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Characterization and Molecular Identification of Lipolytic-bacterial Isolates Forming Biofilm on Polyethylene Plastic

Olivia P. Ariyanti[1], Putri Agustina[1], Sofyan Anif[1], Hernandito A. Seno[1], Alden G. M. P. Hardianto[1], Listy H. Mandiri[1], Dzarifah Zulperi[2], Nazia Jamil[3], Triastuti Rahayu[1], Yasir Sidiq[1*]

[1]Department of Biology Education, Faculty of Teacher Training and Education, Universitas Muhammadiyah Surakarta, Jl. A. Yani, Tromol Pos 1 Pabelan, Surakarta, Indonesia, 57162

[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang Selangor Darul Ehsan, Malaysia

[3]Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore 54590, Pakistan

Corresponding author, e-mail: *ys120@ums.ac.id

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ABSTRACT

Polyethylene (PE) plastic is one of the most widely used for multiple purposes and leading the environmental problems. Lipolytic bacteria are promising agents to reduce the plastic waste. The dry weight of PE plastic was reduced by 33 % in the Winogradsky's column after 45 days of incubation. However, the lipolytic bacteria responsible for those reduction was unknown. This study aimed to characterize and identify the potential lipolytic bacterial isolates forming biofilm on polyethylene PE plastics. Samples of lipolytic bacterial isolates were screened on tributyrin selective media based on the formed clear zone. Moreover, the 16S rRNA genes of the two most potential lipolytic bacterial isolates were amplified. Then, the amplicons of the 16S rRNA gene were sequenced. This study found two potential lipolytic bacteria isolates, AB A-2 and AB M-3, which had the characteristics of round colonies, wavy edges, convex surfaces, and milky white color. The two isolates are gram-positive and have the shape of *Coccobacillus* cells. The molecular identification showed that AB A-2 isolate was *Bacillus* sp., while AB M-3 isolate was *Bacillus amyloliquefaciens*. This finding contributes to novel bacterial isolates that potentially overcoming the plastic waste problem.

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INTRODUCTION

Plastic is composed of alkene polymer compounds with a very large molecular shape. Plastic molecules come from the process of organic condensation and the addition of polymers or other substances (Ratnawati, 2020). Based on the constituent materials, there are 7 types of plastic codes, including 1-PET (polyethylene terephthalate), 2-HDPE (high density polyethylene), 3-PVC (polyvinyl chloride), 4-LDPE (low density polyethylene), 5-PP (polypropylene), 6-PS (polystyrene), and 7-others derived from SAN (styrene acrylonitrile), ABS (acrylonitrile butadiene styrene), PC (polycarbonate), and nylon (Arwini, 2022).

Polyethylene (PE) plastic is the most common type of plastic used by the community in everyday items, such as cracker bags, food wrappers, and other thin plastics (Mogot et al., 2020). This plastic consists of a type of polymer derived from ethylene compounds and is very commonly used due to it is lightweight, strong, water-repellent, resistant to chemicals, and ease to recycle. Polyethylene (PE) plastic has low gas permeability, making it ideal to wrap or store foodstuffs that require protection against oxygen and moisture (Deglas, 2023). Although plastic can provide benefits for humans, it is also detrimental to the environment due to its resistance to decomposition. The strong molecular structure possessed by PE plastic makes it difficult to decompose (Abidin et al., 2023). One of the alternative solutions that can be done to overcome the problem of plastic waste degradation is the use of the biodegradation methods (Asmi et al., 2022).

The process of plastic biodegradation begins with the formation of biofilms. This biofilm is formed by colonizing bacteria, which easily attaches to plastic surfaces. With the help of the intra- and extracellular depolymerase enzymes, bacteria will degrade plastic complex polymers into simpler compounds making them more accessible as a source of carbon and energy (Octavia et al., 2022). Biodegradation is a technique for degrading a chemical by utilizing microorganisms, particularly bacteria, where the bacterial metabolic process can damage the structure of polymers into monomers by utilizing enzymes, such as lipase enzymes, esterases, and serine hydrolase (Sriningsih & Shovitri, 2015). Lipolytic bacteria are widely used in the biodegradation process to overcome the problem of plastic waste in the environment (Halim & Rahayu, 2024). Lipolytic bacteria are microorganisms that produce lipase enzymes that can degrade fats into fatty acids and glycerol (Khairani & Manalu, 2023).

