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Characterization and identification of potential cellulolytic bacteria for bio-degradation of durian shell waste in Mekong Delta, Vietnam

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Abstract. *Tat TQ, Khanh ND, Thi QVC. 2024. Characterization and identification of potential cellulolytic bacteria for bio-degradation of durian shell waste in Mekong Delta, Vietnam. Biodiversitas 25: 4284-4291.* Microorganisms play crucial roles in the decomposition of plant biomass. This study aimed to isolate, screen, and identify potential mesophilic cellulose-degrading bacterial (CDB) strains from natural compost from durian shells (DS). Results revealed that a total of fifteen aerobic strains were isolated at 37°C, thirteen of which exhibited enzymatic degradation of cellulose, with cellulolytic index (CI) values ranging from 0.34 to 3.22. Five strains with CI values greater than 2.0 presented potential extracellular cellulase enzyme activity, with carboxymethyl cellulase (CMCase) at 0.18-0.31 U/mL and filter paper cellulase (FPase) at 0.03-0.19 U/mL. The DSC.03 and DSC.04 strains showed the highest CMCase and FPase activities, respectively. Subsequently, molecular identification and phylogenetic analysis confirmed DSC.03 (PP851408.1) and DSC.04 (PP851410.1) isolates as *Bacillus subtilis* and *Bacillus velezensis*, respectively. The maximum bio-degradation percentages were 86.85% for filter paper and 48.72% for dry DS powder, which occurred in the mixed-culture treatment of the two selected strains after 15 days of incubation under shaking conditions at 150 rpm and 37°C. These findings indicate for the first time that natural durian shell compost contains potential cellulose-degrading bacterial communities with high extracellular enzyme-producing ability and active interactions with each other, which can be beneficial for the fermentation or composting of DS waste.

Keywords: *Bacillus subtilis*, *Bacillus velezensis*, bio-degradation, cellulolytic bacteria, durian shell waste

INTRODUCTION

Vietnam, being an agricultural country, has the potential to develop various industries for cultivation, forestry, husbandry, and food processing. Specifically, in agricultural production and food processing, numerous by-products produce approximately 156 million tonnes, of which by-products after harvesting and processing account for approximately 88.9 million tonnes (Chu-Ky et al. 2022). Among agricultural byproducts, durian fruit waste is a potential material source (Nguyen et al. 2024). In 2022, Vietnam's durian output reached 642.600 tons (Tran et al. 2022). However, the ratio of edible flesh and seeds of durian is low at approximately 30-40%, and 60-70% of the fruit is an inedible shell (Ly et al. 2024). Thus, with high production and consumption rates, durian shell (DS) is an available, large, and low-cost raw material source. Typically, people burn or dispose of these by-products in landfills, which can cause environmental pollution. However, they can be utilized for multiple purposes, such as fertilizer and energy production and the extraction of biochemical and nutrient components, to increase their economic value (Tu Nguyen et al. 2022). The main compound in these materials is cellulose, a linear polymer made of glucose subunits linked by a β-1,4 glucosidic bond with a chain length that varies between 100 and 14,000 residues. According to Juturu and Wu (2014), under the activity of cellulase enzyme complexes, which include three synergistic enzyme types (endoglucanase-EC 3.2.1.4, exoglucanase-EC 3.2.1.91, and β-glucosidase-EC.2.1.21), the glucosidic bonds in cellulose fibers are cleaved, releasing glucose monomers (cellobiose and glucose) as the end product. Many microorganisms can naturally synthesize cellulase enzymes on cellulosic materials.

Over the past few years, there has been a significant increase in the isolation and identification of cellulose-degrading microorganisms. These microorganisms play crucial roles in the organic carbon cycle and exist in various natural environments (Liu et al. 2021), including the soil, water, gastric juice, and feces of herbivores, as well as the guts of invertebrates (termites, snails, caterpillars, and bookworms) (Gupta et al. 2012; Dewiyanti et al. 2022; Mandic-Mulec et al. 2016). These microorganisms can be aerobic, anaerobic, mesophilic, or thermophilic and are identified primarily as bacteria or fungi. In the past, research has focused mainly on cellulase production from filamentous fungi, such as *Penicillium*, *Trichoderma*, *Aspergillus*, *Talaromyces*, *Phanerochaete*, and *Fomitopsis* owing to their abundant production of cellulase enzymes. However, the high production costs of these enzymes have led researchers to shift their focus toward bacteria (Korsa et al. 2023), which have several advantages, such as high natural diversity, expression of multienzyme complexes, rapid growth rates, and resistance to extreme environments

