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Screening and identification of thermophilic cellulolytic bacteria isolated from sawdust compost

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Abstract. Composting process mainly depends on the metabolic pathways of the microorganism and involves the activity of different enzymes. Thermophilic cellulase-producing bacteria isolated from sawdust compost were tested for formation of a visible zone around the colonies on the agar plates medium containing carboxymethyl cellulose at 50ºC. Screening of carboxymethyl cellulase producing isolates was further realized on the basis in liquid medium by DNS method. Among 29 isolates investigated, V1 and V11 strains exhibited maximum enzyme activity of 1.9 and 2.3 U/mL, respectively. These isolates were selected for morphological, physiological and biochemical studies and 16S rRNA gene analysis. They were found a Gram-positive, rod-shaped spore forming cells, which were identified as *Bacillus megaterium* (V1) and *Bacillus subtilis* (V11) based on cell morphology, nucleotide homology and phylogenetic analysis. The optimal temperature for activity of endoglucanases (CMCase) ranged from 35–45°C (strain V1) and 40–50ºC (strain V11). Our findings showed that *Bacillus megaterium* (V1) and *Bacillus subtilis* (V11) cellulase demonstrate thermophilic characteristics within wide range of temperature and meets the requirements for commercial enzymes.
Keywords: composting, sawdust compost, thermophilic cellulolytic bacteria, *Bacillus megaterium*, *Bacillus subtilis*

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INTRODUCTION

Composting is the process of biological decomposition of the organic substrates and considered as a promising source of new thermophilic bacteria and thermostable enzymes (Alfreider et al., 2002). During the process of thermophilic composting, the temperature

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can reach up to 45–80°C and many pathogenic microbes could be eradicated in this stage. For all kind of compost, the most recognized group being able to grow well at temperature ranged from at 30 to 50°C are various members of the genus *Bacillus* belonging to thermophilic Bacilli (Bhattacharya, Pletschke, 2014). In addition, thermophilic Bacilli are promising by capability to utilize a variety of compounds as carbon source and to breakdown different of complex organic substrates including proteins, fats, cellulose, lignins, pectins (Maheshwari, 2014).

A number of thermophilic cellulolytic bacteria isolated from diverse sources of compost were reported. Genus *Bacillus* was reported as the predominant representatives during the thermophilic phase of compost (Cihan et al., 2012). The various of thermophilic bacterial strains of garden (76.1%) with two strains identified as *B. thermodenitrificansi* and *B. licheniformis* indicated by Bhattacharya and Pletschke (2014). Maheshwari (2014) indicated a greater number of species of *Bacillus* from thermophilic compost samples containing manure, corn and sawdust. By using DGGE to analysis of the microbial community from mushroom compost, Zhang et al. (2014) demonstrated the presence of bands corresponding to the genus *Bacillus*. Some thermophilic cellulase-producing bacteria strains from industrial waste compost also were found and identified as *B. amyloliquefaciens* B13C, *B. licheniformis* 1, *B. subtilis* subsp. *spizizenii* 6, *B. subtilis* subsp. *subtilis* B7B (Amore et al., 2013 *a*, *b*).

Cellulases are a group of enzymes including mainly three types are exoglucanases (FPase), endoglucanases (CMCase) and betaglucosidases. These enzymes are secreted by different microorganisms such as bacteria, fungi, and actinomycetes. However, fungi and actinomycetes have several disadvantages, such as long generation time and poor heat tolerance so unsuitable for industrial-scale fermentation, in ones turn thermophilic cellulolytic bacteria could grow well at a broad range of temperature, so they are good object for intensive fermentation process Dees and Ghiorse (2001). Therefore, the aim of this study was: (1) to isolate cellulase-producing bacteria from the thermophilic composting process, (2) to identify potential cellulolytic bacteria strains and (3) influence of temperature on the CMCase (endoglucanases) activity.

MATERIAL AND METHODS

Materials

Strains isolated from sawdust compost. The bacteria used for the test antimicrobial activity of selected strains were as follows: *Pseudomonas aureginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella*.

