057	This study

* positive control
ND denotes 'not determined'

Morphology of the bacterium strain BR0302 showed creamy white colonies, flat with entire margin and 5–6 mm in diameter. Bacterial cells were 1 × 3 µm, rod-shaped, Gram-positive, endospore-forming and motile. The cells were arranged singly or in short chains. The endospores were round or oval-shaped, located at

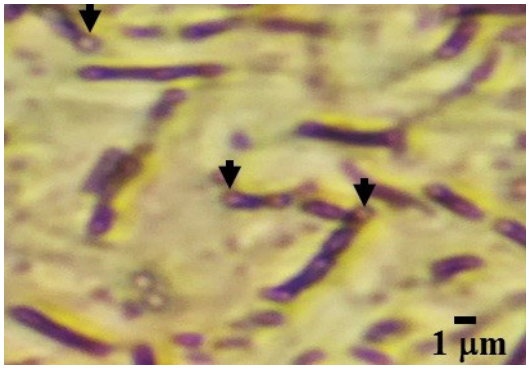

Figure 3: Micrograph of bacterium BR0302 with Gram staining (× 400), arrows indicate the endospores.

the middle and end of the cells. Cell morphology and arrangement is shown in Figure 3. Catalase and oxidase tests were positive. The bacterium was a facultative anaerobe with a salinity tolerance range of 0–5% NaCl. The 16S rDNA sequencing analysis showed that the BR0302 strain was identified as a bacterium in the *Bacillus* genus. It was homologous with *B. thuringiensis*, *B. toyonensis* and *B. cereus* with 100% similarity. The classification of the bacterium strain BR0302 from other closely related species of *Bacillus* genus required identification by unique characteristics, using microscopic methods and antimicrobial activity.

B. thuringiensis (Bt) is a rod-shaped Gram-positive bacterium which exhibits various biological activities including insecticidal, nematocidal and anticancer activity [23]. Lee *et al.* reported that Bt was able to produce unique parasporal crystals after growth on a nutrient agar plate at 30°C for 5 days [24]. The bacterium strain BR0302 did not produce the parasporal crystals after 5 days under microscopic investigation. *B. toyonensis* is a rod-shaped Gram-positive bacterium and its viable spores are used as the active ingredient of the feed additive TOYOCERIN® [25], [26]. TOYOCERIN® has demonstrated inhibition of the growth of pathogenic bacteria such as *Salmonella* sp. and *E. coli*. The antimicrobial activity showed that the bacterium strain BR0302 gave a zone of inhibition against *Salmonella* sp. of 2.0 mm around the paper discs, but had no antimicrobial activity against *E. coli*. From morphology, 16S rDNA sequencing data and antimicrobial activity analysis, the bacterium strain BR0302 could be identified as *B. cereus*. For preliminary application on biotechnological conversion, the agro-residues and paper waste were hydrolyzed by *B. cereus* strain BR0302 to produce reducing sugar. After 48 h of incubation, rice straw, rubber tree leaves and paper waste contained reducing sugars of 0.135 ± 0.001, 0.170 ± 0.000 and 0.123 ± 0.004 mg/mL respectively.

Several strains of *Bacillus* were reported as cellulase-producing bacteria [27]. The cellulase-producing *B. cereus* was isolated from oil palm meal, Phetchaburi Province, Thailand [22]. *B. cereus* strain PH4-39 showed cellulase hydrolysis activity on CMC agar plates by 2.12 after 48 h of incubation and yielded 0.039 ± 0.002 U/mL of CMCase activity. In this study, *B. cereus* strain BR0302 isolated from coastal wetland soil showed more efficient cellulase activity and could be used in the hydrolysis of cellulosic biomass and agro-residues to produce reducing sugars, the prerequisite of the bioethanol fermentation process. Furthermore, it could be utilized in various industries such as food, textiles and fabrics, laundry detergents, pulp and paper and animal feeds.

4 Conclusions

The research of microbes isolated from coastal wetlands has been limited due to frequent flooding conditions. In this study, eighty-seven cellulase-producing bacteria were isolated from coastal wetland soil samples in Rayong Province, Thailand. The bacterium strain BR0302, identified as *B. cereus*, showed maximum cellulolytic activity compared to other isolated bacteria. The *B. cereus* strain BR0302 could be used for the biotechnological conversion of agro-residues and municipal waste, to produce glucose and reducing sugars for production of bioethanol and other biofuels.

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