Cellulase activity of *Bacillus velezensis* isolated from soil in a dairy farm

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**Abstract.** The aim of the present study was to isolate and screen cellulase producing bacteria from soils including characterizing the cellulase activity of the identified bacterial strain. Screening of the cellulase producing bacteria was performed on a carboxymethylcellulose (CMC) agar (pH 7.0) plate at 37°C for 3 days. Bacterial isolate, namely CC1-1, showed the highest cellulase activity on the CMC agar plate. Subsequently, it was identified as *Bacillus velezensis* based on 16S rDNA gene sequence analysis. Cellulase production was carried out under submerged fermentation. The maximum CMCase and FPase activity of 0.030 U/ml and 0.047 U/ml were obtained after 20 h and 32 h, respectively. The optimal pH and temperature of CMCase activity were 6.0 and 60°C, respectively. The cellulase activities were active in a broad pH and temperature range. The CMCase activity was stable at pH 6.0-7.0, 37°C for 120 min and the stability of CMCase was revealed at 50-60°C for 90 min with over 80% remaining activity. However, the optimum pH and temperature of FPase activity were 7.0 and 50°C, respectively. The FPase activity was stable at pH 6.0, 37°C for 90 min and its activity was stable at 60°C for 60 min with over 80% remaining activity. Due to its particular properties, cellulase-producing *B. velezensis* could be a potential candidate for the composting process.

1. Introduction

Cellulose is the most abundant carbohydrate in the plant cell wall. It is a linear polymer of D-glucose residues linked with β-1, 4 glucosidic bonds [1]. Cellulases have been applied in various industries, such as the paper and pulp industry, textile industry, detergent industry, fruit juice extraction, food processing, and animal feed additives as well as in bioethanol production [2]. In nature, complete cellulose degradation requires the synergy of three major types of cellulases: endoglucanase, exoglucanase and β-glucosidase. Endoglucanases randomly cleave the β-1,4 bonds at internal amorphous points of the cellulose polysaccharide chain, generating various lengths of oligosaccharides. Exoglucanase is essential for the cutting of the non-reducing or reducing end of a cellulose chain and liberating either cellobiose or glucose as main products. β-glucosidase catalyzes the hydrolysis of cellobiose to glucose [3].

A number of microorganisms are able to hydrolyse cellulose such as bacteria, actinomycetes and fungi. Most cellulase production has been explored in fungi [4-8]. Compared to fungi, bacteria have considerable benefits for industrial utilization, i.e. easy cultivation, high growth rate and adaptability to substantial genetic manipulations [9]. Extracellular cellulase production from bacteria such as *Cellulomonas* sp. YJ5 [10], *Brevibacillus* sp. DUSELG12, *Geobacillus* sp. DUSELR7, *Paenibacillus*
terrae ME27-1 [11], Bacillus sp., Pseudomonas sp., Serratia sp. [12], B. brevis [13], B. subtilis [14-16], B. thuringiensis [14, 16], B. mojavensis [15] and B. pumilus [16] etc. Cellulose degrading bacteria have been surveyed to obtain more proficient cellulases from various sources such as soils, decomposed plant materials, feces of ruminants, hot springs, and composts [17]. Researchers have been looking for microorganisms with higher cellulase activity. Therefore, the objectives of this study were to isolate and screen the cellulase producing bacteria from soils in the agricultural area and animal farms in a variety of places in Thailand. Moreover, the most active strain from preliminary screening was identified and its cellulase properties were studied. The selected cellulase producing strain has potential benefits for the composting process.

2. Material and methods

2.1. Collection of soil samples
Twenty soil samples were obtained and collected from several sites in Chumphon, Prachuap Khiri Khan and Phetchaburi province, Thailand. The sample was randomly taken to a depth of 10 cm from the area of the lime, banana, pineapple, durian and oil palm tree field including the soil samples from the goat and dairy farms.

2.2. Isolation and screening of cellulolytic bacteria
One gram of sample was added to 9 ml of 0.85% NaCl and serially diluted and the spread plate technique was done using nutrient agar (NA). The culture was grown on 37°C for 24 h and streak plate technique was used for the isolation to obtain the pure colonies. The bacterial isolate was further point inoculated on a carboxymethylcellulose agar (CMC agar) containing (g/L): peptone 10; K₂HPO₄ 2; MgSO₄.7H₂O 0.3; (NH₄)₂SO₄ 2.5; CMC 10 and agar 15 and cultivated at 37°C for 3 days. A 1% Congo red solution was poured onto the agar plate and left for 15 min at room temperature. It was then destained with 1 M NaCl solution for 15 min. A clear zone was shown around bacterial colony displaying cellulose degradation [18]. The maximum ratio of the clear zone diameter to colony diameter was investigated so as to choose the best cellulase producing strain.

