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Identification of Polyester Hydrolase (PETase) Enzyme-Producing Bacteria Based on 16S rRNA Gene Sequences from Bantar Gebang Landfill Soil

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ABSTRACT

A significant portion of polyethylene terephthalate (PET) plastic waste generated from human activities accumulates in landfills due to inadequate waste management, posing serious risks to the environment and public health. This study aimed to identify and characterize bacteria capable of producing polyester hydrolase (PETase), an enzyme known for its ability to degrade PET. Bacterial isolates were obtained through a screening process conducted at the Bantar Gebang landfill site in Bekasi, West Java, Indonesia. The bacterial isolates were qualitatively characterized by Gram staining and biochemical assays, and quantitatively assessed using selective polycaprolactone (PCL) media. Three isolates P3, P4, and P6 exhibited potential PETase enzyme activity. Among these, isolates P3 and P4 demonstrated measurable enzymatic activity, as indicated by clear zone diameters of 1.4 mm and 5.2 mm, respectively, on selective media. PCR amplification of the PETase gene resulted in a DNA fragment of approximately 1000 bp. Molecular identification based on 16S rRNA gene amplification (~1500 bp) and sequence analysis revealed that the isolates were closely related to *Pseudomonas* sp. (97.76% similarity, isolate P3), *Pseudomonas fluorescens* (88.75% similarity, isolate P4), and *Bacillus subtilis* (93.31% similarity, isolate P6). Among the three, isolate P4 (*P. fluorescens*) demonstrated the highest potential as a PETase-producing bacterium.

***Keywords**:* 16S rRNA, PETase, Polycaprolactone, PET Plastic, Biodegrading Bacteria

INTRODUCTION

Plastics are composed of long-chain organic polymers with favorable properties such as being inexpensive, lightweight, durable, and corrosion-resistant. Most plastics are made of PET (polyethylene terephthalate). PET-based plastics generally consist of densely packed polymers that can persist in the environment for extended periods [1]. PET plastic waste has accumulated in various landfills across Indonesia, such as the Bantar Gebang landfill, increasing the potential for water and soil pollution and significantly depleting fossil resources. Ultimately, this leads to environmental issues and global challenges. Indonesia is recognized as the second-largest contributor to marine plastic pollution globally, after China, with an estimated 1.29 million tons annually [2].

One of the best methods to address PET plastic waste is recycling [3,4]. Various strategies have been employed, including chemical, physical, and biological approaches. Among these, biological recycling is preferred due to its simplicity, environmental friendliness, energy efficiency, and minimal waste generation [5,6,7]. This preference is driven by the discovery of microorganisms capable of degrading plastics [5]. The ability of bacteria to degrade PET plastic waste is attributed to the enzyme polyester hydrolase (PETase), which they produce. PETase is secreted by plastic-degrading bacteria [8,9,10] Research by Purba et al reported that the abundance of bacteria associated with PET plastic waste, based on Next Generation Sequencing (NGS) analysis, was higher in the Proteobacteria group and less so in *Cyanobacteria, Actinobacteria, Chlorobi, Bacteroidetes, Firmicutes, Chloroflexi, Thaumarchaeota, and Gemmatimonadetes* [11]

Given the critical issue of PET plastic waste,

eco-friendly approaches are necessary to achieve the Sustainable Development Goals (SDGs), particularly goals 14 and 15, which emphasize healthy and sustainable aquatic and terrestrial ecosystems. Recycling PET plastic waste using PETase enzymes offers an innovative solution. Previous studies have reported PET-degrading microbes primarily from bacterial groups (56.3%) across 36 genera, followed by fungi, microalgae, and invertebrates associated with microbiota [3]. This study focuses on screening potential PETase-producing bacteria for development as a plastic waste management and degradation method.

METHOD

Field Sampling

Microbial sampling was conducted at the Bantar Gebang landfill in zone 4, a fully loaded landfill site. Two types of locations were sampled: dry soil from surface and subsurface layers (±5 cm deep) and wet soil from surface and subsurface layers (±5 cm deep). Samples were wrapped in aluminum foil, placed in silk plastic bags, and stored in a cool box with ice packs to prevent contamination and maintain microbial viability. Samples were then immediately stored in a refrigerator at 4°C [12].