Culture Media. Carboxymethyl cellulose (CMC) agar medium (w/v): 1% CMC, 0.25% $(NH_4)_2SO_4$, 0.025% $K_2HPO_4.3H_2O$, 0.01% NaCl, 0.0125% $MgSO_4.7H_2O$, 1.5% agar.

Nutrient broth medium (NB) (w/v): 0.5% glucose, 1% peptone, 0.5 NaCl, 0.3 beef extract and 1 L of distilled water at pH 7.0–7.2.

Nutrient agar medium (NA): adding 2% agar to NB. The medium was used to scan the antimicrobial activity of the tested bacterial strains.

Methods

Isolation of Cellulase Producing Thermophilic Bacteria. Cellulolytic bacterium was isolated from compost samples obtained in southern region of Vietnam and extracted at 7th day of sawdust composting process (50–60ºC). A 1 kg representative samples were extracted from the internal core (at depth about 30–50 cm) and external zona of the pile (at depth about 5–10 cm). The samples were exposed before use at 70°C for 24 h to exclude non-sporeforming bacteria (Baharuddin et al., 2010). To each of the 90 mL samples were added 10 g (w/v) of sterile water in Erlenmeyer flasks (250 mL) and it have been shaking to make suitable suspensions dilutions. A 100 µL dilutions was expand onto CMC agar plates and then incubated at temperature 50°C for 24 h. The cellulolytic bacteria clones were traced by good colony development and visible clear zone around the colonies after Lugol drops on CMC agar. Selected grown colonies were harvested and transferred to NB agar plates and incubated at 37°C for 24 h. Colony morphological characteristics such as colony size, growth, form and color were tested.

Morphological, *Physiological and Biochemical Studies of Bacterial Isolates.* For the observation of selected isolates, colony morphology such as form of colony, size, surface, pigmentation, Gram reaction and their rate of growth was studied. Catalase test, Hydrolysis of casein, Hydrolysis of Gelatin, Hydrolysis of starch tests, Growth response of the isolates at different concentrations of NaCl, growth pH and growth temperature range were performed in agreement with standard procedures following Bergey's Manual of Systematic Bacteriology. Cell morphology was studied with an Axio Image 2 (Imager.Z2) microscope with integrated Zeiss Axiocam 503 Color Camera Unit under 100× magnification, by Zen 3.3 software (blue version).

Identification of isolates by 16S rRNA gene analysis. Genomic DNA was extracted and purified from cells grown on NB agar by using a Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit (UK). The 16S rRNA gene fragments were amplified as previously described (Do et al., 2021) by using two pairs of primers 27F: 5′-AGAGTTTGATCATGGCTCAG-3′ and 1492r: 5′-TACGGYTACCTTGTTACGACTT-3′. The PCR reaction was run by GeneAmp™ PCR System 9700 (Life Technologies Applied Biosystems, Singapore). PCR protocol was performed as follows: 5 min denaturation at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min 30 s at 72°C, and, finally, extension incubated at 72°C for 5 min. PCR products were analyzed on 1% agarose gel (product size 1420 bp) and were sent for directly sequencing at 1st Base Laboratories Sdn. Bhd., Malaysia. The 16S rRNA gene sequencing data were confirmed and compared with GenBank sequences by the basic local alignment search tool (BLAST).

Preparation of Crude Enzyme Solutions. Initial cultures were prepared by transferring cells by inoculation loop into 100 mL of CMC liquid medium with initial pH 7.0. After 24 h cultivation by shaking at 37ºC, 5 mL starter culture was transfer into 500 mL of CMC liquid medium (in 1000 mL flasks). This medium was the same as the medium using before cellulolytic bacteial isolation; yeast extract (1.0 g/L) was added to provide additional nitrogen source and increase the growth rate. These flasks were again incubated at 37ºC for 24 h on a shaker at 180 rpm. The crude enzyme solutions were harvested after centrifugation of cell-free supernatants from cultures at 4ºC (8,000×g, 10 min) and investigated for cellulolytic activities (Meng et al., 2014).

