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Detection of homologous plastic PET-degrading enzyme-encoding DNA from enriched plastic-contaminated soil samples

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ABSTRACT *Piscinibacter sakaiensis*, first isolated in Japan, is the only well-characterized bacterium known to possess both PETase and MHETase, enabling complete polyethylene terephthalate (PET) degradation. To date, no additional habitats for the species have been reported. This study aims to identify homologous PETase and MHETase DNA from plastic-contaminated landfill soils in Indonesia. Enrichment cultures were established from soil samples collected at Galuga (Bogor) and Cipeucang (South Tangerang). PCR amplification and sequencing revealed a full-length MHETase homolog (G2MHETase, 1,812 bp) from Galuga, showing 99.4% and 99.3% nucleotide identity to MHETase from *P. sakaiensis* and *Delftia* sp. respectively. The deduced amino acid sequence shared 98.5% identity with both. In contrast, a partial PETase homolog (502 bp of 873 bp) was amplified from the Cipeucang sample, displaying 96 and 93% amino acid identity to PETase from *P. sakaiensis* and *P.gummiphilus* respectively. Nanophore NGS analysis of bacterial diversity indicated distinct microbial community profiles between the two sites. Rare taxa potentially associated with the detected genes included *P. gummiphilus*, *Delftia* sp., *Delftia tsuruhatensis* and *Xenophilus aerolatus* from Galuga, and *Piscinibacter* and *Acidovorax* from Cipeucang. These findings demonstrate the feasibility of detecting homologous PET degrading enzyme genes from plastic-contaminated soils using PCR-based approaches.

KEYWORDS MHETase; PCR; PETase; Plastics contaminated soil sample; Sequence homology

1. Introduction

Changes in human lifestyles that favor practicality support an increase in the usage of plastics, particularly polyethylene terephthalate (PET), which is commonly used as packaging for single-use consumer goods. This contributes to a rise in the volume of PET waste, which has recently become a major concern in waste management (Amobonye et al. 2021; Maity et al. 2021; Raheem et al. 2019). Discarded plastics can be degraded and fragmented into smaller bits in the environment when exposed to ultraviolet (UV) light and other environmental degradation processes. These fragments may consist of large microplastics (1 mm–5 mm), microplastics (1 µm–1 mm), and nanoplastics (1 nm–1 µm) (Atugoda et al. 2023; De-la Torre 2020; Waller et al. 2017). According to Nizzetto et al. (2016), microplastics are the primary source of plastic pollution in freshwater, marine, agroecosystems, and soil (Nizzetto et al. 2016). In addition to being ineffective, the traditional methods of addressing the microplastic issue through chemical and physical means are not sustainable.

PET can generally be degraded into its constituents. However, this process requires extreme conditions, such as acidic conditions at high temperatures or alkaline conditions utilizing extremely hazardous chemical catalysts (Amobonye et al. 2021). Several approaches have been undertaken to address the PET waste problem, one of which is to use an enzymatic method to accelerate PET decomposition under milder conditions (Danso et al. 2019; Kaabel et al. 2021; Ru et al. 2020).

The discovery of *Ideonella sakaiensis* (IsPETase) was a vital breakthrough in plastic biodegradation research (Yoshida et al. 2016). This *I. sakaiensis* now is currently known under the nomenclature *Piscinibacter sakaiensis* (Oren and Göker 2023). Compared to previously published enzymes that can degrade PET, PETase from *P. sakaiensis* has a considerably higher enzymatic activity and specificity for PET under ambient conditions (Joo et al. 2018). Accordingly, it has a very promising potential as a biocatalyst that can accelerate the PET decomposition process.

P. sakaiensis has synergistic enzyme system, PETase and MHETase, so that it can degrade PET into its monomers. During enzymatic hydrolysis by *P. sakaiensis*, a PET molecule degrades into a heterogeneous mixture of monomers (mono(2-hydroxyethyl) terephtha-

late (MHET)), dimers (bis(2-hydroxyethyl) terephthalate (BHET)), terephthalic acid (TPA), and ethylene glycol. BHET and MHET are the esters of ethylene glycol and TPA, respectively. MHET can be further degraded into TPA and ethylene glycol by MHETase, while BHET can also depolymerize into MHET by PETase (Maity et al. 2021). It is very interesting to find out whether the same species of *P. sakaiensis* that have similar ability to degrade plastic PET could be found in other habitats in different countries other than Japan.