Isolation of Bacteria from Soil Samples

The isolation process followed the method by Din et al. with minor modifications. Five grams of soil were weighed and ground using sterilized tools to avoid contamination. The soil sample was subjected to serial dilution with 9 mL of sterile distilled water. From dilutions of $10^2, 10^3,$ and 10^4, 100 µL was plated on nutrient agar (NA) in Petri dishes using the spread plate method. Plates were incubated at 37°C for 48 hours [13].

Biochemical Testing of Bacterial Cells

Bacterial morphology, including colony shape, colony count, and colony color, was observed for isolates that grew on Petri dishes. Subsequently, a series of biochemical tests was conducted, following Eashur & Jasim [12] with slight modifications. These tests included Gram staining, oxidase test, catalase test, TSIA (Triple Sugar Iron Agar), indole test, methyl red and Voges-Proskauer (MR-VP) tests, Simmons' citrate agar (SCA) test, urease test, and sugar fermentation tests for glucose and sucrose.

Screening of Potential PETase-Producing Bacteria Using Selective Media

Qualitative selection of potential PETase-producing bacteria was performed using Polycaprolactone (PCL) selective medium, following the method first developed by Nawas et al, with slight modifications. The streak plate method was used for bacterial inoculation on the selective medium [14].

Bacterial Genomic DNA Isolation and DNA Concentration Measurement

Bacterial DNA was isolated using protocols from GF-1 Vivantis and SYNC DNA Extraction Kit Geneaid. DNA concentration was measured using an Infinite 200 PRO NanoQuant Microplate Reader.

Phytase Gene Detection Employing the Specific Primer SM14est

Genomic DNA from the isolated bacterial strains was subjected to PCR amplification using the specific primer pair SM14est (SM14est_fw: CATATGTTTCAGCGGGTCTGGGCGCTG and SM14est_rev:CTCGAGTTAGTGGTGATGGTGG TGATGGC), following the protocol described by Almeida et al. for the detection of the PETase gene [8]. The PCR conditions were as follows: initial denaturation at 98 °C for 3 minutes; denaturation at 98 °C for 1 minute; annealing at 60 °C for 30 seconds; extension at 72 °C for 1 minute, for a total of 35 cycles."

Bacterial Identification Based on 16S rRNA

DNA isolates were subjected to PCR using universal 16S rRNA primers (27F "AGAGTTTGATCMTGGCTCAG" and 1492R "CGGTTACCTTGTTACGACTT") for bacterial identification.

Sequencing Analysis

Initial sequencing was conducted by Genetics Sciences using a Genetic Analyzer. Sequence data were analyzed with Bioedit and MEGA X software. The sequencing results were assembled using BioEdit and subjected to multiple sequence alignment. The obtained 16S rRNA gene sequences were then compared with reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTn (*Basic Local Alignment Search Tool for nucleotides*) algorithm. Phylogenetic analysis to determine the

closest relationships was subsequently performed using MEGA X to construct a phylogenetic tree.

RESULT AND DISCUSSION

Gram Staining and Biochemical Tests

From the streaking process, nine bacterial samples cultured from single colonies underwent Gram staining for identification. Seven isolates were found to be Gram-positive: C1, C3, K1, K4, P2, P6, and P7. The remaining two isolates, P3 and P4, were Gram-negative. The morphology of the bacteria varied, as shown in Table 1.

Gram-positive bacteria were stained purple, while Gram-negative bacteria were stained red. Morphologically, the isolates included two cocci (round-shaped), two chain-shaped bacilli, one chain-shaped isolate, three rod-shaped bacilli, and one flagellated bacillus. Most isolates were rod-shaped (bacilli), indicating that many bacteria from the landfill soil exhibited this morphology.

Biochemical test results revealed differences and similarities among isolates (Table 2). Most isolates tested positive for catalase, except for K4 and P7. For the oxidase test, all isolates were positive except K1. All isolates were positive for urease and methyl red (MR) tests but negative for Voges-Proskauer (VP). In the sucrose fermentation test, all isolates except P3 were positive. Conversely, in the glucose fermentation test, all isolates except P2 were positive. The indole test was positive for all isolates except P4 and P6, while the Simmons' Citrate Agar (SCA) test was negative for all except P4 and P6. The Triple Sugar Iron Agar (TSIA) test revealed various carbohydrate fermentation profiles across isolates, as follows: yellow-red (C1 and K4), red-yellow (C3 and P6), red-red (K1, P3, P4, and P7), and yellow-yellow (P2).