2.3. Molecular identification of bacterial isolate
The selected cellulolytic bacterial isolate was sent to Apical Scientific Sdn Bhd., Malaysia for sequence analysis. Molecular identification of the bacterial isolate was investigated from the nucleotide sequence of the 16S ribosomal DNA (rDNA). Sequence homology was analyzed using the nucleotide BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic construction was performed using the neighbor-joining method using MAGA 7.0 with 1,000 replicates of bootstrap values.

2.4. Cellulase production
The bacterial isolate was inoculated in 150 ml CMC broth containing (g/L): CMC 10; FeSO₄.7H₂O 0.02; MgSO₄.7H₂O 0.2; KNO₃ 0.75; K₂HPO₄ 0.5; CaCl₂ 0.04; yeast extract 2; and D-glucose 1. The culture was then cultivated at 37°C, 150 rpm for 24 h for inoculum preparation. The inoculum (5%) was transferred into the same medium in a 5 L fermenter. The bacterium was grown at 37°C, airflow rate of 1.0 vvm, stirring rate of 200 rpm, for 72 h and the sample was withdrawn every 4 h. The crude enzyme was harvested by centrifugation at 14,000×g, 4°C for 10 min. The culture filtrate was analyzed for cellulase activity assay.

2.5. Cellulase assay
Endocellulase (CMCase) activity and filter paper activity (FPase) were analyzed using the modified method proposed by Wood and Bhat [19]. The CMCase activity was investigated by mixing the enzyme solution (0.5 ml) with 1.0 (w/v) CMC in 0.05 M phosphate buffer (pH 7.0) 0.5 ml and incubated at 37°C for 30 min. The reducing sugar liberated was assayed using the dinitrosalicylic (DNS) method [20] with glucose as the sugar standard. The total cellulase activity or FPase activity assay in the culture filtrate
was the same as the CMCase assay method while for the substrate a 1x6 cm strip of Whatman No.1 filter paper immersed in 1.0 ml of 0.05 M phosphate buffer (pH 7.0) was used and incubated at 37°C for 30 min. The reducing sugar was assayed using the DNS method. One unit of cellulase (CMCase or FPase) was the quantity of enzymes which produced 1 µmole of glucose or reducing sugar per minute under the specified conditions. All experiments of cellulase activity assay were performed in three replications and the results were reported as mean±SD.

2.6. Characterization of crude cellulase activity

2.6.1. pH profile and stability. The various buffers such as 50 mM of glycine-HCl buffer (pH 3.0), citrate buffer (pH 4.0-5.0), phosphate buffer (pH 6.0-7.0), Tris-HCl (pH 8.0) and glycine-NaOH (pH 9.0-10.0) were used to investigate the influence of pH on the activity and stability of CMCase and FPase. Crude enzyme was mixed with 1% CMC or a 1x6 cm strip of Whatman No.1 filter paper prepared in each buffer solution. Determination of CMCase and FPase activities was done after the reaction assay was kept at 37°C for 30 min. The highest activity was defined as 100% relative activity and the others were calculated by comparison with the highest activity.

pH stability was performed by keeping crude enzyme in different buffers as above described at 37°C for 3 h. The sample was taken every 30 min for determination of the residual CMCase and FPase activities according to the standard assay method.

2.6.2. Temperature profile and stability. Crude enzyme was mixed with 1% CMC or a 1x6 cm strip of Whatman No.1 filter paper prepared in a suitable buffer solution. The CMCase and FPase activities were assayed after the reaction mixture was kept in the temperature range of 30-90°C for 30 min. The highest activity was defined as 100% relative activity and others were calculated by comparison with the highest activity.

Regarding the thermostability measurement, the crude enzyme was incubated in a variety of temperatures as described above for 3 h. The sample was taken every 30 min for determination of the residual CMCase and FPase activities according to the standard assay method.

