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Selection and Identification of Potential Hydrolytic *Bacillus* from Litter Composting

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ABSTRACT

Microbe study on degraded trash has been widely conducted, with the intention of improving the efficiency and effectiveness of waste processing. The purpose of this study was to select and identify hydrolytic *Bacillus* from litter composting. The samples were the litter composting outcomes with five different treatments, namely the inclusion of nutrient broth (NB), commercial formula, molasses, tryptic soy broth (TSB), and talcum, with each formula containing a microbial consortium. Bacteria were isolated using the serial dilution method. Each isolate was evaluated for its capacity to lyse red blood cells on blood agar media. Cellulolytic, amylolytic, and proteolytic bacteria were grown on nutritional agar media containing carboxy-methyl cellulose (CMC), starch, and skim milk. The antagonistic test involved testing isolates that hydrolyze all three types of substrates. Isolates that were not antagonistic to one another were identified based on phenotypes (colony and cell morphology using Gram staining) and genotypes (16S rRNA gene). Fourteen isolates of *Bacillus* sp. had cellulolytic, amylolytic, and proteolytic properties. Four isolates were chosen; two of them were comparable to *Bacillus stercoris*, while the other two, 5.5 and 5.9R, are similar to *Calidifontibacillus erzurumensis* and *Bacillus amyloliquefaciens*. Therefore, litter can be used to determine the hydrolytic capability of *Bacillus* isolates.

Keywords: amylolytic, Bacillus, cellulolytic, proteolytic

INTRODUCTION

Indonesia produces 19.4 million tons of waste per year, with household waste accounting for 38.7% of the total and food waste accounting for 41.4% (MenLHK 2023). Various efforts have been undertaken to minimize waste volume, particularly organic waste, which can naturally breakdown into CO_2, CH_4, and other simple organic chemicals with the help of microbes (Sailer *et al*. 2021; Wang *et al*. 2022). Composting, biodigesting, pelleting, or animal feeding are examples of such efforts (Dantroliya *et al*. 2022; Maliki *et al*. 2023), as is biodrying, which yields goods with economic and potential value (Qonitan *et al.* 2021). However, these efforts are still hampered by the lengthy process, high operational expenses, and poor product quality (Nanda and Berruti 2021). Previous studies has attempted to develop answers via biostimulation and bioaugmentation of potential microorganisms (Kinet *et al*. 2015; Fan *et al*. 2018; Quan *et al*. 2023).

Bacillus is known for its potential as a decomposer. According to Zhou *et al*. (2021), inoculating a consortium consisting of *Bacillus amyloliquefaciens* B59, *B. haynesii* A31, *B. amyloliquefaciens* B11, and *B. licheniformis* B58 in the organic material decomposition process was more stable and efficient because it could reduce volatile materials by up to 46.90%, solid mass by 76.16%, and material reduction stability until the end of the process, as well as increase the important beneficial substances. *Bacillus* is also widely dispersed, especially in severe environments, due to its ability to create endospores, an adaptive generative form, as well as numerous enzymes and antimicrobials that have been widely used and developed in a variety of disciplines (Sorokin *et al*. 2017; Ruginescu *et al*. 2019).

Bacillus strains have been extensively studied in many ecosystems, particularly soils with diverse distributions (Sulistiyani *et al*. 2021; Alyousif 2022; Yuliana and Hidayati 2022), although composting has received less attention. If the same *Bacillus* sp. strain from diverse habitats is unclear to have the same capabilities (Sorokin *et al*. 2017; An *et al*. 2018), then the search for superior prospective strains is critical. The purpose of this work was to isolate and identify *Bacillus* sp. with hydrolytic capacity from the litter composting process.