(stability at extremes of temperature and pH). In addition, bacterial cellulases are highly effective and potent catalysts for industrial and environmental conservation applications (Barzkar and Sohail 2020). In addition, bacteria are easily genetically modified to improve cellulase production yield (Adebami and Adebayo-Tayo 2020). As a result, the search for new cellulose-degrading bacterial strains has increased, and new species have been isolated from various natural habitats. Bacteria belonging to the genera *Saccharophagus*, *Bacillus*, *Clostridium*, *Thermobifida*, *Cytophaga*, and *Fibrobacter* have been screened and shown to produce different types of cellulase under both anaerobic and aerobic conditions (Liu et al. 2021). Thapa et al. (2020) reported that the cellulolytic activity of bacteria depends on their source of occurrence. Recent studies have reported successful bioconversion of various complex cellulosic waste materials, such as coffee exocarps (Bui 2014) and paper cup waste (Karthika et al. 2020). However, there is no research on bacterial cellulolytic activity in the composting of DS waste. Therefore, the aim of this study was to isolate and screen cellulolytic bacteria from DS waste and construct a phylogenetic tree.

MATERIALS AND METHODS

Sample collection and preparation

Approximately 10 kg of fresh DS of the 'RI6' cultivar was collected from local markets in the My Tho city area, Tien Giang Province (Mekong Delta, Vietnam), and separated into two parts. In the first part, approximately 8 kg of material was cut into 1-1.5 cm thick small pieces, and the natural composting process was carried out for 30 days in plastic boxes for bacterial isolation. The remaining DSs were approximately 2 kg, washed with tap water, cut into small pieces, dried at 80°C to <12% moisture, and ground into a fine powder (0.2 mm). The powder was subsequently soaked in 1% (w/w) sodium hydroxide solution at a ratio of 1:10 (w/v) for 2 hours at room temperature. Alkaline pretreatment causes the lignocellulose in the material to become swollen and porous, thereby increasing the efficiency of enzymatic hydrolysis (Ojumu et al. 2003). After that, DS powder was filtered and washed with distilled water until the wash water became neutral. Finally, the residue was redried at 80°C until constant weight and stored for further experiments at 4°C.

Culture media and chemicals

Carboxymethyl cellulose (CMC-Na) agar medium for bacterial isolation comprised of (10 g of CMC-Na, 4 g of KH_2PO_4, 4 g of Na_2HPO_4, 2 g of tryptone, 0.2 g of $MgSO_4.7H_2O$, 0.004 g of $FeSO_4.7H_2O$, 0.001 g of $CaCl_2.2H_2O$, and 15 g of agar/L), and the pH was adjusted to 7.0. Luria-Bertani (LB) broth medium for bacterial growth comprised (10 g of peptone, 5 g of yeast extract, and 10 g of NaCl/L). The mineral salt medium (MM) for the cellulose substrate degradation tests comprised (3.5 g of $Na_2HPO_4.2H_2O$, 1 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.1 g of $MgCl_2.6H_2O$, 50 mg of $Ca(NO_3)_2.4H_2O$, 1 mL of vitamin B12, and 1 mL of trace salt solution/L), and the pH was adjusted to 7.2. The trace salt solution contained (0.07 g of $ZnSO_4.5H_2O$, 0.01 g of MoO_3, 0.01 g of $CoCl_2.6H_2O$, 0.01 g of H_3BO_3, 0.01 g of $MnSO_4.5H_2O$, 0.01 g of $NiSO_4.7H_2O$, and 0.005 g of $CuSO_4.5H_2O$) in 100 mL of water. All chemicals used were of high purity (>90%), including CMC-Na, agar, and peptone, they were supplied by Merck (Darmstadt, Germany); other chemicals were obtained from Xilong (Shantou, China).

Isolation and primary screening of cellulolytic bacterial strains

Isolation of cellulose-degrading bacterial (CDB)

For this, 10 g of durian shell compost (DSC) sample was mixed with 90 mL of distilled water and then shaken at 250 rpm at 37°C for 60 minutes on an orbital shaker to homogenized the samples, this was considered as 10^{-1} dilution, which was subsequently diluted tenfold to achieve 10^{-5} dilutions. Next, 1 mL of each of the 10^{-3}, 10^{-4}, and 10^{-5} dilutions were placed on agar nutrient plates containing CMC-Na media and incubated aerobically at 37°C for 48-72 hours. After incubation, all single colonies with different colony morphologies were sub-cultured onto nutrient plates to obtain pure strains, and stored at 4°C until subsequent experiments (Bui 2014).