The unpublished data of our previous study reported that indigenous lipolytic bacteria from cemetery soil are able to degrade PE plastic. This was demonstrated from the reduction in the dry weight of plastic in the Winogradsky, SEM, and FTIR columns. In another study, PE plastic degradation tests were also carried out in Winogradsky columns using indigenous lipolytic bacteria from grave soil, river sediments, and garbage disposal soil. The previous study (unpublished) showed a decrease in the dry weight of PE plastic by 33% in the Winogradsky's column after 45 days of incubation, which is suspected to be caused by indigenous lipolytic bacteria from several types of soil that were then collected. However, the collection of bacteria has not been characterized and molecularly identified based on the 16S rRNA gene. According to research by Pertiwi et al. (2015), the easiest locus to amplify is the 16S rRNA locus because it is conserved within a species, and undergoes minimal mutations or nucleotide changes. The advantages of identifying bacteria using the 16S rRNA gene includes its ability to identify uncultured bacteria, high accuracy, and relatively short processing time. The 16S rRNA gene is widely utilized in various fields, especially in species identification (Akihary & Kolondam, 2020). This study aimed to characterize and identify the potential lipolytic bacteria associated with PE plastic in the Winogradsky column. Molecular identification was conducted using 16S rRNA gene sequences, which were compared against NCBI database. The findings could have significant implications for beneficial bacterial exploration to solve environmental plastic problems.

MATERIALS AND METHODS

Materials

This exploratory research was carried out from July to December 2024 at the Biology Education Laboratory, Universitas Muhammadiyah Surakarta. Samples of lipolytic bacterial isolate were obtained from the laboratory collection. A total of 8 bacterial isolates were selected, including isolates with codes AB A-1, AB A-2, AB A-3, AB A-4, AB M-1, AB M-2, AB M-3, and AB S-5. The bacterial isolate sample were derived from the biodegradation of polyethylene (PE) plastic in Winogradsky's column.

Media Preparation

In this study, 3 types of media were used, including nutrient agar (NA) media, tributyrin lipolytic selective media, and nutrient broth (NB) media. A 20 g/L NA media was used to rejuvenate the collection of lipolytic bacterial isolates. While tributyrin media was used to select lipolytic bacterial isolates based on the clear zone produced by each bacterial isolate. This medium was made by dissolving 5 g of pepton, 3 g of yeast extract, 10 g of tributyrin, and 20 g of bacteriological agar in 1 L of aquades then heated in erlenmeyer until dissolved (Halim & Rahayu, 2024). NB media was used to grow selective isolate lipolytic bacteria by dissolving 8 g of NB in 1 L of aquades. All media were sterilized using an autoclave for 15 minutes at 121°C before being dispensed into a test tube or petridish.

Selection of Lipolytic Bacterial Isolates

The selection of lipolytic bacteria isolate began with the rejuvenation of the collection of lipolytic bacteria into the Nutrient agar media oblique. The lipolytic bacteria were then incubated at room temperature for 48 hours (Simamora & Sukmawati, 2020). Furthermore, the selection of lipolytic was carried out by the diffusion method using a blank disk (Intan, 2021). The initial stage was to prepare a liquid culture from lipolytic bacterial isolates to be tested in 3 days old NB medium with shaking. A blank disc was placed on the surface of the tributyrin media and then dripped with a liquid culture of lipolytic bacteria to be tested. Furthermore, it was incubated in a room temperature incubator for 24 hours. The lipolytic index (IL) was obtained by measuring the diameter

of the colonies and clear zones that were formed and included in the following formula:

$$Lipolytic\ Index = \frac{Diameter\ of\ Clear\ Zone}{Diameter\ of\ Bacterial\ Colony}$$

Chairunnisa et al., 2019

The larger the area of the clear zone formed, the greater the ability of bacteria to produce lipase enzymes to degrade lipids (Mandiri, 2023). The higher the index value produced, the higher the lipolytic activity (Rini et al., 2023).

Characterization of Potential Isolates

Characterization of potential isolates included observation of colony morphology and Gram staining. Observation of the morphology of a colony of lipolytic bacteria was done by observing the shape, surface (elevation), edge of bacteria (margin), and color of the colony. In the Gram staining process, a bacterial smear was made on the glass object by adding enough aquades and taking a colony of pure culture bacteria on the tilted NA agar and then fixating. Next, the smear was inundated with a solution of crystal violet, iodine, 90% ethanol, and safranin, then rinsed and dried (Talaro & Chess, 2018). The colony morphology and Gram staining results were observed under a microscope.