METHODS

This study was carried out from August 2023 to January 2024 at the Microbiology Laboratory,

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Composting Preparation

Before usage, the microbial consortium of *Bacillus sonorensis* Bac 1, *Lysinibacillus halotoleran* Pink 45.2, *Pichia manshurica* Ks3 DIY, *Lactobacillus rhamnosus* N4, and *Enterobacter ludwigii* BT 111 was tested for optical density (OD) of up to 0.8 (10^8−10^9 CFU/mL). A total of 25 kg of garbage gathered from the Bogor Agricultural University dump was packed into five wooden boxes measuring 60 cm × 50 cm × 40 cm. Each container received different treatments, (1) inoculated with 1.25 L of molasses media containing a microbial consortium, *Rhodopseudomonas* sp., *Lactobacillus* sp., *Streptomyces* sp., yeast, and *Trichoderma* sp., (2) added with 1.25 L of NB media without a microbial consortium, (3) inoculated with 1.25 L of TSB media which also contained a microbial consortium, and (4) inoculated with a commercial formula containing *Rhodopseudomonas* sp., *Lactobacillus* sp., *Streptomyces* sp., yeast, and *Trichoderma* sp. as much as 3% (v/w) or 0.15 L (according to usage procedures), and (5) mixed with 100 g of talcum containing 10% microbial consortium in distilled water (Swandi *et al*. 2019).

Isolating *Bacillus* sp. from Litter

On day 0 (the first day of waste microbial inoculation), samples were collected from each 500 g composting container. Samples were collected from each corner at half the depth of the container, placed in labeled plastic clips, and stored in the refrigerator (± 8 °C) to delay microbial growth and death until ready for analysis. A total of 10 g samples from each treatment were suspended in 90 mL of physiological NaCl solution in a stomacher bag container and crushed with a stomacher for 20 sec. A total of 1 mL of filtered water was suspended in 9 mL of physiological NaCl solution, boiled in water at 80 °C for 15 min, then cooled and diluted up to 10^{-5}. The dilutions ranging from 10^{-3} to 10^{-5} were taken up to 1 mL and put to NA medium using the pour plate method. Colonies produced at 10^{-3} and 10^{-4} dilutions were isolated on NA media and purified by subculture until a single colony was obtained (Chhetri *et al*. 2022). Macroscopic characteristics examined included colony morphology (shape, color, border, and elevation), while microscopic traits were determined using Gram staining.

Selecting on Test Media

To test for hemolysis, the isolate was grown on blood agar and incubated for 24-48 h at room temperature (± 27 °C). A clean zone around the colony indicated positive hemolysis (Zhang *et al*. 2021). The hemolysis-negative isolates were examined further. The cellulose hydrolysis test was carried out by growing the isolate on NA media and 1% carboxyl methyl cellulose (CMC), culturing it for 24−48 h at room temperature, then pouring 1% Congo-red solution for 15 min and washing with 0.5 M HCl. A yellow zone around the colony suggested a positive outcome (Khatiwada *et al*., 2016). The starch hydrolysis test was carried out by growing the isolate on NA media with 1% starch and culturing it for 24−48 h at room temperature. A positive result was indicated if a yellow zone appeared around the colony after pouring the iodine solution. The protein hydrolysis test was carried out by growing the isolate on NA media with 2% skim sterilized with a separate autoclave for 5 min before incubating for 48−72 h at room temperature, with a clear zone forming around the colony indicating positive results (Olanbiwoninu and Fasiku 2015).

$$\text{Hydrolytic index} = \frac{(\text{clear zone diameter} + \text{colony diameter}) - \text{colony diameter})}{\text{Colony diameter}}$$

The hydrolytic capacity was divided into four types: very strong (ratio ≥ 2), medium (ratio >1 up to <2), weak (ratio ≤ 1), and no reaction (Chao 2005).