Primary screening

The pure isolates were inoculated onto CMC-Na agar nutrient plates with three spots per plate, and then incubated at 37°C for 48 hours. After incubation, 1% (w/v) Congo red reagent was added to cover the entire medium, and the mixture was left for 15 minutes before washing with 1 M NaCl. A clear zone around the colonies indicated cellulose degradation, resulting in the isolation of CDB. The diameter (mm) of clearance zone (D) relative to the diameter of the colony (d) was measured via a caliper to determine the cellulolytic index (CI) value for the CI categories of low ($CI \leq 1.0$), medium ($1.0 < CI < 2.0$), and high ($CI \geq 2.0$). The isolates with high CI values were selected for further study. The value was determined via the following formula (Ferbiyanto et al. 2015):

$$CI = \frac{D-d}{d}$$

Determination of cellulase activity of potential cellulolytic bacterial strains

Crude cellulase production

Three colonies of each test strain were cultured in a 150 mL Erlenmeyer flask containing 50 mL of LB broth medium at 150 rpm and 37°C overnight. Next, 2 mL of the suspension was transferred to a 150 mL Erlenmeyer flask containing 40 mL of MM medium, which consisted of 0.1 g of filter paper (two 1×6 cm strips, 0.05 g per strip) as the sole carbon source. As a negative control, 2 mL of the bacterial culture was replaced with distilled water. The cultures were then incubated at 150 rpm and 37°C for 72 hours. After incubation, the cultures were centrifuged at 5,000 rpm for 15 minutes at 4°C, supernatant was collected as a crude enzyme for enzyme assays (Bui 2014).

Cellulase activity assays

The 3,5-dinitrosalicylic acid (DNS) reaction assay was conducted to determine the activity of cellulase enzymes, specifically carboxymethyl cellulase (CMCase) and filter paper cellulase (FPase). To determine CMCase activity, 0.5 mL of the collected crude enzyme was mixed with 0.5 mL of 50 mM citrate buffer (pH 4.8) solution containing 2% (w/v) CMC. In contrast, for FPase activity, 0.5 mL of the crude enzyme was mixed with 1 mL of 50 mM sodium acetate buffer (pH 4.8) solution containing a dry filter paper strip (0.05 g). The mixtures were then incubated at 50°C for 30 minutes for the CMCase assay and 60 minutes for the FPase assay. After incubation, 3 mL of the DNS reagent was added to 1 mL of the reaction mixture, which was subsequently heated in boiling water for 5 minutes. The reactions were then cooled to room temperature by placing the tubes in an ice-water bath, and absorbance was measured at 540 nm via a UV spectrophotometer. The amount of reducing sugars was determined via a standard graph of glucose. One unit of CMCase/FPase refers to the number of enzymes that can liberate 1 µmol of reducing sugars (expressed as glucose) per minute under standard conditions (Lokapirnasari et al. 2015). The cellulase activity was determined via the following formula:

$$\text{Enzyme activity} = \frac{C \times 1000}{V \times t}$$

Where:
C: glucose production (mg)
V: enzyme dosage (mL)
t: reaction time (minutes)

Identification of potential cellulolytic bacterial strains

Preliminary identification

The bacterial strains that showed high cellulose-degrading ability were selected for morphological, physiological, and biochemical studies to identify them at the genera and species level. Biochemical tests, namely Gram staining, catalase, oxidase, indole, fermentation, and starch hydrolysis tests were conducted via standard methods. The results of their physiological and biochemical characteristics were compared to Bergey's Manual of Determinative Bacteriology (Bergey 1994).

Molecular identification

The bacteria with the greatest cellulase activity were cultured in an LB broth medium enriched at 150 rpm and 37°C for 48 hours. The suspension was centrifuged at 10,000 rpm for 10 minutes to harvest the cells, which were subjected to total genomic DNA extraction (Ihrmark et al. 2012). The 16S rRNA gene fragment was amplified via universal bacterial primers with the forward primer 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and the reverse primer 1492R (5´-TACGGYTACCTTGTTACGACTT-3´). The polymerase chain reaction (PCR) mixture consisted of a 25 µL mixture containing distilled water, 1X PCR buffer, 2.0 mM $MgCl_2$, 150 µM dNTPs, 10 pmol of each primer, 2.0 UI of Taq DNA polymerase, and 40 ng of DNA sample. The PCR cycle was performed in a thermal cycler (Eppendorf) with the following thermal conditions: initial denaturation at 95°C for 3 minutes; 30 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds; and a final extension at 72°C for 10 minutes. The resulting PCR product was then electrophoresed on a 1.5% agarose gel and purified before being sent to Nam Khoa Biotek Company (Vietnam) for sequencing. The nucleotide sequences obtained were compared via the BLAST program on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence homology analysis of the 16S rRNA gene was conducted via GenBank data. A phylogenetic analysis with the neighbour-joining model was performed via the MEGA version 11 program. The reliability of the branching and clustering patterns was estimated with 1,000 bootstrap replicates. The 16S rRNA data for identification of strains DSC.03 and DSC.04 were deposited in the NCBI database under the following serial numbers: PP851408.1 and PP851410.1, respectively.