Molecular Identification by 16S rRNA Gene

The molecular identification process based on the 16S rRNA gene included DNA extraction, amplification of the 16S rRNA gene, electrophoresis, and amplification of the 16S rRNA gene. Lipolytic bacterial isolate cultures grown in 4 ml of Nutrient Broth medium were then harvested by centrifugation at 15000 rpm for 1 min. DNA extraction was performed by following the procedures outlined in the Geneaid PrestoTM Mini gDNA Bacteria Kit. The extracted DNA was amplified by the 16S rRNA gene with two universal primers, including 27F and 1492R using mini-PCR. The modified PCR program used were: initial denaturation of 96 °C for 120 minutes, denaturation at 96 °C for 30 minutes, annealing at 50 °C for 40 minutes, elongation at 72 °C for 60 minutes, and final extention at 72 °C for 240 minutes (Oktavia & Wibowo, 2016). The PCR product was loaded into agarose gel at 0.8 % and run for electrophoresis at 100 voltages for 30 minutes. The results of electrophoresis were observed under UV light. PCR products resulting from the amplification of the 16S rRNA gene were then sequenced using the Sanger method, a commonly used method in DNA sequencing in the laboratory (Al-Shuhaib & Hashim, 2023). After the sequencing stage, identification or search for similarity of sequencing results with existing databases was carried out using the *Basic Local Alignment Search Tool* (BLAST) technique using the www.ncbi.nlm.nih.gov online server (Noer, 2021). The alignment of some sequencing results was depicted in the phylogenetic tree through the MEGA 11 program (Tamura et al., 2021).

Data Analysis

The characterization of lipolytic bacterial isolates was analyzed using quantitative descriptive by calculating the lipolytic index based on the clear zone formed around the colony. Therefore, the molecular identification of lipolytic bacterial isolates was analyzed using qualitative descriptive methods with DNA sequencing techniques based on the 16S rRNA gene using the online server https://blast.ncbi.nlm.nih.gov/Blast.cgi, and the phylogenetic tree was constructed using the MEGA 11 program.

RESULTS AND DISCUSSION

Selection of Potential Lipolytic Bacterial Isolates

The lipolytic bacteria isolate sample collected by the Biology Education Laboratory of FKIP, University of Muhammadiyah Surakarta were obtained from the biodegradation polyethylene (PE) plastic in the Winogradsky column. A total of 8 bacterial isolates were selected, namely isolates with codes AB A-1, AB A-2, AB A-3, AB A-4, AB M-1, AB M-2, AB M-3, and AB S-5. The selection of potential lipolytic bacterial isolates was carried out using the diffusion method with a blank disk to assess the ability of each isolate to form a clear zone around the colony on the tributyrin medium.

The clear zone formed is the result of a reaction between bacteria and tributyrin media, where tributyrin is a type of lipid commonly found in natural vegetable fats and animal oils. The tributyrin is hydrolyzed by the lipase enzyme from lipolytic bacteria, which produces water-soluble butyric acid and makes the medium transparent, forming a clear zone around the colony (Mandiri et al., 2023). The larger the clear zone that forms around the colony, the greater the ability of the lipolytic bacteria to produce lipase enzymes to

degrade lipids. The lipolytic index (IL) is obtained by measuring the diameter of the colonies and clear zones and then calculating them with the lipolytic index formula.

The results of the ability of lipolytic bacterial isolates collected by the Biology Education Laboratory of FKIP, University of Muhammadiyah Surakarta in hydrolyzing fats in tributyrin media determined by the calculation of the lipolytic index (IL) are preseted in Table 1. From the results of the calculation of the lipolytic index, bacterial isolates with an average lipolytic index above 1.5 were selected to be further characterized and identified. Table 1showed that two potential isolates, namely isolates with codes AB A-2 and AB M-3 were identified. The lipolytic index values of both isolates are relatively higher compared to those of landfill soil (1.51), palm oil liquid waste (0.48) (Khairani & Manalu, 2023), and mangrove soil (1.05) (Remijawa et al., 2020). The larger the area of the clear zone formed, the greater the ability of bacteria to produce lipase enzymes to degrade lipids (Mandiri, 2023). The higher the index value produced, the higher the lipolytic activity (Rini et al., 2023).