Table 1. Morphology of Isolates Based on Gram Staining

No.	Isolate Code	Colony Shape	Gram Type	Source
1.	C1	Cocci	+	Dry, Subsurface
2.	C3	Bacilli	+	Wet, Subsurface
3.	K1	Bacilli, Flagellated	+	Dry, Subsurface
4.	K4	Cocci	+	Wet, Surface
5.	P2	Bacilli	+	Wet, Subsurface
6.	P3	Bacilli	-	Wet, Subsurface
7.	P4	Bacilli, Chain	-	Wet, Subsurface
8.	P6	Bacilli, Chain	+	Dry, Surface
9.	P7	Chain	+	Wet, Surface

Table 2. Biochemical Characterization of Bacterial Isolates from Bantar Gebang Soil

Sample	Catalase	Oxsidase	TSIA		Urease	Sucrose	Glucose	Indole	SCA	MR	VP
			Slant	Butt							
C1	+	+	yellow	red	+	+	+	+	-	+	-
C3	+	+	red	yellow	+	+	+	+	-	+	-
K1	+	-	red	red	+	+	+	+	-	+	-
K4	-	+	yellow	red	+	+	+	+	-	+	-
P2	+	+	yellow	yellow	+	+	-	+	-	+	-
P3	+	+	red	red	+	-	+	+	-	+	-
P4	+	+	red	red	+	+	+	-	+	+	-
P6	+	+	red	yellow	+	+	+	-	+	+	-
P7	-	+	red	red	+	+	+	+	-	+	-

Table 2 also presents the results of Triple Sugar Iron Agar (TSIA) testing, which can be interpreted based on the color changes observed in the slant and butt of the medium. When a bacterial isolate is capable of fermenting glucose, lactose, and sucrose, both the slant and the butt turn yellow. Isolate P2 (2) exhibited the ability to ferment all three sugars. Conversely, if both the slant and butt remain red, it indicates that the isolate is incapable of fermenting glucose, lactose, or sucrose [15]. Isolates K1, P3, P4, and P7 showed no sugar fermentation activity. Meanwhile, a red slant with a yellow butt

indicates limited carbohydrate fermentation [16]. Isolates C3 and P6 demonstrated carbohydrate fermentation; however, peptone catabolism still occurred, resulting in an alkaline reaction due to ammonia production [16]. On the other hand, a yellow slant with a red butt suggests carbohydrate fermentation without subsequent peptone catabolism. Therefore, isolates C1 and K4 were able to ferment carbohydrates but did not undergo peptone catabolism

In the urease test, the results were evaluated based on color change. A pink coloration indicates a positive result, signifying that the isolate is capable of hydrolyzing urea. All bacterial isolates in this study were able to hydrolyze urea, as shown in Table 2. Subsequently, sugar fermentation tests using glucose and sucrose were performed. A positive result, indicating the production of acid and gas, is evidenced by a color change to yellow and the presence of gas bubbles in the Durham tube. Isolate P3 was the only isolate that did not ferment sucrose, while isolate P2 was the only one that did not ferment glucose (Table 2). Indole production was assessed using Kovac's reagent. A red ring at the surface of the medium indicates a positive result. Most isolates produced a red ring, except for isolates P4 and P6. The next biochemical test was Simmons's Citrate Agar (SCA), with results interpreted based on a color change. As shown in Table 2, only two isolates—P4 and P6—tested positive for citrate utilization. Bacterial isolates that produce sodium carbonate (Na_2CO_3) convert the medium from green to blue, indicating citrate metabolism. In the Methyl Red–Voges Proskauer (MR–VP) test, results were determined by the presence or absence of a red to purplish ring at the surface of the culture medium. A visible ring indicates a positive result [17]. All isolates produced a positive result in the MR test; however, none of the isolates showed a positive reaction in the VP test.