Enzyme Activity Assay. The cellulolytic reaction was carried out in glass tubes, by adding 0.5 mL of crude enzyme solution to 1 mL of 1% (w/v) CMC, readied in a buffer (sodium acetate 0.1 M (pH 5.0) and 1.0 mL of sodium acetate 0.05 M (pH 4.8). This mixture was incubated for 30 min at 50°C. The reducing sugars liberated estimation were made by DNS method with some modifications. DNS reagent was prepared as follow: $C_7H_4N_2O_7$ (3,5-dinitrosalicylic acid) 10.6 g, NaOH, 19.8 g, $C_4H_4KNaO_6$ (potassium sodium tartarate) 306 g, C_6H_6O (phenol) 7.6 mL, $Na_2O_5S_2$ (Natri metabisulfite) and distilled water up to 1416 mL). The enzyme reaction was stopped by inclusion of 3.0 mL, heated in closed glass tubes for 5 min and then cooled in frozen water. This solution was measured for optical density (OD) at 540 nm using a spectrophotometer. One unit (IU) of CMCase activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar per min (Islam, Roy, 2018).

Influence of temperature on the CMCase activity. To screen the influence of temperature on the activity of the CMCase, the mix of the crude enzyme extract and CMC 1% was incubated (w/v) in the buffer (pH 4.8) for 30 min. The different temperatures values were tested in range of 20 to 60°C with 5°C intervals. CMCase activity was determined by the DNS as described by Li et al. (2016). The results reported obtained as mean values of the three independent replicates.

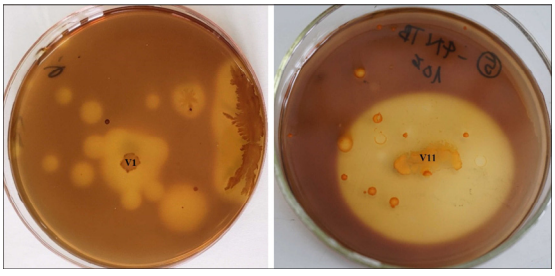
Antimicrobial activities of bacterial strains. Antimicrobial activity on all bacteria of study was tested by the agar-well diffusion method. The fermentation broth of each bacterial strain (100 mL) was extricated with ethyl acetate, then the ethyl acetate phase was recalled and evaporated at 50°C by using rotary evaporator to obtain 1 ml concentrated extract (Amin et al., 2015; Chu et al., 2019).

The bacteria investigated were incubated on NB medium at the temperature 30ºC for 24 h. After that, 100 µl suspensions of each strain were spread on the nutrient agar medium plates. Eight mm diameter wells in agar plates were punched by using a steel borer. After that, 100 µl concentrated extract of each sample were directly contended into the wells. These plates were incubated for 24 h at 30ºC, and antimicrobial activity was observed as a clear disk-like zone of inhibition around the wells (Balouiri et al., 2016).

Statistical and genetic analyses. Statistical data analyses were performed using the software packages MS Excel 2000 (Microsoft Corp.), PAST 2.17c (Hammer et al., 2001) and Statistica 6.0 (Statsoft Inc., OK, USA). The Neighbor-Joining phylogenetic tree was constructed using a MEGA7 software based on 16S rRNA gene sequences.

RESULTS AND DISCUSSION

Isolation of thermophilic cellulose degrading bacteria. In this study, cellulolytic bacterial isolates were obtained from 9 different samples collected from sawdust composts. After incubation at 50°C for 24 h, the bacterial isolates were selected based on morphological colonies characteristics and a visible clearing zone on the agar plate. A total of 29 bacterial isolates were selected for cellulose degradation in the first round of screening. Cellulase activity were confirmed by both the halo zones around the colonies on CMC agar medium and by using the crude cellulase extract obtained from the liquid cultures by the lugol-staining method on agar medium with CMC as an exclusive carbon source. Of all positive isolates, strain V1 and V11 from the sawdust compost showed