Characterization of Potential Isolates

After selection, the potential lipolytic bacterial isolates AB A-2 and AB M-3 were then characterized through colony morphology and Gram staining. Morphological observations were made by observing a single colony under a microscope of each potential isolate growing on the tributyrin medium. The morphology of a colony of lipolytic bacteria can be observed from the shape, surface (elevation), bacterial edges (margins), and color of the colony. Lipolytic bacteria can have circular or irregular shapes flat or undulate margins, flat or convex surfaces, and yellowish-white, or green (Khairani & Manalu, 2023). The results of the morphological observations of the isolation colonies of AB A-2 and AB M-3 showed similarities, where the two isolates were round, with undulate margin, convex surfaces, and milkywhite coloration (Table 2, Figure 2). Both potential isolates also exhibited shiny and slimy colony surfaces.

Table 1. Result of the selection of bacterial isolate lipolytic ability

Lipolytic Bacteria Isolates Code	IL Average ± Standard Deviation
AB A-1	1.19 ± 0.09
AB A-2	**1.57 ± 0.33**
AB A-3	1.44 ± 0.17
AB A-4	1.39 ± 0.15
AB M-1	1.15 ± 0.00
AB M-2	1.35 ± 0.22
AB M-3	**1.57 ± 0.77**
AB S-5	1.32 ± 0.08

Note: The bold is selected bacterial isolate lipolytic ability

Table 2. Results of characterization of potential isolates AB A-2 and AB M-3

Isolates Code	Gram Staining Results and Cell Shape	Colony Characters			
		Colony Form	Margin	Elevation	Colony Colors
AB A-2	Positive Coccobacillus	Round	Undulate	Convex	Shiny milky white and slimy
AB M-3	Positive Coccobacillus	Round	Undulate	Convex	Shiny milky white and slimy

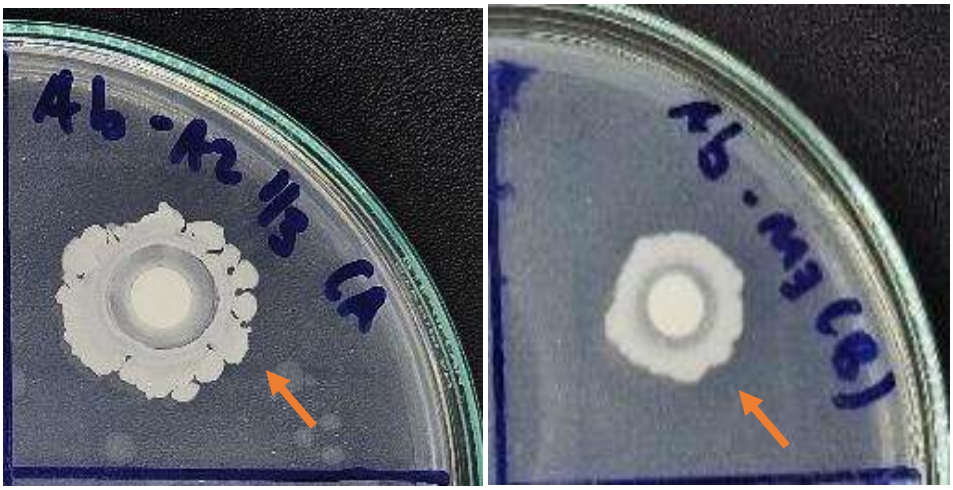

Figure 1. Clear zone of lipolytic bacteria in tributyrin media. Note: oranye arrow: clear zone detected