In addition to PETase and MHETase from *P. sakaiensis*, another PET degrading enzyme, leaf-branch compost (LCC) cutinase, was identified from metagenomic DNA derived from compost sources. LCC cutinase is capable of hydrolyzing PET and has been studied alongside PETase to explore its potential for biotechnological applications (Sulaiman et al. 2012, 2014). This enzyme shares similar motifs and identical active sites with *P. sakaiensis* PETase. The same study also reported that a newly identified PET degrading esterase mined from a metagenomic database exhibited similar motifs and conserved active sites (Helianti, unpublished data). Taken together, these reports suggest that although PET hydrolase sequences show some diversity, the consensus regions remain relatively conserved.

Another report described that mono(2-hydroxyethyl) terephthalic acid hydrolases (MHETases) have been identified in several bacterial species with high sequence homology, indicating a greater degree of conservation and broader distribution of MHETase (Watts et al. 2024). This showed that PET hydrolases and MHETases do not always coexist within the same bacterial cell. To date, *P. sakaiensis* remains the only species known to harbor both of these synergistic enzymes (Yoshida et al. 2016).

Furthermore, certain natural molecules found in the environment are difficult to degrade. Water-insoluble macromolecules in the environment include lignin, starch, chitin, and cellulose. Despite their durability against chemical and physical degradation, these polymers can be degraded by enzymes that nature has produced (Andlar et al. 2018; Chen et al. 2020; Wang et al. 2020). It seems possible that specific hydrolytic enzymes found in nature could bioremediate PET because ester linkages connect PET monomers with a hydrolysable functional group in their C-C backbone (Maurya et al. 2020). Under ideal growth conditions, several bacteria have been shown to grow exclusively on plastic waste and rely only on plastics as carbon sources (Austin et al. 2018; Belabbas et al. 2025; Dubey and Thalla 2025; Yoshida et al. 2016).

In addition, only a few microbes are effectively grown in a lab laboratory setting. The majority are uncultured microorganisms, which are potential sources for industrial enzyme genes. From these uncultured sources, target genes encoding enzymes could be screened and further produced and characterized via recombinant DNA technology (Yusof 2015).

Therefore, this study aimed to detect alternative genes encoding PET degrading enzymes (PETase and MHETase homologs) from plastic contaminated soils. Samples were collected from the Galuga landfill (Bogor) and the Cipeucang landfill (South Tangerang), Indonesia. Because direct genome extraction from soil often yields low quality DNA due to co-extracted inhibitors such as humic substances, an enrichment culture strategy was used to obtain microbial genomic DNA suitable for PCR analysis (Bertrand et al. 2005). PET was supplied as a selective substrate to preferentially stimulate microorganisms potentially associated with PET degradation and to increase the likelihood of recovering relevant hydrolase genes (Yoshida et al. 2016). Degenerate primers based on known PET hydrolase, such as *P. sakaiensis* PETase and LCC cutinase were designed and employed; along with a pair of specific primers derived from the *P. sakaiensis* MHETase DNA sequence. In addition, bacterial diversity was analyzed using nanopore technology NGS of 16S rDNA from the enriched samples to evaluate the relationship between the detected DNA fragments and the microbial community composition.

2. Materials and Methods

2.1. Primer design

The MHETase primers were designed from the NCBI reference sequence NZ_BBYR01000104.1. Meanwhile, degenerate primers for partial PETase detection were designed based on NZ_BBYR01000074.1 and LCC cutinase. Degenerate primers came from the consensus region of the enzyme sequences. The list of primers is shown in Table 1.

2.2. Soil sampling

Topsoil (± 15 cm) from a landfill area in South Tangerang (Cipeucang, 6°19’34.7”S 106°39’43.2”E) and Bogor (Galuga, 6°33’53.2”S 106°38’33.6”E) was collected aseptically using sterilized tubes and plastic bags. The samples were kept in a cold box while being transported to the laboratory.

2.3. Soil enrichment culture

The soil samples were dried in a laminar flow hood for approximately 1.5 h, then crushed using a sterile mortar and transferred into a 50 mL tube, to which 0.85% NaCl was added. The mixture was vortexed with sterile beads

TABLE 1 Primer design based on Ideonella sakaiensis strain201-F6 (Genbank GAP38373.1) and other PET hydrolases.