Cellulosic substrate disintegration experiments

The gravimetric determination method was conducted to estimate cellulose degradation in DS powder by the identified bacterial strains according to Bui (2014) with mild modifications. In eight separate 250 mL Erlenmeyer flasks each containing 100 mL of MM four of them supplemented with 0.25 g of filter paper while the others supplemented with 0.25g of DS powder were added to 5 mL of overnight culture, while the negative control flask was replaced with 5 mL of distilled water. In particular, 5 mL culture including 2.5 mL for each pure bacterial culture was used in the mix-culture flasks. All the flasks were sealed with aluminum foil and incubated at 150 rpm and 37°C for 15 days. After incubation, the mixtures were centrifuged at 5,000 rpm for 15 minutes at 4°C to collect the pellets. The resulted pellets were washed repeatedly with diluted nitric acid and hydrochloric acid, distilled water, and then dried at 80°C until a constant weight was achieved. The bio-degradation rate (%) was determined via the following formula:

$$\text{Bio-degradation rate (\%)} = \frac{m_1 - m_2}{m_1} \times 100$$

Where:
m_1: The weight of the original filter paper or DS powder (g)
m_2: The constant weight of pellets (g)

Statistical analysis

All the experiments were performed in triplicates. All the data were entered, calculated, and graphed via Microsoft Excel 2019 software. One-way analysis of variance was performed via Minitab 16 software to determine the significance of differences at $p<0.05$, and Tukey's test was applied.

RESULTS AND DISCUSSION

Isolation and screening of cellulolytic bacteria

A total of 15 strains with varying colony morphologies were isolated and purified from a DSC sample after 30 days of natural composting. The nutrient media used for isolation contained CMC as the sole source of carbon.

Among the 15 isolates, only 13 (approximately 86.67%) were positive for cellulase activity according to the Congo red staining method. The diameter of each colony, clearance zone, and cellulolytic index are presented in Table 1. These values were significantly different ($p<0.05$) among the isolates. The isolates with a cellulolytic index greater than 2.0 were named DSC.03, DSC.04, DSC.11, DSC.13, and DSC.15 were selected for further analysis (Figure 1). According to Dewiyanti et al. (2022), habitats containing cellulosic materials are the best sources for finding cellulose-degrading microorganisms, as the degradation of these substrates is a complex process that requires the participation of various microbial enzymes.

Previous studies have widely used the Congo red staining method for screening cellulose-degrading microorganisms. Among the 5 selected strains, DSC.03 showed the highest potential, with a cellulolytic index of 3.22. The range of cellulolytic index values obtained in the present study (from 2.32 to 3.22) is greater than the range reported by Dewiyanti et al. (2022) (from 0.07 to 0.8) and similar to the range reported by Li et al. (2020) (from 2.50 to 3.25) for cellulolytic aerobic bacterial isolates from soils in industrial and agricultural areas in Kerala and silkworm excrement samples, respectively.

Table 1. The cellulolytic activity of different isolates

Isolates	d (mm)	D - d (mm)	CI	CMCase (U/mL)	FPase (U/mL)
DSC.01	2.87^{efg}	3.50^{de}	1.24^{de}	NT	NT
DSC.02	4.60^{abc}	7.40^{c}	1.58^{d}	NT	NT
DSC.03	5.43^{a}	17.47^{a}	3.22^{a}	0.31^{a}	0.16^{b}
DSC.04	4.87^{ab}	13.93^{b}	2.86^{abc}	0.27^{ab}	0.19^{a}
DSC.05	2.33^{gh}	3.53^{de}	1.52^{d}	NT	NT
DSC.06	2.20^{gh}	0.67^{fg}	0.34^{fg}	NT	NT
DSC.07	3.43^{def}	0.00^{g}	0.00^{g}	NT	NT
DSC.08	4.23^{bcd}	0.00^{g}	0.00^{g}	NT	NT
DSC.09	3.67^{cde}	0.07^{fg}	0.02^{g}	NT	NT
DSC.10	1.87^{h}	2.80^{ef}	1.49^{d}	NT	NT
DSC.11	3.07^{efg}	7.43^{c}	2.43^{bc}	0.24^{b}	0.05^{c}
DSC.12	3.13^{efg}	2.60^{efh}	0.82^{ef}	NT	NT
DSC.13	2.60^{fgh}	6.00^{cd}	2.32^{c}	0.18^{c}	0.08c
DSC.14	4.87^{ab}	1.63^{efg}	0.34^{fg}	NT	NT
DSC.15	2.63^{fgh}	7.93^{c}	3.02^{ab}	0.25^{b}	0.03^{d}

Notes: The values are the means (SDs). Representative data from three biological replicates are shown. Means followed by the same letters in each column are not significantly different according to the least significant difference (Tukey) test ($p<0.05$). NT: Not tested