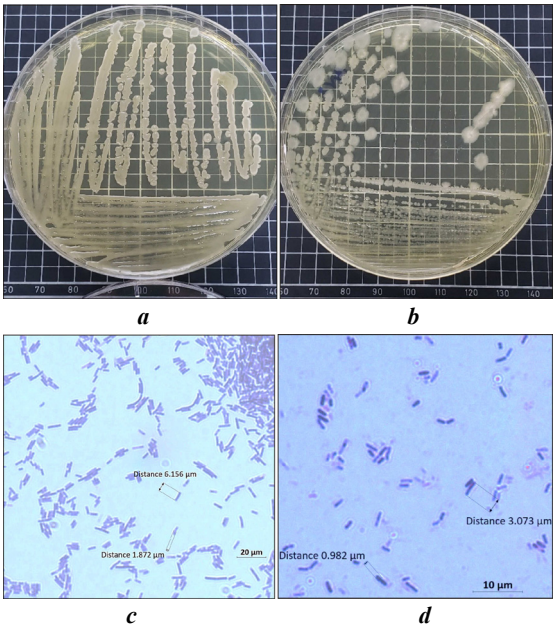
a *b*

Fig. 1. Growth of bacterial V1 (*a*) and V11 (*b*), isolated from sawdust compost, and CMCase activity on CMC agar plates with 1% CMC and pH 7.0 at 50°C. Cellulase activity could be detected by clear halos around the colonies

both maximum zone of clearance (Fig. 1) and the highest activity (exhibited maximum enzyme activity of 1.9 and 2.3 U/mL) so was selected for further study.

Morphological, biochemical and physiological characterization of strain V1 and V11 and its 16S rRNA identification. The morphology of strain V1 and V11 is shown in Figure 2. Both of them were Gram-positive, sporulating rod–shaped bacteria. The cells of V1 strain ranged in size from 4.0 to 6.5 µm in length and from 1.5 to 2.0 µm in width. The cells of V11 strain ranged in size from 2.0 to 3.5 in length and from 0.5 to 1.0 µm in width when grown in NA media at 30ºC.

They were also found catalase-positive, moderate thermophiles, able to growth at varying NaCl concentration (0–9%), hydrolysis of gelatin, casein, starch. Besides that, these strains showed effective antibacterial activity against other bacteria tested. Biochemical, physiological and morphological characterizations of the two strains showed minor differences from each other (see Table).



a *b*

c *d*

Fig. 2. Morphological characterizations of the two strains. The colony morphologies of V1 (*a*) and V11 (*b*) strains on NB agar and the Gram-stained V1 (*c*) and V11 (*d*) cells observed under 1,000× light microscope, respectively

Partial 16S rDNA gene sequences obtained (~1420 bp) of isolates V1 and V11 were submitted to GenBank and deposited with the accession numbers MW093073 and MW093074. The results of comparative phylogenetic analyses of the two strains based on BLAST search (Fig. 3) determined the attribution of the strain V1 as a single clade to *Bacillus megaterium* strain FJAT and *B. megaterium* strain NBRI13G (the highest identity at 99.79%). The strain V11 was the most relative (99.93%) to *B. subtilis* strain DSM10 and *B. subtilis* strain UCMB5021. Therefore, based on

morphological, biochemical and physiological characteristics, along with 16S rRNA identification, our isolates were attributed to *Bacillus megaterium* (V1) and *Bacillus subtilis* (V11).

Table. Morphological, biochemical and physiological characteristics of the strain V1 and V11

Characteristics	Bacterial isolates	
	V1	V11
Gram stain	Gram-positive bacteria	Gram-positive bacteria
Cell shape	Rod	Rod
Size in length, μm	4.0–6.5	2.0–3.5
Size in width, μm	1.5–2.0	0.5–1.0
Spore-forming	+	+
Colony morphology (on nutrient agar plate)	Irregular, smooth, cream-colored, opaque	Irregular, dry, cream-colored, opaque colonies
Growth temp range, °C	20–60	20–60
Growth pH range	4.0–8.5	5.5–8.0
NaCl, g/l	0–7	0–9
Catalase	+	+
Hydrolysis of		
Gelatin	+	+
Casein	+	+
Starch	+	+
Antimicrobial activity		
Escherichia coli	+	+
Salmonella typhimurium	+	+
Staphylococcus aureus	+	–
Pseudomonas aureginosa	–	+

Note. +, positive; –, negative.