Primer name	Primer sequence
IsMHETase-For	5′-ATG CAG ACA ACA GTC ACC ACG ATG CT-3′
IsMHETase-Rev	5′-TCA GGG AGG CGC CGC GCA GGC GAA-3′
Deg-PETase-1 For	5′-CGC GGY CCS AAC CCS AC-3′
Deg-PETase-2 For	5′-TGG CMT CGC ACG GCT TCG T-3′
Deg-PETase-Rev	5′-GTA GCG SGT RTC GTT ATC CAC-3′

until homogeneous and centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant obtained was then used as a starter (10%) to be grown in 20 mL of Nutrient Broth media in a 100 mL Erlenmeyer flask (150 rpm, room temperature, overnight). The initial culture was then used as a starter for the next culture using minimal media containing a pre-sterilized PET film (ø = 1.1 mm) as the main carbon source (Yoshida et al. 2016). Cultures were prepared by adding 10% starter to 20 mL of minimal media in a 100 mL Erlenmeyer flask and were then grown in a shaker (100 rpm) at room temperature for 2 weeks. The minimal media were composed of 0.05% yeast extract, 0.2% ammonium sulfate, 0.1% trace elements (0.1% $FeSO_4 \cdot 7H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% $CuSO_4 \cdot 5H_2O$, 0.01% $MnSO_4 \cdot 5H_2O$, and 0.01% $ZnSO_4 \cdot 7H_2O$), and 10 mM phosphate buffer (pH 7.0).

2.4. DNA Genome Extraction

Microbial cells were harvested by centrifuging the enriched culture medium (8,000 rpm, 30 min). The pellets were then extracted using the Thermo Scientific™ K0721 GeneJET Genomic DNA Purification Kit according to the manufacturer's instructions. The purified genomic DNA from all samples was confirmed by agarose gel electrophoresis.

2.5. PCR Amplification, Cloning of PCR Product, and DNA sequencing

MHETase and partial PETase genes were detected using the touchdown PCR technique. MyTaq™ Red Mix Bioline was used as the polymerase enzyme. The PCR cycling conditions (except the annealing step) were set as follows: initial denaturation at 95 °C for 1 min, denaturation at 95 °C for 15 s, and extension at 72 °C for 10 s. To amplify the MHETase gene using MHETase primers, an annealing temperature from 70 °C to 60 °C was set. On the other hand, two forward primers were used to amplify the PETase gene sequence. An annealing temperature from 63 °C to 53 °C was used for the first primer, Deg-PETase-1 For. The amplified DNA was then subjected to a nested PCR using the Deg-PETase-2 For primer from 64 °C to 54 °C. All results were visualized by electrophoresis using 1% agarose gel. The desired amplified products were isolated from the gel using the GenepHlow™ Gel Extraction Kit. The purified DNA was then ligated to the pJET1.2 plasmid (CloneJET PCR Cloning Kit, Thermo Scientific), followed by DNA transformation to into *E. coli* competent cells. Plasmids from transformants were then extracted using the Presto™ Mini Plasmid Kit (Geneaid). The extracted plasmids were verified using agarose gel electrophoresis and were sent for DNA sequencing (Genetika Science, Indonesia).

For the analyses of bacterial diversity of the enriched samples, the PCR of 16S rDNA using extracted DNA from each enriched sample as a template and a pair of 27F and 1492R primers were conducted. The reaction was as follows. After initial 5-min hot start incubation at 94 °C, the mixture was introduced to 30 cycles, each cycle including 1 min at 94 °C, 35 s at 50 °C, and 2 min at 72°C, then 5 min extension at 72 °C. Full length 16S barcoding for metagenomics using Oxford Nanopore Platform NGS was performed by Genetika Science, Indonesia.

2.6. DNA Sequence Analysis and Deduced Amino Acid Analyses

DNA sequencing results were first analyzed using either the National Center for Biotechnology Information (NCBI) BLAST tool (https://www.ncbi.nlm.nih.gov/) or Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers and Higgins 2014; Tatusova and Madden 1999). Then, the sequences were translated into amino acids and subsequently subjected to a protein BLAST to retrieve related sequences. Multiple sequence alignment of the obtained sequences was undertaken using the MUSCLE algorithm on MEGA 11 software (Edgar 2004; Tamura et al. 2021).