3. Results and discussion

3.1. Isolation and screening of cellulase producing bacteria
Thirty-six bacterial isolates were obtained from 20 soil samples. After selection of the cellulase producing strains on the CMC agar plates, the results showed that three bacterial isolates (CC1-1, CC2-1 and GF-4) produced extracellular cellulase for the hydrolysis of CMC. The highest ratio of clear zone size to colony size was found in CC1-1 isolated from soil taken from the dairy farm in Phetchaburi province.

3.2. Identification of bacterial isolate by using molecular technique
The selected bacterial isolate, CC1-1, was sequenced approximately 1,500 bp of 16S rDNA gene. The nucleotide sequence was aligned and compared with others from GenBank using the nucleotide BLAST tool. CC1-1 was demonstrated to have 99% similarity with Bacillus sp. Phylogenetic analysis showed that CC1-1 was identified as Bacillus velezensis as shown in figure 1. Several cellulose degrading bacteria have been found in different ecological niches. In addition, Bacillus was reported to be the dominant cellulose degrading bacteria in samples collected from soils such as B. brevis [13], B. subtilis [14-16], B. cereus [14-15], B. thuringiensis [14, 16], B. mojavensis [15] B. pumilus [16].

3.3. Production of cellulase under submerged fermentation
Cellulase production from B. velezensis was carried out in CMC broth at 37°C for 72 h. The highest CMCase and FPase activities of 0.030 U/ml and 0.047 U/ml were obtained after 20 and 32 h, respectively. In the previous reports, several bacterial cellulases activities were similar to this study.
CMCase and FPase activities were 0.079 U/ml and 0.011 U/ml, respectively in *B. pumilus* EB3 after 24 h fermentation [21]. Subsequently, Rastogi et al. [22] reported that CMCase activity of 0.020 and 0.058 U/ml was received from *Brevibacillus* sp. DUSELG12 and *Geo bacillus* sp. DUSELR7 after 10 and 7 days of cultivation, respectively. However, the higher CMCase activities were found in *B. cereus* B7 (0.440 IU/ml/min), *B. cereus* B37 (0.410 IU/ml/min), *B. subtilius* B20 (0.357 IU/ml/min), and *B. thuringiensis* B49 (0.334 IU/ml/min) [14].

![Figure 1. Phylogenetic analysis of bacterial isolate, CC1-1 based on 16S rDNA gene sequences was created by the neighbor-joining method. Numbers represent bootstrap values and the accession numbers of the strains are given in brackets.]

3.4. Characterization of crude cellulase activity

3.4.1. pH profile of CMCase and FPase activity. The effect of pH value on cellulase activity was studied in a wide pH range of 3.0-10.0 as shown in figure 2. The highest CMCase activity was achieved at pH 6.0. Remaining activities of 98%, 80% and 72% was found at pH 5.0, 4.0 and 3.0, respectively. At pH 8.0-9.0, the enzyme activities remained at 64% with the remaining activity of 24% at pH 10.0. The optimum pH of CMCase from *B. velezensis* in this study was similar to *B. licheniformis* 2D55 [23]. Several research works revealed that the optimum pH of CMCase from bacteria was 5.0-5.5. The CMCase activity from the composite microbial system (FH3) showed the highest value at pH 5.0 [24]. The maximum CMCase activities of *Geo bacillus* sp. DUSELR7 and *Brevibacillus* sp. DUSELG12 were pH 5.0 and pH 5.5, respectively [22]. Moreover, the optimum pH of CMCase activity from *P. terrae* ME27-1 was 5.5 [11]. However, the optimum purified CMCase from *Cellulomonas* sp. YJ5 was pH 7.0 [10]. The highest FPase activity of *B. velezensis* was at pH 7.0 and its activity decreased in both the acidic and basic pH ranges. In contrast, the optimum pH of FPase activity from the composite microbial system (FH3) was pH 4.5 [24] and that from *B. licheniformis* 2D55 was pH 6.0 [23].
Figure 2. pH profile of CMCase and FPase activities from B. velezensis at 37°C for 30 min.