Screening of Potential PETase Producing Bacteria with Selective Media

Among the nine bacterial isolates, only three showed indications of possessing PETase enzymatic activity. Screening with Polycaprolactone (PCL) medium revealed that only two isolates, P3 and P4, produced inhibition zones (clear zone), However, the inhibition zone observed for isolate P3 was too small (Figure 1). P6 did not produce any inhibition zones. The presence of inhibition zones around P3 and P4 colonies indicated the likelihood of PETase enzyme production. The inhibition zone diameters for P3 and P4 were measured and are presented in Table 3.

The selective medium used in this study was Polycaprolactone (PCL). The use of this medium was first introduced by Nishida and Tokiwa in 1993 and was later adopted by other researchers (14). Polycaprolactone (PCL) is a synthetic polyester commonly used as a substrate to assess the enzymatic activity of PETase and cutinase (8, 18)). This medium consists of several main components, namely water, agar, and Lactose Broth (LB). Based on Figure 1, only two isolates, P3 and P4, exhibited PETase enzyme activity. This was indicated by the formation of inhibition zones. An inhibition zone is a clear area surrounding the bacterial growth medium where no bacteria grow (19). The formation of an inhibition zone indicates that the bacterial isolate produces the PETase enzyme, thus demonstrating enzymatic activity in degrading the substrate. The diameter of the inhibition zone was calculated by subtracting the colony zone diameter from the clear zone diameter. The inhibition zone for isolate P3 measured 1.4 mm, while isolate P4 had a larger inhibition zone of 5.2 mm (Table 3). This indicates that isolate P4 has the highest potential for PETase enzymatic activity.

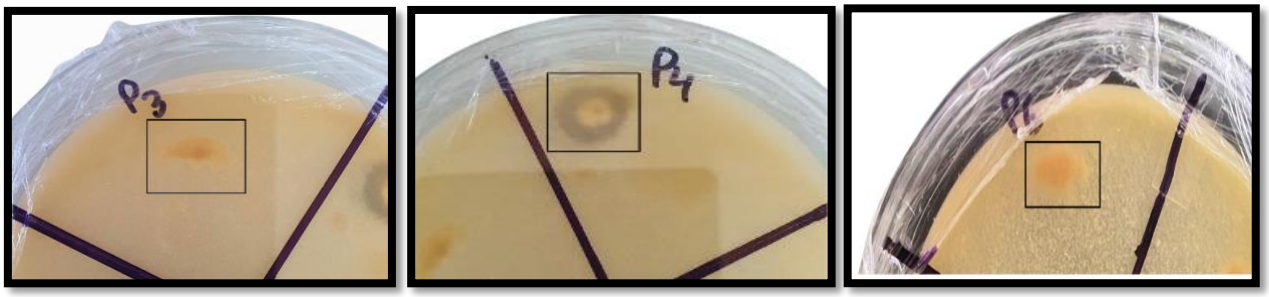

Figure 1. PCL Media Test on P3, P4, P6 after incubation at 28ºC for 14 days.

Table 3. Inhibition Zone Sizes of Isolates

No.	Sample Code	Colony Zone (mm)	Clear Zone (mm)	Inhibition Zone (mm)
1.	P3	11,3	12,7	1,4
2.	P4	4,5	9,7	5,2
3	P6	5	0	0

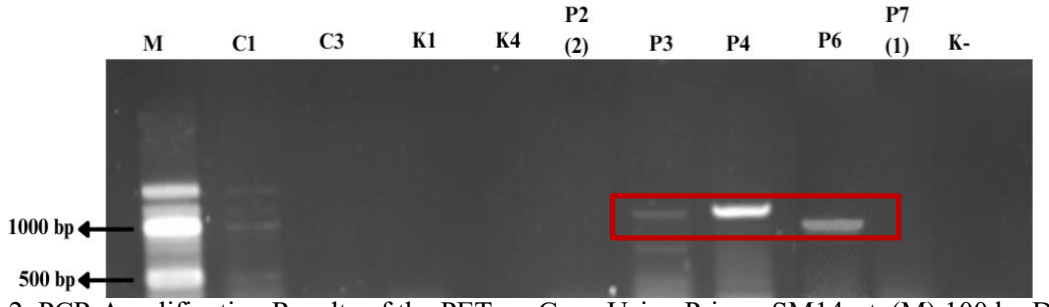

Figure 2. PCR Amplification Results of the PETase Gene Using Primer SM14est. (M) 100 bp DNA Ladder; (C1) Isolate C1; (C3) Isolate C3; (K1) Isolate K1; (K4) Isolate K4; (P2-2) Isolate P2; (P3) Isolate P3; (P4) Isolate P4; (P6) Isolate P6; (P7-1) Isolate P7 (1); (K–) Negative Control

Phytase Gene Detection Employing the Specific Primer

The results of the qualitative assay using selective media were validated by detecting the PETase-encoding gene through PCR using the specific primer SM14est. This primer selectively binds to DNA sequences containing the PETase gene. Therefore, the presence of an amplified DNA band in the PCR electrophoresis indicates a positive result for the presence of the PETase enzyme (Figure 2).