The bacteria of genus *Bacillus* isolated from thermophilic compost were reported recently. Xie et al. (2021) reported about the isolation and identification *Bacillus megaterium* L2 strain. This strain showed good cellulose degradation activity and antibacterial bioactive compounds. A number of thermophilic bacterial strains including *B. subtilis*, *B. licheniformis*, *Bacillus* sp., *Paenibacillus* sp. and *Geobacillus* sp. were isolated and identified from hot compost in Nepal by Acharya et al. (2012). Among these strains, *B. subtilis* indicated highest enzyme activity on carboxymethylcellulose (CMC) in the pH 7.2 and 50°C. Alfreider et al. (2002) reported the isolation bacteria belonging to the genus *Bacillus* from organic waste of Innsbruck town. These strains *B. subtilis* and *Bacillus* sp. showed an optimal performance at 70 and 80°C, respectively. By using CMC as substrate, Kim et al. (2012) isolated thermophilic bacilli from composting vegetable wastes and investigated enzymatic activities of ferments, such as CMCase, avicelase, β-glucosidase, and xylanase. These bacteria were identified as *B. licheniformis* 1, *B. subtilis* subsp. *subtilis* B7B, *B. megaterium* 6, and *B. amyloliquefaciens* B31C.

Influence of temperature on the CMCase activity. Screening the influence of temperature on the activity of the CMCase was performed under different temperatures conditions ranging from 20 to 60°C. The results shown in Figures 4 demonstrate that both strain V1 and V11 appear considerable CMCase activity in the crude enzyme solution in a wide temperature range and there exists a strong influence of temperature on

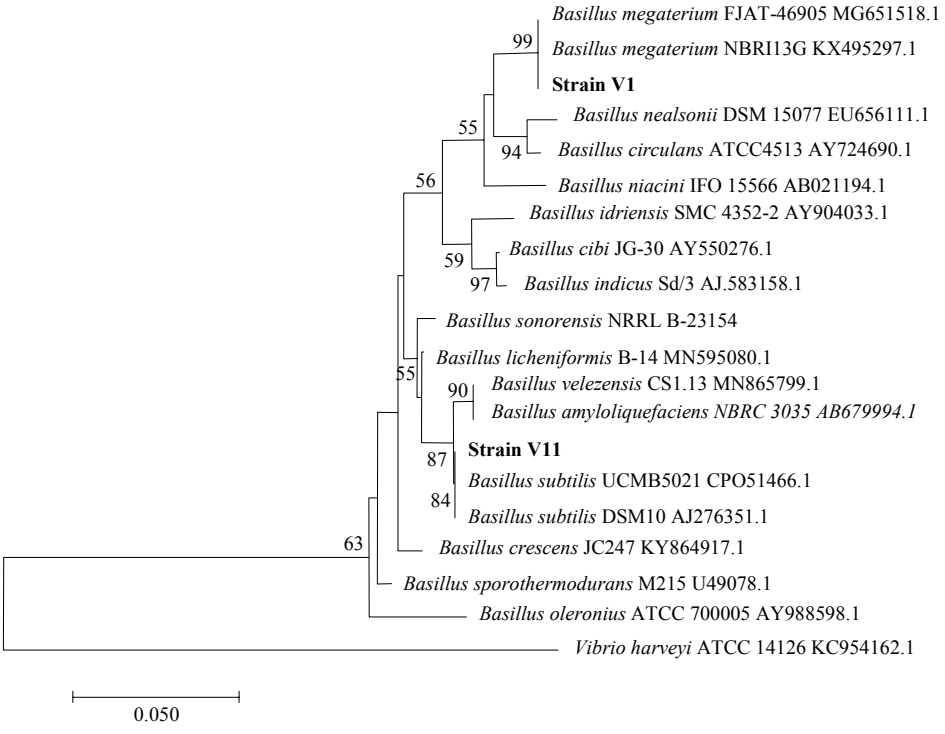


Fig. 3. Phylogenetic tree (NJ) of *Bacillus* and positions of strains V1 and V11. The scale bar refers to a phylogenetic distance of 0.050 nucleotide substitutions per position. Numbers at the node are bootstrap values (1,000 replicates)

enzyme activity. The results of the test at different temperatures showed that the temperature optimum for cellulase activity of strain V1 was found between 35 and 45°C, reaching maxima at 40ºC (2.1 U/mL) while temperature optimum for cellulase activity of strain V11 was found between 40 and 50°C, reaching maxima at 45ºC (2.52 U/mL) after 24 h cultivation. When the temperature increased above 50ºC or decreased below 30ºC, the results showed that the enzyme activity decreased.