Identifying 16S rRNA of Selected Isolates

A commercial DNA extraction kit (Zymo Research's Quick-DNA Fungal/Bacterial Miniprep Kit, USA) was used to isolate DNA. DNA was amplified using universal 16S rRNA primers 63F (5'- CAGGCC TAA CAC ATG CAA GTC -3') and 1387R (5'- GGG CGG WGT GTA CAA GGC -3') under PCR conditions of 5 min of pre-denaturation at 94 °C, 45 sec of denaturation at 94 °C, 1.5 min of elongation at 72 °C, and 10 minu of post-elongation at 72 °C. The denaturation to elongation phases were repeated 35 times. To amplify the 16S rRNA gene in a 50 µL volume, we used 25 µL MyTaq HS Red Mix 2×, 4 µL primer 63F (10 pmol), 4 µL primer 1387R (10 pmol), 4 µL DNA template, and 13 µL nuclease-free water (Mursyida *et al*. 2015). The amplification findings were electrophoresed and sequenced at the Genetika Sains Indonesia laboratory. The sequencing products were processed with GeneStudio, and BLAST-N was used to identify species with homology on the Genbank site of the National Center for Biotechnology Information (NCBI) at https://www.ncbi.nlm.nih.gov. A phylogenetic tree was then constructed using MEGA 11's Neighbor-Joining method with a bootstrap value of 1000× to represent the evolutionary relationship between isolates that have similarities.

RESULTS AND DISCUSSION

***Bacillus* Abundance**

Bacillus abundance was highest in container 5 (talcum), with an average of 8.6 × 10^5 CFU/mL, and lowest in container 1 (molasses), with an average of 0.1 × 10^5 CFU/mL (Table 1). A total of 56 *Bacillus* isolates

were successfully isolated from all composting treatments due to differences in colony morphology. The initial composition of *Bacillus*, which plays a key role in waste degradation, was expected to be determined by sample collection on the first day of composting. The significant abundance of *Bacillus* in container 5 demonstrates that talcum is an effective carrier medium for *Bacillus* development during composting by providing favorable physicochemical conditions (temperature, humidity, and pH). Litter comprises complex organic materials (cellulose, lignin, pectin, and protein) as plentiful substrates, creating favorable environmental conditions for the growth of numerous bacteria (Zhan *et al*. 2023).

Hydrolytic Activity of *Bacillus* sp. Isolates

A total of 28 (50%) isolates tested negative for hemolysis; their hydrolytic abilities were next evaluated, and 22 isolates (78.6%) revealed hydrolytic activity; however, only 14 isolates had all three activities, amylolytic, proteolytic, and cellulolytic (Table 1). In this study, the number of *Bacillus* isolates with all three hydrolytic abilities was significantly higher than in Daza's (2016) study, which successfully isolated only three *Bacillus* strains from six bacterial strains with all three hydrolytic abilities derived from sugar cane composting. Isolates 2.5, 2.10R, and 4.2Bb had the highest levels of cellulolytic, amylolytic, and proteolytic activity (Figure 1). Isolate 2.10R had strong amylolytic ability, but very weak cellulolytic and proteolytic abilities, while isolate 4.2.Bb had very strong proteolytic ability but very weak cellulolytic and amylolytic abilities. Five isolates then selected to represent the three hydrolytic abilities (cellulolytic, amylolytic, and proteolytic) were 2.5, 2.7, 4.7, 5.5, and 5.9R. The hydrolytic index indicates the ability to hydrolyze a substrate as a source of carbon and energy. The greater the hydrolytic ability possessed by a

Table 1 Isolation results of the hydrolytic activity of *Bacillus* isolated from litter with various treatments

Specific information	Composting container					Total
	1	2	3	4	5	
Total plate count (CFU/mL) *Bacillus*	0.1×10^5	6.7×10^5	6.5×10^5	4.1×10^5	8.6×10^5	
Total isolates	6	14	11	13	12	56
Negative hemolysis	1	7	5	9	6	28
Hydrolytic activity						
Negative	-	1	1	2	2	6
Positive	1	6	4	7	4	22
Cellulolytic	-	6	3	5	3	17
Amylolytic	1	6	4	5	4	20
Proteolytic	1	5	4	6	3	19
Total isolates	-	5	3	4	2	14

Remarks: *(1) inoculated y 25% (v/w) or 1,25 L molasse media containing microbe consortium, (2) added by NB media 1,25 L without microbe consortium, (3) inoculated by TSB media 1,25 L containing microbe consortium, (4) inoculated by commercial formula 3% (v/w) or 0,15 L (according to usage procedures), and (5) mix with 100 g talcum containing 10% microbe consortium in distilled water.