The electrophoresis results presented in Figure 2 indicate that only three isolates P3, P4, and P6 were detected to carry the PETase gene, with an amplicon size of approximately ± 1000 bp, consistent with the expected target gene. The DNA band observed in isolate P4 was more intense than that of isolate P6, whereas isolate P3 displayed a fainter band. This variation is likely due to differences in DNA concentration among the isolates. These findings confirm that only isolates P3, P4, and P6 possess the PETase gene and are therefore capable of producing the PETase enzyme. Isolate C1 produced multiple or non-specific DNA bands during PCR amplification, which may indicate non-specific primer binding, partial homology with the target gene. Non-specific amplification can result from suboptimal primer-template binding, low DNA quality, or the presence of secondary structures and inhibitors that interfere with PCR specificity [20].

The detection of the PETase gene in isolates P3, P4, and P6 through PCR amplification, as evidenced by the presence of ~1000 bp amplicons, suggests these isolates possess the genetic potential for polyethylene terephthalate (PET) degradation. The amplicon size aligns with previous reports on PETase gene amplification using the SM14est primer, supporting the specificity of the primer and the validity of the result [8].

The variation in band intensity observed among the three isolates—particularly the stronger band in P4 and the weaker band in P3—may be attributed to differences in initial DNA template concentration or PCR amplification efficiency. Such differences are commonly encountered in environmental isolates due to variable cell lysis efficiency, DNA purity, or the presence of PCR inhibitors [21]. The presence of the PETase gene in these isolates provides strong molecular evidence of their potential enzymatic activity in PET biodegradation. However, gene presence alone does not confirm expression or enzymatic functionality. Therefore, further validation such as gene expression analysis (e.g., RT-qPCR) or enzymatic activity assays would be essential to confirm the actual PET-degrading capability of these strains. Nonetheless, the molecular screening using PCR serves as a crucial first step in identifying promising candidates for plastic biodegradation applications.

Identification of Bacteria Using 16S rRNA Primer

Molecular identification of the three selected isolates was carried out using universal primers targeting the 16S rRNA gene. The results of electrophoresis with

the 16S RNA primer showed DNA bands with a size of approximately ± 1500 bp for isolates P3, P4, and P6, as shown in Figure 3.

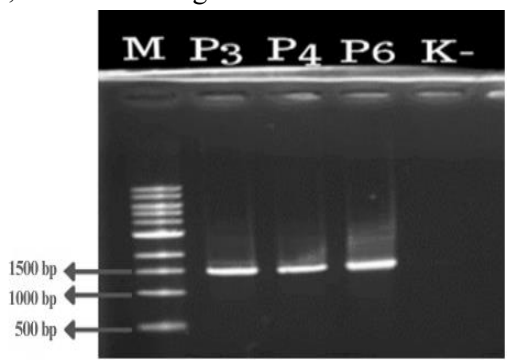

Figure 3. DNA Amplification Results Using 16S RNA Primer; (M) 1 kb DNA Ladder Marker, (P3) Isolate P3, (P4) Isolate P4, (P6) Isolate P6, (K-) Negative Control

The observed amplicon size is consistent with the expected length of the bacterial 16S rRNA gene, indicating successful and specific amplification [13]. This result confirms the presence of amplifiable 16S rRNA gene regions in all three isolates, supporting their suitability for subsequent sequencing and phylogenetic analysis. The use of universal 16S rRNA primers is a widely accepted method for bacterial taxonomic identification due to the gene's conserved and variable regions, which allow discrimination at the genus and, in many cases, species level [22]. These results serve as a foundational step for downstream analysis, such as BLAST comparison with reference sequences in GenBank and the construction of phylogenetic trees, to determine the closest taxonomic affiliations of the PETase-producing isolates