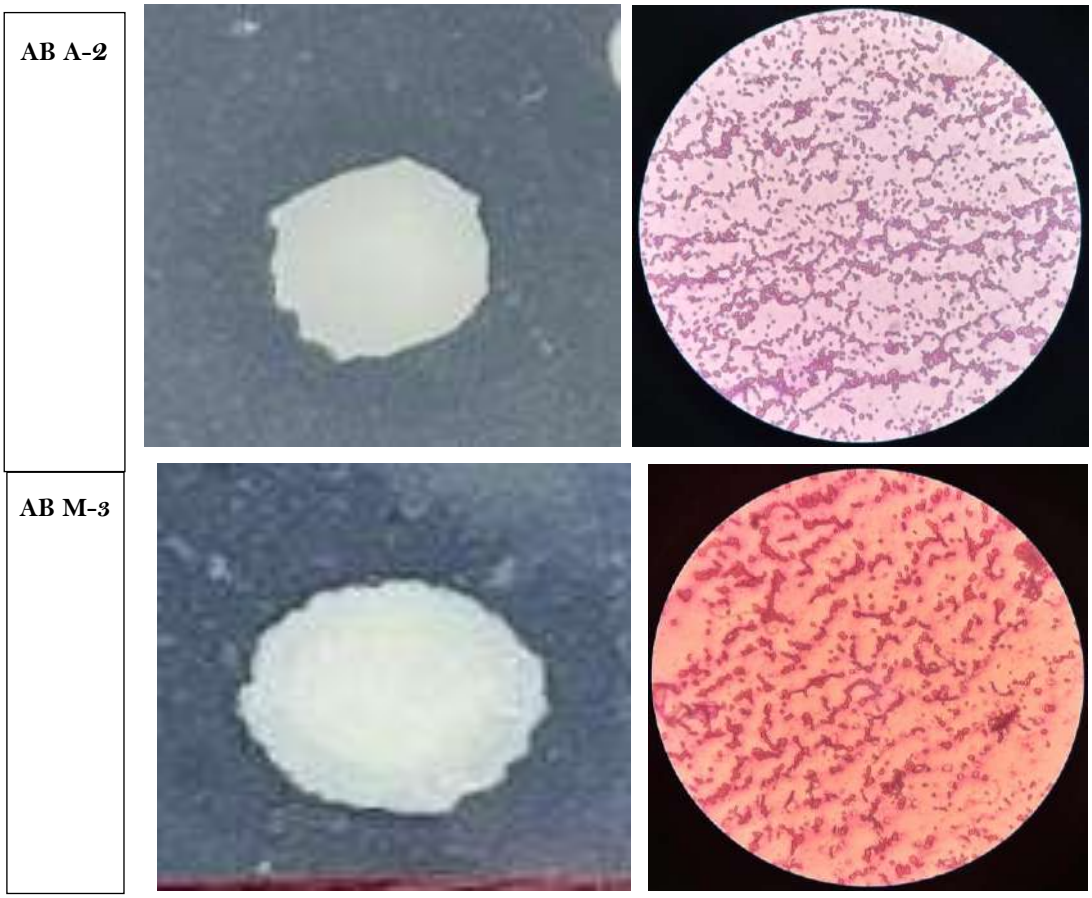

Figure 2. Colony morphology and Gram staining results

The results of characterization of potential isolates through the observation of Gram staining revealed that AB A-2 and AB M-3 isolates are Gram positive bacteria in the form of *Coccobacillus* (Table 2, Figure 2). The classification of positive and negative Grams can be determined from the colors produced after the Gram staining process. If the color produced is purple, the isolate is a Gram-positive bacteria, while the isolate that produces a red color is a Gram negative. Gram-positive bacteria retain the crystal violet dye, whereas Gram-negative bacteria lose the crystal violet dye and take up the safranin dye, which gives them a red color (Susanto, 2016). Gram-negative bacteria appear red, while Gram-positive bacteria appear purple (NauE et al., 2022).

Molecular Identification of Potential Isolates based on 16S rRNA Gene

Currently, bacteria identification can be done using molecular biology methods, which are not only fast but also highly sensitive. The molecular biology method involved the analysis of the 16S rRNA gene sequence (Nurzhulian et al., 2021). The 16S rRNA gene marker is one of the most commonly used methods for bacterial identification. The 16S rRNA gene is a part of prokaryotic organisms, with some regions being 'conserved' (Akihary & Kolondam, 2020). The length of the 16S rRNA gene sequence is about 1,550 bp and consists of conserved regions (Noer, 2021). The 16S rRNA gene has a sustainable component and can be found in every organism. Therefore, the 16S rRNA gene can be used in the PCR process and sequence analysis (Fazri et al., 2019). According to research by Pertiwi et al. (2015), the easiest locus to amplify is the 16S rRNA locus because it is conserved (does not undergo many mutations or nucleotide changes). The advantages of identifying bacteria using the 16S rRNA gene include its ability to identify bacteria that cannot be cultured, its high accuracy, and its relatively short processing time. The 16S rRNA gene is widely utilized in various fields, especially for identification purposes (Akihary & Kolondam, 2020). The stages of bacterial identification using the 16S rRNA gene in general include DNA extraction, amplification of the 16S region through PCR, gene visualization through electrophoresis, sequencing, and processing sequencing data through bioinformatics (Noer, 2021).