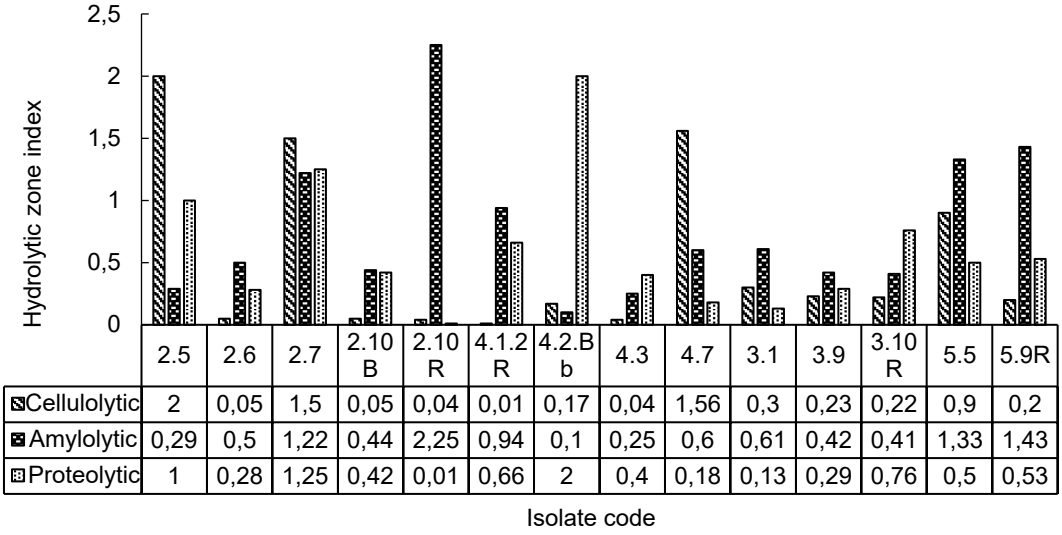


Figure 1 Results of hydrolytic tests (cellulolytic, amylolytic, and proteolytic) of 14 isolates based on their hydrolytic zone index (the first number in the isolate code indicates the composting container).

microorganism indicates a general metabolic ability or high adaptability to diverse environments, making it more competitive in its habitat (Madigan *et al*. 2021). This ability also suggests microbes' potential as primary decomposers, as they may break down complex organic molecules into simpler ones for usage by other species in the food chain (Tortora *et al*. 2023).

VThe antagonist test results showed that two pairs of isolates, 2.5 and 2.7, and 5.5 and 5.9R, were not antagonistic to each other, while one isolate, 4.7, was antagonistic to all other isolates (Table 2). Antagonistic testing was conducted to ensure that the bacteria used in the consortium did not inhibit each other (Liu *et al.* 2022).

Characteristics and Identified *Bacillus* sp. Originating from Litter

The *Bacillus* isolate colonies in this investigation were often round or irregular, with a smooth texture or consistency on the surface and mucoid within, particularly isolate 5.5's colony, which could be lifted to several centimeters off the agar surface (Table 3). Isolates 2.5 and 2.7, despite having distinct morphological appearances based on 16S rRNA molecular identification, were both 99% similar to *B. stercoris* or *B. subtilis*, but the other two isolates, 5.5 and 5.9R, shared 99% similarity with *Calidifontibacillus erzurumensis* and *B. amyloliquefacien*, respectively (Table 4, Figure 2). The 16S rRNA gene is extremely