3. Results and Discussion

3.1. Detecting MHETase and PETase gene sequences

Soil samples from the Galuga Bogor (G) and Cipeucang South Tangerang (C) landfills were cultured for two weeks using minimal media, and an overnight nutrient broth culture was used as a starter. PET film served as the primary carbon source. The microbial community from the culture were was then harvested. The genomic DNA of the microbes were extracted and used as templates for PCR amplification.

Four primers were designed in this study based on *P. sakaiensis* strain 201-F6 (GenBank GAP38373.1) and other related PET hydrolases from GenBank. IsMHETase-For and IsMHETase-Rev were designed to detect the MHETase gene, while degenerate primers were designed to detect the partial PETase gene. The degenerate primers for the PETase gene were designed based on the conserved regions of genes encoding enzymes that degrade PET from several different sources, and were intended to target PETase homolog genes across microbial species (Figure 2).

Genomic DNA extracted from the pellets obtained from the minimal media culture were extracted and then visualized in a 1% agarose gel (Figure 1a). This extract was then used as the template for PCR amplification. The PCR amplification resulted in the expected bands (Figure 1b). A fragment of approximately 1800 bp emerged using the IsMHETase-For and IsMHETase-Rev primers, whereas a DNA fragment of approximately 500 bp was obtained from a nested PCR using the second degenerate PETase primers to amplify specific DNA fragments from the 1:10 diluted first PCR product.

The cultivation conditions largely determined microbial community formation, indicating that environmental structure influenced selective processes. The amount of initial inoculum (i.e., dilution) also affected variations in community structure (Junkins et al. 2022). Using the

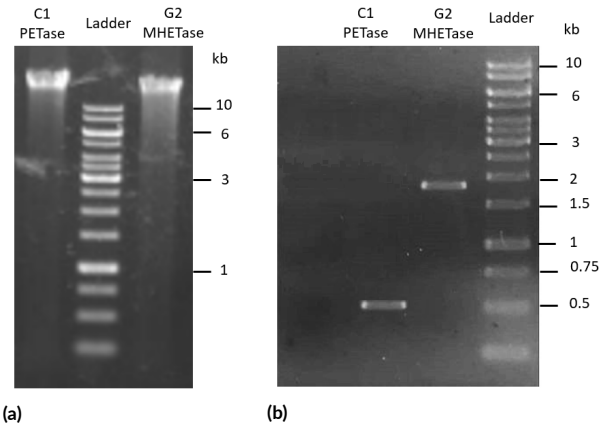

FIGURE 1 (a) Genome extraction of enrichment culture in minimum media and (b) visualization of the amplified fragment of G2MHETase (Galuga) and C1PETase (Cipeucang).

primer pair IsMHETase-For and IsMHETase-Rev, we successfully detected MHETase, yielding the expected ∼1.8 kb DNA fragment. In contrast, partial PETase detection required the use of an additional internal primer to obtain the expected ∼500 bp DNA fragment, suggesting a low abundance of the target DNA template in the genomic DNA pool of the Cipeucang sample. Nested PCR is commonly employed to detect target DNA sequences present at very low concentrations (Leclerc et al. 2025; Panei et al. 2024).

Initially, degenerate primers were employed to detect two genes, PETase and MHETase, from raw environmental samples collected from the Cipeucang and Galuga habitats. DNA was directly extracted from these samples and used as a template for PCR; however, no visible bands were observed for either PETase or MHETase detection. Consequently, the strategy was modified to include enrichment of the environmental samples, as described in this study. Despite several attempts, the degenerate primer approach still yielded no visible PCR bands. Subsequently, a nested PCR strategy using degenerate primers was applied for partial PETase detection (two rounds of PCR), and specific primers were employed for MHETase detection. Using this approach, a partial PETase fragment was successfully amplified only from the Cipeucang sample, while no PETase band was obtained from the Galuga sample. In contrast, MHETase fragments were successfully amplified only from the Galuga sample with the specific primer set. Attempts were also made to amplify PETase using primers designed to span from the start to stop codons, but this strategy was likewise unsuccessful. Two possible explanations may account for these results. First is the low abundance of PET degrading bacteria in these environments, which makes detection challenging and may require more intensive efforts. The second possibility is that the DNA extracted from the environmental samples was highly fragmented rather than intact genomic DNA, thereby reducing PCR efficiency (Wagner et al. 2015).