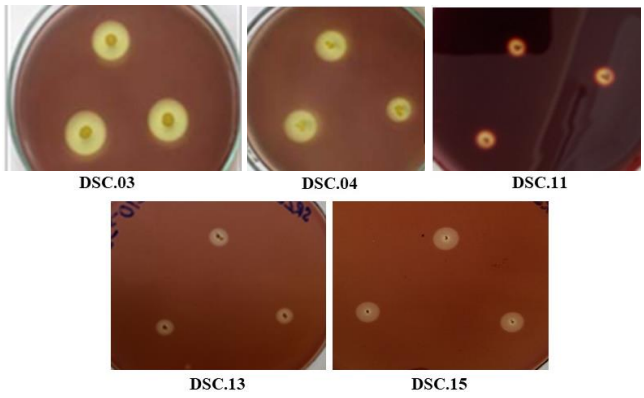

Figure 1. Zone of hydrolysis of cellulose by 5 selected isolates

In addition, approximately 46% of CDB isolates exhibit endoglucanase activity, and only a small proportion of these isolates exhibit exoglucanase (degradation) activity (Khotimah et al. 2020). The cellulolytic indices of bacterial strains vary depending on the genes they possess and the carbon source they use. Typically, the diameter of the clear zone is greater than the diameter of the colony, as cellulase enzymes are secreted into the surrounding environment by cellulose-degrading bacteria. Interestingly, bacteria cannot enter cellulose molecules because their size is smaller than the size of cellulose molecules, so cellulosic substrate degradation is based on an extracellular cellulase enzyme activity (Juturu and Wu 2014). These results suggested that the bacterial strains present in DSCs have the potential for cellulose degradation.

Determination of cellulase activity

The results showed that all five strains selected for enzyme production exhibited extracellular cellulase activity after 72 hours when grown in MM supplemented with filter paper as the sole carbon source. The CMCase and FPase activities ranged from 0.19-0.31 U/mL and 0.03-0.18 U/mL, respectively (Table 1). In addition, the dispersion of bacterial strains on the graph, as shown in Figure 2, indicates significant differences in their cellulolytic activities. These four cellulolytic activity attributes (CMCase, FPase, CI value, and d) can be divided into two distinct regions based on their distributions. Region 1 includes the CMCase, FPase, and CI value properties, with principal component analysis (PCA) eigenvectors of the screening data of 0.572, 0.565, and 0.527, respectively. They were all located on the positive side of the first component axis and had high values, indicating their strong influence on the first principal component (PC1). On the other hand, region 2 only includes the d property (-0.894), which was located on the negative side of the second component axis, suggesting its great contribution to the second principal component (PC2). Furthermore, the CI, CMCase, and FPase values were close vectors, indicating that these three values have a strongly positive relationship. However, the vector direction of the d and CI values was nearly 90°, which suggested that there was no relationship between these two values. These values are not inversely related (the vector direction was 180°) (Putri et al. 2024). Thus, the two strains, DSC.03 and DSC.04 had the most potential and they were similar cellulolytic activities.

The DSC.03 isolate showed the greatest amount of CMCase (0.31 U/mL), while the highest amount of FPase (0.19 U/mL) was detected by DSC.04. Thus, these samples were selected for DS substrate disintegration experiments and identification. The cellulase enzyme activity results revealed that both the strains showed greater CMCase activity than FPase activity. This finding is consistent with previous findings for *Bacillus cereus*, which showed CMCase and FPase activities of 3.52 UmL^{-1} and 0.34 UmL^{-1}, respectively (Karthika et al. 2020). Gupta et al. (2012) reported that the CMCase and FPase activities were 0.40 and 0.20 $IUmL^{-1}$, respectively, for CDB 8 and CDB 10 strains isolated from the guts of termites and snails. However, a study by Rastogi et al. (2009) revealed that the maximum

FPase activity of the *Geobacillus* sp. DUSELR7 strain culture was only 0.043 U/mL on day 8. This difference in intergeneric strains may have impacted the cultivation process and led to variations in cellulase activities. For most strains, the results were consistent with the general understanding that strains selected from CMC-Na as a carbon source tend to have relatively high CMCase activity (Ma et al. 2020). The DSC.03 and DSC.04 strains exhibited advantages in the secretion of cellulases, resulting in high activity of both enzymes. Interestingly, strains with both CMCase and FPase activities did not always appear together in the present study. The CMCase activity was highest for the DSC.03 strain at 0.31 UmL^{-1}, whereas FPase activity was highest for the DSC.04 strain at 0.19 UmL^{-1}. In addition, although CMCase activity was greater in the DSC.15 strain than in the DSC.11 and DSC.13 strains, it had the lowest FPase activity. These trends may have occurred because different strains can produce different cellulases under the same or other carbon sources (Liu et al. 2021). However, according to Ma et al. (2020), wild bacteria typically have low enzyme production capacity and activity. Therefore, it is necessary to use modified technology to improve the ability of these materials in the future.