Table 2 *Bacillus* antagonistic test

Isolate code	2.5	2.7	4.7	5.5	5.9R
2.5	-	-	+	+	+
2.7	-	-	+	+	+
4.7	+	+	-	+	+
5.5	+	+	+	-	-
5.9R	+	+	+	-	-

Remarks: (-) no antagonistic, (+) antagonistic

Table 3 Colony morphology characteristics and selected *Bacillus* isolate cells

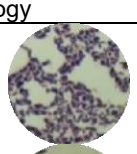
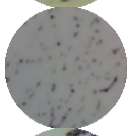
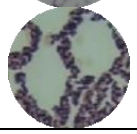
Isolate code	Characteristics of isolate			
	Colony morphology		Cell morphology	
2.5	Irregular colonies, serrated, convex, 1−2 mm in size, dull/lusterless, non-transparent, moist and mucoid inside, beige color		positive Gram, long stem (1−2 µm)	
2.7	Irregular colonies, serrated, convex, 1−2 mm in size, shiny, non-transparent, moist and mucoid inside, beige color		positive Gram, long stem (1−2 µm)	
5.5	Round colonies, smooth edges, convex, 2−3 mm in size, shiny, transparent, mucoid, clear color		positive Gram, long stem (1−2 µm)	
5.9R	Irregular colonies, notched edges, convex, 1−2 mm in size, dull, not transparent, moist and mucoid inside, beige color		positive Gram, long stem (1−2 µm), endospores in the center of the cell	

Table 4 BLAST-n result of 16S rRNA sequence from selected isolate

Isolat code	Reference bacteria	Reference sequence length	*E*-value	Identity value (%)	Reference access number
2.5	*B. stercoris* D7XPN1	1455	0.0	99.54	NR_181952.1
	B. subtilis JCM 1465	1472	0.0	99.54	NR_113265.1
2.7	*B. stercoris* D7XPN1	1455	0.0	99.46	NR_181952.1
	B. subtilis JCM 1465	1472	0.0	99.46	NR_113265.1
5.5	*Calidifontibacillus erzurumensis* P2	1401	0.0	99.62	NR_180225.1
	B. velezensis CBMB205	1445	0.0	99.54	NR_116240.1
5.9R	*B. amyloliquefaciens* MPA 1034	1448	0.0	99.84	NR_041455.1
	B. amyloliquefaciens NBRC 15535	1472	0.0	99.84	NR_117946.1