3.2. DNA and protein sequence analysis

The sequencing was conducted using pJET1.2 forward and reverse primers (Thermo Scientific CloneJET PCR Cloning Kit). DNA sequencing (Sanger sequencing method) resulted in a partial sequence of PETase (502 of 873 bp) and a full-length MHETase sequence (1812 bp).

The 502 bp C1PETase gene sequence was further analyzed using the NCBI Nucleotide BLAST, which revealed close similarity to sequences from *Piscinibacter gummiphilus*, *Acidovorax delafieldii*, and *Schlegelella brevitalea*, with identity scores of 94%, 85%, and 77%, and query coverage of 100%, respectively (Supplementary 1, Table 2). The bacterial diversity of the enriched Cipeucang sample showed a consistent result, as the *Piscinibacter* sp. and *Acidovorax* sp. were detected, although at extremely low abundance (only one read each) (Supplementary 5).

The nucleotide sequence of this partial fragment was aligned with other PETase sequences retrieved from the NCBI BLAST results. *P. sakaiensis* PETase (IsPETase) was also included as a reference, since it did not appear in the BLAST output (Supplementary 1). At the nucleotide level, the partial C1PETase sequence showed the highest similarity to IsPETase, with 97% identity, followed by *P. gummiphilus* with 94% identity (Supplementary 3). The deduced amino acid sequence was aligned with other PET hydrolase sequences, including IsPETase and three selected PETases from *P. gummiphilus*, *A. delafieldii*, and LCC cutinase (Figure 2). The C1PETase fragment showed the highest similarity to IsPETase, with 96% identity. Multiple sequence alignment further revealed that the three catalytic residues and the canonical motif are conserved in the deduced C1PETase sequence (Figure 2). This indicates that the partial C1PETase fragment is unlikely to be a pseudogene, as it contains the key

TABLE 2 NCBI nucleotide BLAST result of C1PETase sequence.

Description	Scientific name	Accession	Query cover (%)	Per. Ident (%)
Piscinibacter gummiphilus strain SBD 7-3 plasmid unnamed1, complete sequence	*Piscinibacter gummiphilus*	CP136337.1	100	94.22
Piscinibacter gummiphilus strain SBD 7-3 chromosome, complete genome	*Piscinibacter gummiphilus*	CP136336.1	100	93.63
Acidovorax delafieldii pbsA gene for PBS(A) depolymerase, complete cds	*Acidovorax delafieldii*	AB066349.1	100	84.58
Schlegelella brevitalea strain DSM 7029. Complete genome	*Schlegelella brevitalea*	CP011371.1	100	77.25