Sequencing Analysis

BLAST analysis of the sequenced isolates revealed the following, Isolate P3 (1484 bp) is closely related to *Pseudomonas sp.* strain STA3 with a 97.76% similarity (Accession Code: KY888133.1); Isolate P4 (1492 bp) was identified as *Pseudomonas fluorescens* strain FP2327 with an 88.75% similarity (Accession Code: CP117438.1); Isolate P6 (1477 bp) was identified as *Bacillus subtilis* strain PUL-A with a 93.31% similarity (Accession Code: EU144043.1). These results were used to construct a phylogenetic tree, as shown in Figure 4.

BLAST analysis of the 16S rRNA gene sequences revealed varying degrees of similarity between the selected isolates and known bacterial strains in the NCBI GenBank database. Isolate P3 (1484 bp) showed a high similarity (97.76%) to *Pseudomonas* sp. strain STA3 (Accession No. KY888133.1), suggesting a close phylogenetic relationship at the genus level. A similarity score above 97% generally indicates affiliation at the species level, although further analysis, such as full-genome sequencing or multilocus sequence typing (MLST), would be required for definitive species identification [23]. Isolate P4 (1492 bp) was identified as *Pseudomonas fluorescens* strain FP2327 (Accession No. CP117438.1) with a lower similarity of 88.75%. This relatively low percentage suggests that P4 may represent a more distantly related species or even a potentially novel strain within the *Pseudomonas* genus. Typically, similarity values below 95% indicate the possibility of a new species [24]. Isolate P6 (1477 bp) showed 93.31% similarity to *Bacillus subtilis* strain PUL-A (Accession No. EU144043.1), indicating its likely affiliation with the *Bacillus* genus. Similar to P4, the moderate similarity score suggests potential taxonomic novelty or significant genomic divergence from known reference strains.

Based on the phylogenetic tree, isolates P3, P4, and P6 were identified as *Pseudomonas sp.*, *Pseudomonas fluorescens*, and *Bacillus subtilis*, respectively. According to Iskandar et al. [25], a similarity percentage of ≥97% but <99% indicates the bacteria belong to the same species, while percentages <95% suggest identification at the genus level [26]. The findings align with previous studies, such as those by Vague et al which reported the presence of *Pseudomonas fluorescens* and *Bacillus subtilis* in landfill soils [27]. These bacteria have shown the ability to degrade LDPE plastic based on weight reduction. For instance, *Pseudomonas fluorescens* degraded up to 22% of plastic weight, while *Bacillus subtilis* achieved a 14% reduction within one month. Studies also demonstrate *Pseudomonas fluorescens* to be more effective in PET degradation compared to other bacteria, such as *Streptomyces sp.* [12].