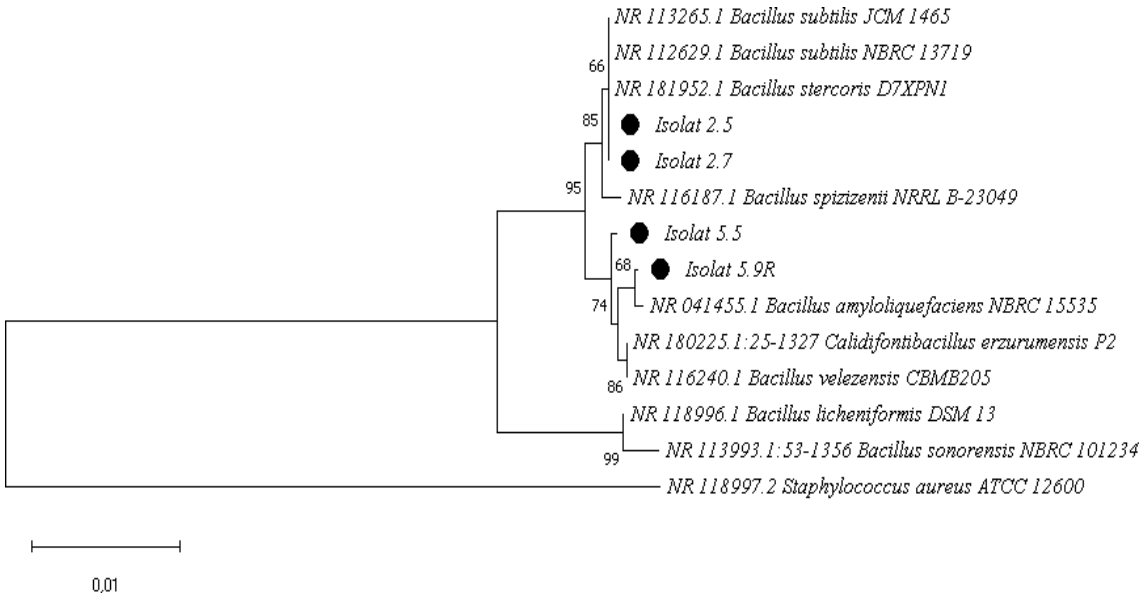


Figure 2 Phylogenetic tree of the four selected isolates constructed using the Neighbor-Joining method using the Kimura 2-parameter model with bootstrap value 1000×. *Staphylococcus* sp. was used as the outgroup.

conservative, allowing for very small changes between species.

Bacillus is a genus of Gram-positive, rod-shaped, aerobic and facultative anaerobic bacteria that are highly adaptable (psychrophilic to thermophilic, acidophilic to alkaliphilic, salt-tolerant to halophilic) and hence widespread. *Bacillus* is distinguished by its ability to create endospores as a cell's adaptive response to environmental stress (Logan 2009). The mucoid consistency generated by the production of mucus or extracellular polysaccharides (EPS) is frequently connected with the propensity to form biofilms (Flemming *et al*. 2016; Jautzus *et al*. 2022). Isolates 2.5 and 2.7 were on the same branch of the phylogenetic tree as *B. stercoris* D7XPN1 and *B. subtilis* JCM 1465 (Table 4, Figure 2), indicating a high level of genetic similarity.

Adelskov and Patel (2016) discovered that *B. stercoris* D7XPN1, effectively isolated from a commercial-scale food waste composter, was a distinct subspecies of *B. subtilis* utilizing the e-DDH (estimated DNA-DNA hybridization) analytical method. The e-DDH study compares a bacterium's entire genome to specified genes. Isolate 5.5 showed branching similar to *C. erzurumensis* and *B. velezensis* CBMB205 rather than *B. amyloliquefaciens* NBRC 15535. The isolate of *C. erzurumensis* P2 isolated from a hot spring in Turkey lacks the ability to hydrolyze starch or casein (Adiguzel *et al*. 2020), whereas *B. velezensis* CBMB205 successfully isolated from the rice rhizosphere in Korea has different proteolytic abilities from this study (Hwangbo *et al*. 2016). Different habitat circumstances can have an impact on the capabilities of bacterial strains within a certain niche. This study also found that the isolate 5.9R, which is closely related to *B. amyloliquefaciens*, may produce hydrolytic enzymes. Shahid *et al*. (2021) investigated the metabolites of *B. subtilis* and *B. amyloquefaciens*, which colonize plant roots and create proteases and cellulases. Su *et al*. (2019) have even begun to develop endospore production and bioactive metabolites of *B. amyloliquefaciens*, which produces amylase, protease, and cellulase, indicating the *Bacillus* sp. group's overall ability to create enzymes.

CONCLUSSION

Fourteen hydrolytic *Bacillus* sp. isolates, including amylolytic, proteolytic, and cellulolytic, were successfully isolated from litter composting. Isolates 2.5, 2.7, 5.5, and 5.9R were chosen because they had the greatest amylolytic, proteolytic, and cellulolytic index. Molecular identification of the four isolates using the 16S rRNA gene revealed that isolates 2.5 and 2.7 shared similarities with *Bacillus stercoris*, isolate 5.5 with *Calidifontibacillus erzurumensis*, and strain 5.9R with *B. amyloliquefaciens*. Further testing of the four isolates' degrading ability in waste is critical for product development and widespread use, and whole genome analysi

s (whole genome sequencing) is also required to validate the isolate's identity.

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