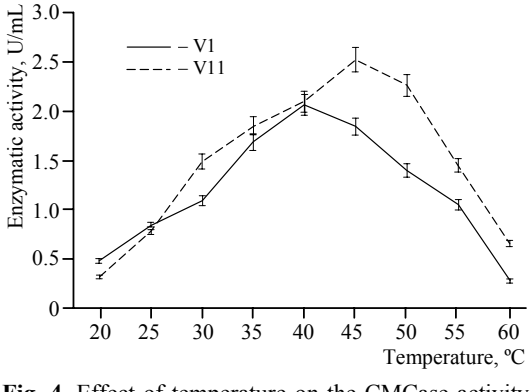


Fig. 4. Effect of temperature on the CMCase activity of the V1 and V11 cellulase

There are a few studies that show the potential of cellulolytic enzymes of bacteria isolated from compost. De Marco et al. (2017) re-

ported *B. licheniformis* 380 isolated from compost producing a thermostable alkaline cellulase and identified favorable temperature conditions for maximal CMCase activity at 50°C in the presence of different substrates. Siu-Rodas et al. (2018) identified of *B. subtilis* isolated in the thermophilic phase from composting coffee remains (piles). Endocellulase and exocellulase activities of this strain were shown. Partanen et al. (2010) isolated and identified bacteria including the phyla Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria and Deinococcus-Thermus. Most of them belong to *Bacillus* genus. These strains showed the cellulases exhibited optimum temperature at 50–60°C. *B. subtilis*, *B. megaterium*, *B. licheniformis* and *B. amyloliquefaciens* were considered to be tolerant to thermophilic conditions and the enzyme allowed these strains to be treated as an ideal candidate for application in biofuel and food industry.

CONCLUSIONS

Bacillus megaterium V1 and *Bacillus subtilis* V11 are Gram-positive, sporulating rod-shaped bacteria, both isolated from sawdust composting, had the ability to produce thermophilic cellulase at wide range of relatively high temperatures (20–60°C). Beside that, these strains can hydrolysis of gelatin, casein, starch and tolerate against some other bacteria. These strains can be successfully applied for degradation of organic substances in compost.

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Скрининг и идентификация термофильных разлагающих целлюлозу бактерий, выделенных из компостированных опилок

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Аннотация. Процесс компостирования в основном зависит от метаболических путей микроорганизмов и включает в себя активность различных ферментов. Термофильные бактерии – продуценты целлюлазы, изоляты компоста из опилок исследовали на образование видимых зон вокруг колоний на чашках с агаром. Среда содержала карбоксиметилцеллюлозу при 50°C. Кроме того, дальнейший скрининг изолятов, продуцирующих карбоксиметилцеллюлазу, на жидкой среде был проведен с использованием метода DNS. Среди 29 исследованных образцов изоляты V1 и V11 показали максимальную ферментативную активность – 1.9 и 2.3 ед./мл соответственно. Данные изоляты были отобраны для морфологических, физиологических и биохимических исследований и анализа последовательности гена 16S rRNA. Это оказались грамположительные палочковидные спорообразующие бактерии. На основании клеточной морфологии, гомологии нуклеотидных последовательностей и филогенетического анализа изоляты были идентифицированы как штаммы *Bacillus megaterium* (V1) и *Bacillus subtilis* (V11). Оптимальная температура активности эндоглюканазы (CMCase) составляла от 35 до 45°C (штамм V1) и от 40 до 50°C (штамм V11). Наши результаты показали, что целлюлазы *Bacillus megaterium* (V1) и *Bacillus subtilis* (V11) демонстрировали термофильность в широком диапазоне температур и соответствовали требованиям коммерческих ферментов.
Ключевые слова: компостирование, компост из опилок, термофильные целлюлолитические бактерии, *Bacillus megaterium*, *Bacillus subtilis*

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