Based on the analysis of the results of bootstrap phylogenetic tree reconstruction using the Neighboor-Joining method, it is evident that potential isolates with codes AB A-2 and AB M-3 are very close to the genus *Bacillus*. The potential isolate of AB A-2 was identified as a species of *Bacillus* sp. (Table 3, Figure 3), while the potential isolate of AB M-3 was suspected to be *Bacillus amyloliquefaciens* (Table 3, Figure 3). One type of lipase enzyme-producing lipolytic bacteria is the genus *Bacillus* (Royanti et al., 2023). This is also supported by the research of Nurzhulian et al. (2021), where lipolytic bacteria isolated from wadi fermentation products (eel digestive organs) were also bacteria from the genus *Bacillus*.

There are several types of bacteria capable of producing the enzyme lipase, one of which is *Bacillus*. According to research by Royanti et al. (2023), five isolates were obtained from Kebun Raya Tanah Liwa, with the highest lipolytic index (IL) found in *Bacillus* BP 16, reaching a value of 6.34. Lipase production activity ranging from 0.014 to 3.231 U/mL has also been identified in several *Bacillus* species from fermented foods in Thailand, with the highest activity observed in *Bacillus subtilis* (Tanasupawat et al., 2015). Lipolytic activity was also observed in *Bacillus* isolates from coffee plantations, with the highest index recorded at 6.01 in one isolate (Ervina et al., 2020). Moreover, lipolytic bacteria from the genera *Bacillus*, *Pseudomonas*, and *Klebsiella* have been isolated from palm oil mill wastewater, with lipolytic index values ranging from 0.16 to 0.48 mm (Khairani & Manalu, 2023).

The Bacillus bacteria type is one of the lipolytic bacteria that can be used as a biodegradation agent, biocontrol, and biodetergent (Royanti et al., 2023). The ability of these bacterium to produce lipase enzymes can be used to degrade the structure of objects that contain lipid content. In the biodegradation process, *Bacillus* bacteria use carbon as an energy source. This ability is also proven in the plastic biodegradation process, where the *Bacillus* sp. can use plastic polymers as a source of carbon providing bacteria with energy for cell division (Oktavia et al., 2022). The previous studyalso proves that indigenous lipolytic bacteria from grave soil can degrade PE plastic. This was confirmed by the reduction in the dry weight of plastic in the Winogradsky, SEM, and FTIR columns.

Bacillus amyloliquefaciens is a Gram-positive aerobic bacteria that commonly found in soil. The physiological characteristics of *Bacillus amyloliquefaciens* includes easy metabolic adaptation and inexpensive cultivation costs. *Bacillus amyloliquefaciens* plays an important role in various industries due to its ability to produce a wide variety of antimicrobial enzymes and compounds. Its ability to produce various enzymes, such as amylase, protease, lipase, makes this bacterium widely applied to advance sustainable and environmentally friendly solutions in various sectors (Kolsi et al., 2023). In several different strains of *Bacillus amyloliquefaciens*, cyclic lipopeptides, such as surfactin, fengycin, iturin A, and bacillomycin D have been identified, each encoded by the genes *srfABCD*, *fenABCDE*, *ituD*, and *bmyC-BAD*, respectively (Luo et al., 2022). A mixture of *Bacillus amyloliquefaciens* and *Staphylococcus saprophyticus* in a Winogradsky column has demonstrated plastic degradation

capability, where the degradation rate of polystyrene (PS) plastic is higher than that of polyethylene (PE) plastic (Rahayu et al., 2024).