Identification of cellulolytic bacteria

Two potential isolates of cellulase-producing enzymes, DSC.03 and DSC.04, were identified based on their morphological, biochemical, and physiological characteristics and their 16S rRNA gene sequences. On morphological observation, single colonies of the DSC.03 strain grown on nutrient media supplemented with CMC appeared to be circular and whitish and had a smooth, convex surface with entire edges. In contrast, the DSC.04 strain had irregular, white colonies with flat, wrinkled, and undulating surfaces (Figure 3). The results for further physiological and biochemical tests were collected in Table 2. Based on their physiological and biochemical characteristics, the morphology of their colonies, and Bergey's universal methods, DSC.03 and DSC.04 could preliminarily belong to the genus *Bacillus*.

The agarose gel electrophoresis results of the 16S rRNA gene sequences of strains DSC.03 and DSC.04 are shown in Figure 4. The DNA fragments amplified via PCR were single bands weighing approximately 1500 bp each. The PCR products of strains DSC.03 and DSC.04 were sequenced to obtain the full-length 1424 bp and 1442 bp gene sequences, respectively. The phylogenetic trees of the two isolates were constructed based on their 16S rRNA gene sequences, as depicted in Figure 5. The results of phylogenetic analysis revealed that *Bacillus* sp. is closely related to DSC.03 and DSC.04 strains and had 100% similarity to *Bacillus subtilis* strain FUA2233 (accession no. CP154923.1) and *Bacillus velezensis* strain B268 (accession no. CP053764.1), respectively. Combined with the physiological and biochemical results, DSC.03 and DSC.04 were identified as *B. subtilis* and *B. velezensis*, respectively.

Table 2. Biochemical tests for the identification of bacterial isolates

Tests	Bacterial isolates	
	DSC.03	DSC.04
Colony morphology		
Whole colony	Circular	Irregular
Edge	Entire	Undulate
Elevation	Convex	Flat
Surface	Smooth	Wrinkles
Color	Whitish	White
Morphological tests		
Cell shape	Rods	Rods
Gram staining	+	+
Spore formation	+	+
Motility	+	+
Biochemical tests		
Catalase	+	+
Oxidase	+	+
Indole production	-	-
Nitrate reduction	+	+
Methyl red test (MR)	-	-
Voges-Proskauer test (VP)	+	-
Gelatin hydrolyzing	+	+
Starch hydrolyzing	+	+
Sugar fermentation tests		
D-Glucose	+	+
D-Lactose	+	-
Results	*Bacillus* sp.	*Bacillus* sp.

Notes: (-): Negative, (+): Positive

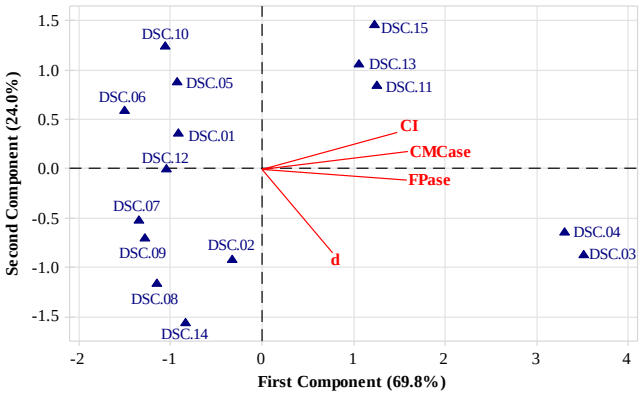

Figure 2. Principal component analysis of isolated bacteria. D: Colony diameter; CI: Cellulolytic index; CMCase: Carboxymethyl cellulase; Fpase: filter paper cellulase