3.4.2. Temperature profile of CMCase and FPase activity. The optimum temperature of cellulase was studied ranging from 30-90°C as shown in figure 3. The optimal temperature of CMCase activity was 60°C. The remaining CMCase activity was even 95% at 70°C, but its activity rapidly decreased at 80-90°C. The maximum temperature of the purified CMCase activities from B. pumilus EB3 [21] and Cellulomonas sp. YJ5 [10] were similar to this study. However, those of CMCase activities from P. terrae ME27-1, Brevibacillus sp. DUSELG12 and Geobacillus sp. DUSELR7 were 50°C, 55°C and 75°C, respectively [11, 22]. The optimum temperature of FPase activity of B. velezensis was 50°C and its activity sharply decreased after 50°C, whereas that of B. licheniformis 2D55 was 80°C [23].

Figure 3. Temperature profile of CMCase and FPase activities from B. velezensis at optimum pH for 30 min.

3.4.3. Effect of pH on CMCase and FPase stability. Incubation of cellulase at various times in buffer solutions with pH values between 3.0-8.0 at 37°C. The cellulase activities were active in a wide pH
range. The CMCase stability was at pH 6.0-7.0 for 120 min and the activity was maintained at over 80% as shown in figure 4. The residual activity of CMCase was almost 70% under pH 5.0 and its activity maintained 50% at pH 8.0 after 120 min. However, CMCase from Cellulomonas sp. Y15 was stable at pH 7.5-10.5 [10]. The CMCase activity from P. terrae ME27-1 displayed stability in a wide pH range of 5.0-9.5 [11]. The FPase stability of all pH ranges gradually decreased after a longer incubation period. The remaining activity of approximate 66-70% was found in the pH range of 5.0-7.0 for 180 min as shown in figure 5. At pH 3.0 and pH 8.0, the residual FPase activity was approximately 50% after 120 min. On the contrary, the FPase activities of Bacillus strain MAM-29 and Bacillus strain MAM-38 were the best at high pH values but low at low acidic pH [25].

![Figure 4](image4.png)

**Figure 4.** Effect of pH on CMCase stability after incubation in various pH buffer solutions at 37°C for 180 min.

![Figure 5](image5.png)

**Figure 5.** Effect of pH on FPase stability after incubation in the various pH buffer solutions at 37°C for 180 min.

### 3.4.4. Effect of temperature on CMCase and FPase stability.

The thermostable enzymes are a very important parameter for industrial applications. The cellulase activities were active in the broad range
of temperature. The CMCase activities were stable at 50-60°C for 90 min and their activities decreased with prolonged incubation as shown in figure 6. The remaining CMCase activity was approximately 50% at 70°C for 90 min whereas the activities were unstable at higher temperatures (80°C and 90°C). The remaining FPase activity at approximately 80% was found at 60°C for 60 min as shown in figure 7. On the other hand, FPase activity decreased with the increasing temperature (70-90°C). Regarding the temperature profile of CMCase and FPase, the optimum temperatures of CMCase and FPase were 60°C and 50°C, respectively. Therefore, the remaining CMCase and FPase activities after incubation at 30-40°C were lower than those at 50-60°C for 180 min. The activity of cellulases of *B. velezensis* was more thermostable than previous studies. The CMCase and FPase activities from the composite microbial system (FH3) were stable at 30-45°C for 3 h, and then decreased sharply above 50°C [24]. The purified CMCase activities from *Cellulomonas* sp. YJ5 [10] and *P. terrae* ME27-1 [11] were stable at temperatures below 50°C. Similar results with the present study were observed from *Brevibacillus* sp. DUSELG12, CMCase activity was stable at 50°C above 1 h [22].

![Figure 6](image6.png)

**Figure 6.** Effect of temperature on CMCase stability after incubation at different temperatures for 180 min.

![Figure 7](image7.png)

**Figure 7.** Effect of temperature on FPase stability after incubation at different temperatures for 180 min.
### 4. Conclusions

Bacterial isolate, CC1-1, was isolated from soil collected from the area of a dairy farm in Phetchaburi province, Thailand. It showed the maximum hydrolysis capacity of CMC by cellulase. Subsequently, it was identified as *B. velezensis*. The properties of cellulases were investigated after cellulase production under submerged fermentation process. The optimum pHs of CMCase and FPase activities were pH 6.0 and 7.0, respectively. The optimum temperatures of these activities were 60°C and 50°C, respectively. Because of the ability to retain good activity in a broad pH and temperature range, the strain has tended to be used in the composting process. Further studies are recommended on *B. velezensis* and strain improvement for increasing cellulose hydrolysis in agricultural wastes which reduces composting time.

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