The potential isolates of AB A-2 and AB M-3 were derived from biofilms in the biodegradation study of polyethylene (PE) plastics in Winogradsky columns, sampled from 2 different soil types. AB A-2 isolate was sampled from the landfill soil, while AB M-3 isolate was sampled from the cemetery soil. This recent study might be the first report isolating the bacteria from cemetery soil with unique biofilm producer traits. Lipolytic bacteria can be found in various potential sources, such as landfills, public cemeteries, and river sediments (Halim & Rahayu, 2024). Based on research conducted by Mandiri (2023), out of 36 bacterial isolates collected from

Table 3. Results of identification of monocular isolate of potential lipolytic bacteria

Isolates Code	Nearest Species	Query Cover	Percent Identity	Accession number
AB A-2	*Bacillus* sp.	100 %	99.93 %	OQ472445.1
AB M-3	*Bacillus amyloliquefaciens*	100 %	100.00 %	JQ664685.1

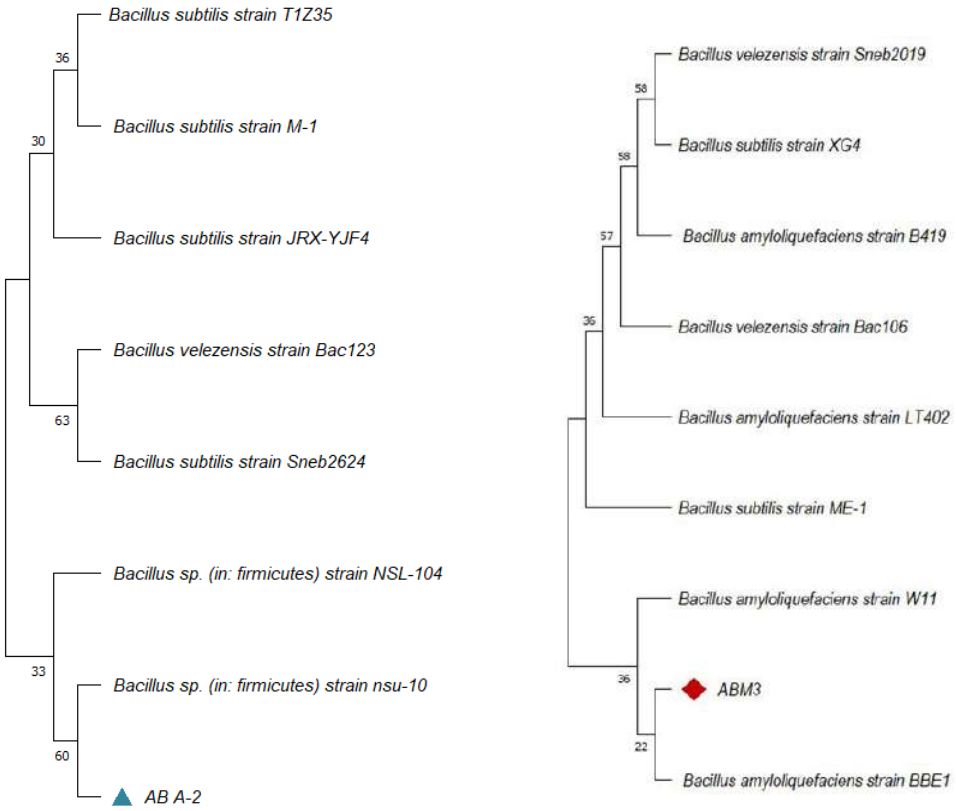

Figure 3. Construction of potential isolate phylogenetic trees AB A-2 and AB M-3

TPU Pracimaloyo, approximately 22.2% (8 isolates) exhibited lipolytic activity. The study by Khairani Manalu (2023) also identified that lipolytic bacterial isolates sampled from river sediment soil belonged to the genera *Bacillus*, *Klebsiella*, and *Pseudomonas*. Soil from public cemeteries (TPU) harbors lipolytic bacteria with a lipolytic index (LI) > 2 (Rini, 2023). *Bacillus* bacteria are known to have the ability to survive in a wide variety of environmental conditions, ranging from highly nutritious environments to extreme and toxic environments (Simamora & Sukmawati, 2020).

CONCLUSION

The AB A-2 and AB M-3 isolates exhibited spherical colonies with undulating margins, convex elevation, and a milky white appearance. Gram staining revealed that both isolates are Gram-positive with a coccobacillary morphology. Molecular identification based on 16S rRNA gene sequencing confirmed that isolate AB A-2 belongs

to the genus *Bacillus sp.*, while isolate AB M-3 was identified as *Bacillus amyloliquefaciens*. As members of the *Bacillus* genus, both isolates possess notable lipolytic activity, reinforcing their potential for industrial and environmental applications, particularly in plastic biodegradation. Their ability to break down complex compounds underscores their significance in sustainable waste management strategies.

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