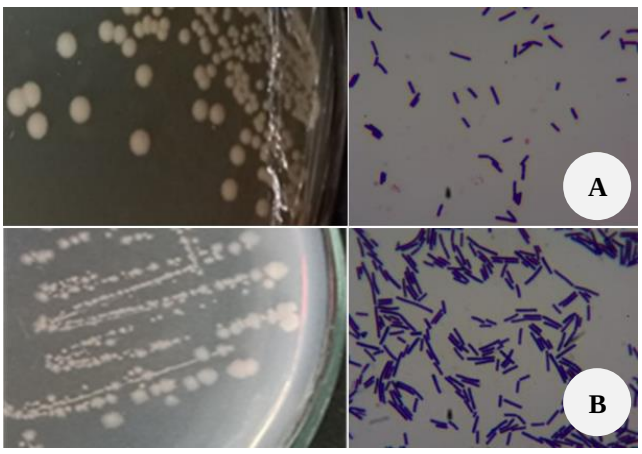

Figure 3. Colony and Gram staining results: A. DSC.03; B. DSC.04

Finally, the 16S rRNA gene sequences of DSC.03 and DSC.04 isolates were submitted to GenBank as *B. subtilis* DSC.03 and *B. velezensis* DSC.04, respectively with the accession numbers PP851408.1 and PP851410.1, respectively. Previously, *B. velezensis* B268 was isolated from rhizosphere soil in Zhejiang, China. This strain has been found to have antagonistic properties against bacterial wilt disease in plants. Its closely related strains have also been reported as endophytic bacteria that promote plant growth and inhibit fungi that cause diseases in plants (Helal et al. 2022). *B. subtilis* FUA2233 belongs to the fermentation microbiota of Mahewu, a traditional fermented cereal beverage from Zimbabwe (Xie et al. 2024). However, its specific properties have not been reported.

The genus *Bacillus* is known for being an aerobic, spore-forming rod that can germinate. These strains also tested positive for Gram, catalase, oxidase, nitrate reduction, hydrolysis (gelatin, starch), acid fermentation from sugars (glucose, lactose), and the VP reaction. However, in comparison with previous reports, *B. subtilis* CECT 39 can undergo acid fermentation from lactose (Ruiz-Garcia et al. 2005), and *B. subtilis* B1 and *B. subtilis* B2 test negative for oxidase (Kadhum and Hasan 2019). Additionally, *B. velezensis* BR-01 was negative for the VP reaction (Zhou et al. 2022). According to Soliman et al. (2022), these strains also tested negative for indole and MR reactions. The Bacillaceae family (phylum Firmicutes), as reported by Mandic-Mulec et al. (2016), is widely found in natural environments such as soil, water, activated sludge, sediment, various fermented foods, and human and animal systems. They have also been found in extreme environments, such as compost. Many members of this family can degrade cellulose. A study by Ma et al. (2020) confirmed that *B. subtilis* strains were the dominant bacteria in rotten wood samples collected from Qinling Mountain in Shaanxi Province, China. Tasaki et al. (2017) reported that this dominance may be due to the robust enzyme secretion system of the strain. Other studies have reported *B. subtilis* bacterial strains in the guts of termites (Gupta et al. 2012), soil (Li et al. 2020), and compost (Siu-Rodas et al. 2018). Similarly, *B. velezensis*, formerly known as *Bacillus amyloliquefaciens*, was first isolated in 1999 from a sample collected from the mouth of the Vélez River in Málaga Province, southern Spain Ruiz-Garcia et al. (2005). This bacterial strain is a promising source of potential protease and cellulase products (Li et al. 2020). Therefore, the strain identified in this study has the potential to be used commercially as a cellulase enzyme producer, for composting cellulose waste, and as a plant protection agent without causing harm to the environment.

Cellulosic substrate disintegration

The degradations of FP and DS powder were observed for two selected strains, *B. subtilis* DSC.03 and *B. velezensis* DSC.04, as well as for mixtures of these bacteria, as shown in Table 3. After 15 days of incubation, gravimetric analysis revealed degradation rates greater than 76.0% and 40.0% for the cellulose supplied as FP and DS powders, respectively, in shaken flasks, whereas minimal weight losses of only 1.13% and 0.79%, respectively, were noted for the control sample without bacterial supplementation. Interestingly, the combined activity of the two bacterial strains was more effective than their activities were, with significantly greater degradation rates of 86.85 and 48.72% for the FP and DS powders, respectively. Greater cellulose bio-degradation with the FP than with the DS powder was observed in all three treatments.

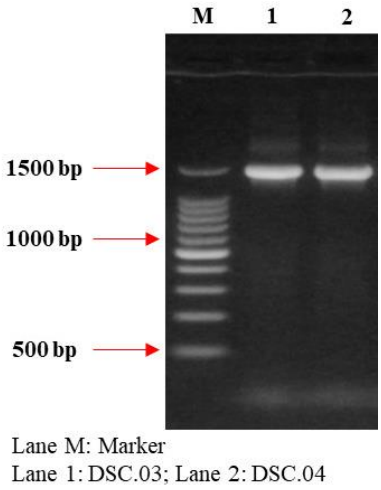

Figure 4. Agarose gel electrophoresis of the amplified products of the 16S rRNA gene