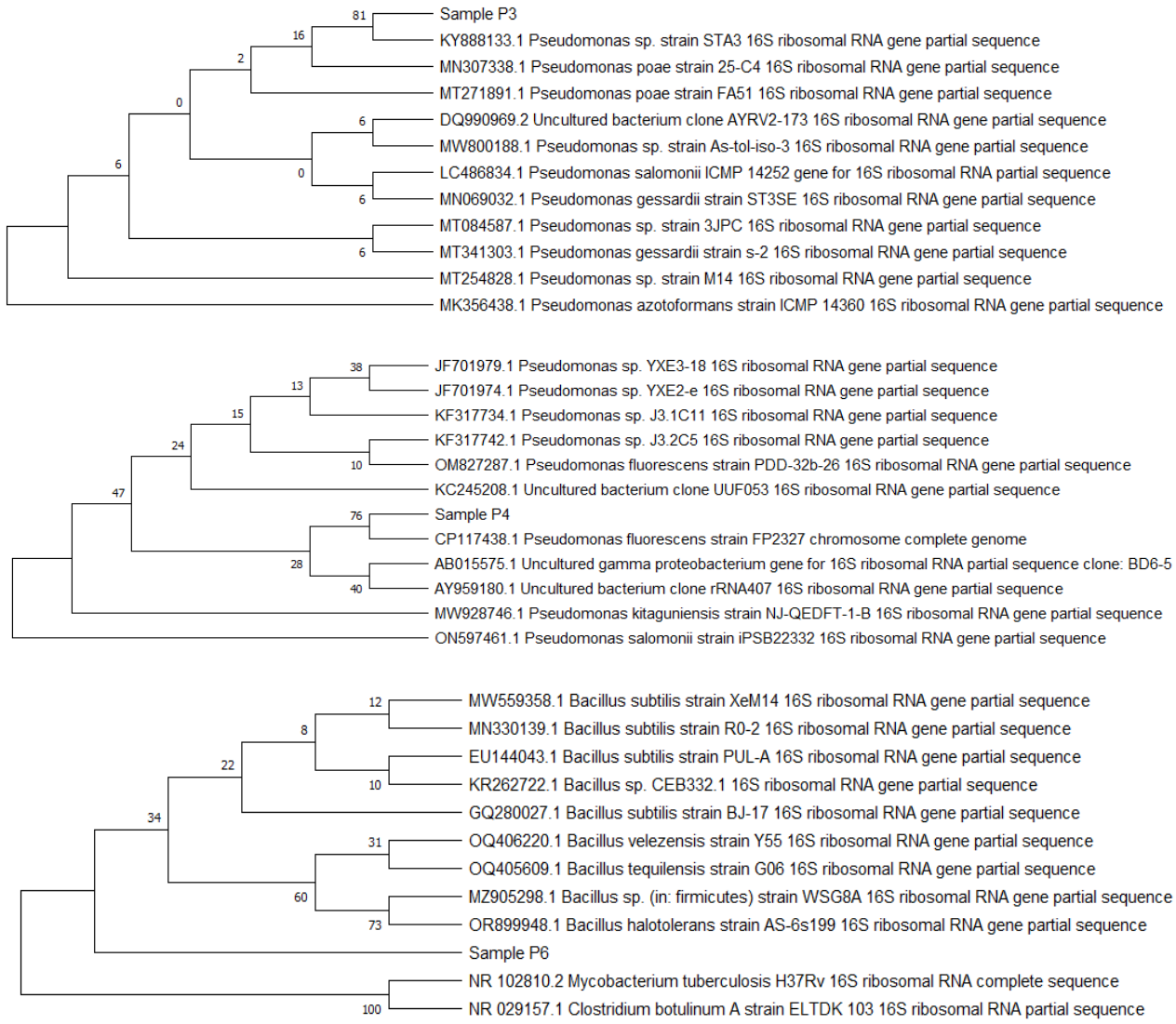

Figure 4. Phylogenetic Tree of Isolates P3, P4, and P6

The bacterial types *Pseudomonas sp.* and *Bacillus sp.* have inherently shown higher degradation capabilities compared to fungi and other bacterial species [28]. Various studies on *Pseudomonas sp.* and its variants have demonstrated significant capabilities in degrading polyethylene (PE) plastics. However, *Pseudomonas sp.* is more frequently reported to be associated with PE and LDPE plastics [29]. Reports on *Pseudomonas sp.* for PET plastic degradation remain limited [27; 30].

One type of *Bacillus* with significant potential for degrading PET plastic is *Bacillus subtilis*. This is also supported by research from Asmita et al. [31], which showed that *B. subtilis* has a greater ability to degrade PET plastic compared to other bacterial species, such as *Pseudomonas aeruginosa*. According to Patil et al [32], *B. subtilis* was able to reduce the weight of plastic by 74.59% in Nutrient Broth (NB) medium after four months. These findings provide a taxonomic framework for further investigation of the PETase-producing capabilities of the isolates. In particular, the phylogenetic positioning of P4 and P6 highlights their potential as novel environmental strains with biotechnological relevance in PET biodegradation. Further genomic and enzymatic characterization will be essential to elucidate their metabolic pathways and to evaluate their practical applications in plastic waste bioremediation.

CONCLUSION

This study identified three bacterial isolates capable of producing the PETase enzyme, making them potential agents for the biodegradation of PET plastic waste. The isolates were isolate P3 which identified as *Pseudomonas sp.*, isolate P4 which identified as *Pseudomonas fluorescens*, and isolate P6 identified as *Bacillus subtilis.* Among these, P4 (*Pseudomonas fluorescens*) exhibited the highest potential for enzymatic activity and could be further developed for plastic waste management.

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