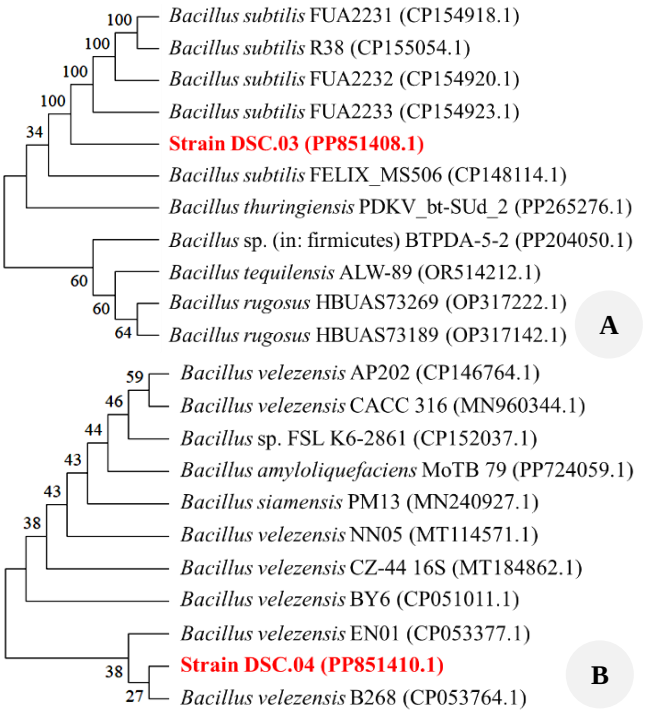

Figure 5. Phylogenetic analysis: A. DSC.03 strain; B. DSC.04 strain

Table 3. Bio-degradation rates of DS powder by selected isolates in liquid culture medium at the end of a 15-day incubation period

Treatments	Residual substrate (g)		Bio-degradation (%)	
	FP	DS powder	FP	DS powder
Control (non-bacterial)	0.2472[a]	0.2480[a]	1.13[d]	0.79[c]
B. subtilis DSC.03	0.0521[c]	0.1381[bc]	79.17[b]	44.77[ab]
B. velezensis DSC.04	0.0580[b]	0.1466[b]	76.81[c]	41.35[b]
The mix-culture	0.0329[d]	0.1282[c]	86.85[a]	48.72[a]

Notes: Means followed by the same letters in each column are not significantly different according to the least significant difference (Tukey) test ($p<0.05$)

The mixed culture, consisting of two selected isolates, showed potential for the biodegradation of cellulose substrates (FP and DS powder), with average degradation rates of 5.79% and 3.25% per day, respectively. This is in line with the findings of Bui (2014), who reported that their CDB isolates were able to digest more cellulose from FP than from coffee exocarps, with average digestion rates of 9.62% and 1.25% per day of incubation, respectively. These isolates were also used to aid in the biodegradation of coffee exocarps in the coffee processing areas of Vietnam. In another study by Egwuatu and Appeh (2018), the highest average degradation rates of FP were observed for bacteria isolated from termite guts, specifically the *Chryseobacterium luteola* and *Pseudomonas mendocina* strains, with rates of 3.17% and 3.0%, respectively, per day of incubation. Interestingly, compared with that of the cellulolytic fungi, the FP digestion activity of the *B. subtilis* DSC.03 and *B. velezensis* DSC.04 strains in the present study were significantly greater than that of the best cellulolytic fungus, *Chaetomium dolichotrichum* BY-20, which degraded only 1.42% of the FP disc weight per day of incubation (Yadav and Bagool 2015). The difference in degradation rates could be attributed to various factors, such as species, substrate type, and culture conditions. For example, submerged cultures involve better contact between cells and cellulose fibers in liquid media, allowing faster metabolism (Bui 2014). Chen et al. (2020) reported that plant biomass is a complex substrate that is difficult to degrade and requires a diverse range of cellulases for enzymatic hydrolysis by microorganisms. In nature, many species of microorganisms coexist and have positive interactions, with the most effective degradation occurring when they are present in a community (Sarsan et al. 2021). This cooperative activity has been observed in lignocellulose degradation, where multiple cellulolytic microorganisms work together (Cortes-Tolalpa et al. 2017). Additionally, a mixed culture of a cellulolytic microorganism with a non-cellulolytic microorganism is crucial for effective cellulose degradation (Kato et al. 2004).

In conclusion, a total of fifteen CDB strains were isolated from a DSC sample collected after 30 days of natural composting. The most promising candidates for cellulolytic activity were identified as *B. subtilis* DSC.03 and *B. velezensis* DSC.04. These strains showed the highest CMCase and FPase activities, with values of 0.31 and 0.19 U/mL, respectively. Furthermore, the disintegration performance was significantly greater when a mixed culture of the two identified strains was used, compared to using a pure strain for both filter paper and DS powder treatments. The degradation rate of DS powder in the mixed culture was 48.72%, which was significantly higher than that of pure *B. subtilis* DSC.03 (44.77%) and pure *B. velezensis* DSC.04 (41.35%). These findings suggest that the mixed culture of *B. subtilis* DSC.03 and *B. velezensis* DSC.04 had great potential for efficiently treating cellulose-rich by-product waste, such as DS waste. Further studies should be conducted on the optimal conditions for cellulase production and composting processes using mixed cultures of *B. subtilis* DSC.03 and *B. velezensis* DSC.04.

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