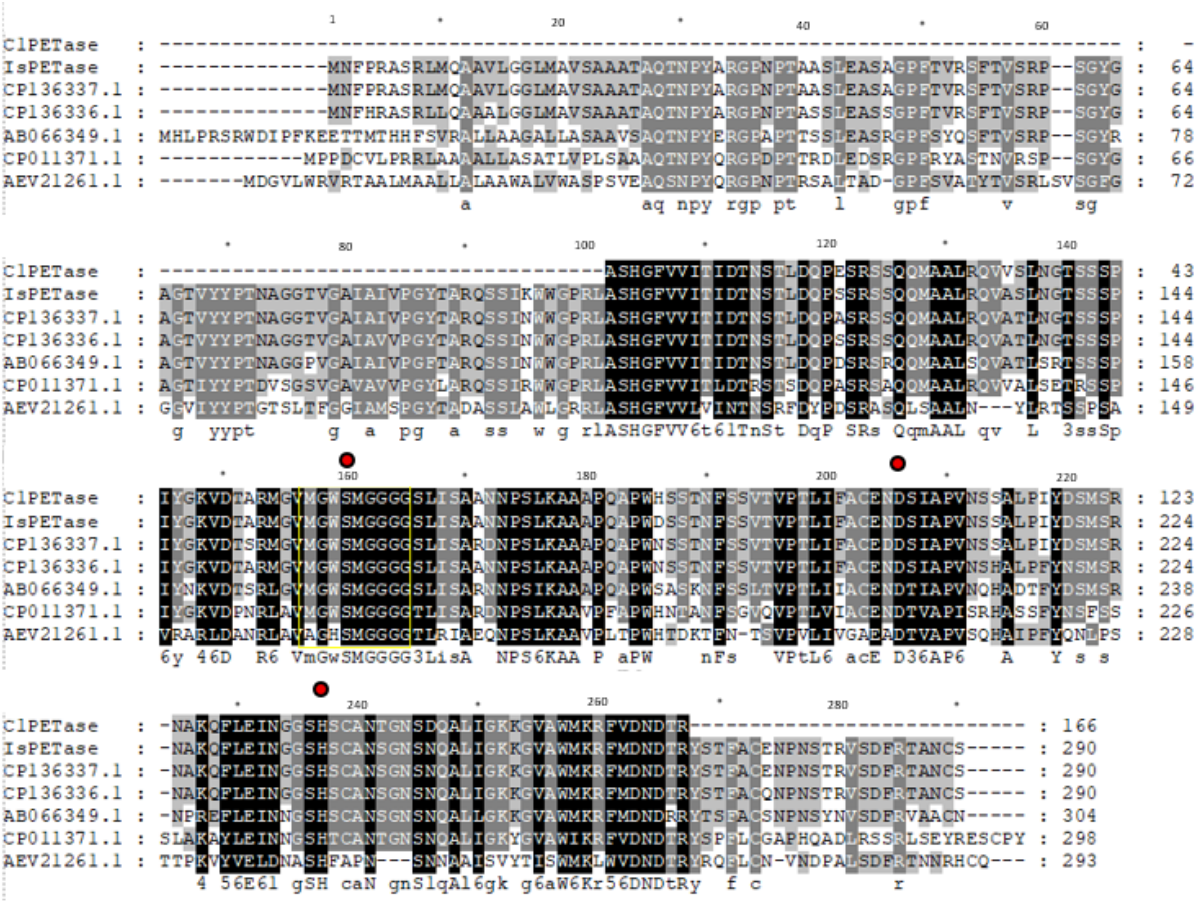

FIGURE 2 Multiple sequence alignment of amino acid sequences of PETase homolog enzyme detected in soil sample of Cipeucang (C1PETase) in comparison to PETase of *I. sakaiensis* (IsPETase, 96.4%), dienelactone hydrolase family protein of *Piscinibacter gummiphilus* strain SBD 7-3 plasmid (CP136337.1, 93.4%), dienelactone hydrolase family protein of *Piscinibacter gummiphilus* strain SBD 7-3 genome (CP136336.1, 94%), PBS(A) depolymerase of *Acidovorax delafieldii* (AB066349.1, 80.7%), triacylglycerol lipase of *Schlegelella brevitalea* strain DSM 7029 (CP011371.1, 71.1%), and LCC of uncultured bacterium (AEV21261.1, 39.2%). Active sites are indicated by red filled circle (●), and consensus motif was shown in yellow box. The catalytics motifs or catalytic triad Ser160 –His237 –Asp206 of IsPETase was conserved. Degenerated Primers (P) positions are indicated by arrows (→).

active-site residues and conserved motif characteristic of functional PET hydrolases.

For the detection of PETase, degenerate primers were designed based on conserved regions of PETase and other PET hydrolases; therefore, they amplify only partial sequences of PETase or related PET hydrolases. We acknowledge that missing regions of the PETase sequence may influence enzyme secretion, structural stability, or substrate-binding efficiency. However, the primary objective of this study was to detect homologous DNA as an initial indication of PETase presence. Further experiments to obtain the full open reading frame will be required in future studies. Nevertheless, because the aim of this work was the detection of homologous fragments, partial PETase sequences were sufficient to indicate a high probability of the presence of PETase or related PET hydrolases in the samples. This approach serves as a preliminary screening parameter.

Furthermore, the bacterial DNA in this study was extracted from a mixed community (as confirmed by NGS data). Pseudogenes are relatively rare in bacteria (Kuo and Ochman 2010) and can coexist with functional homologs (Lerat and Ochman 2005). Although further experiments, such as obtaining the full ORF via inverse PCR, are required for confirmation, the detection of this partial PETase gene fragment provides strong evidence for the presence of PET degrading enzymes in this habitat.

Unlike PETase, which required nested PCR with degenerate primers to detect a partial gene sequence, successful MHETase detection was achieved using specific primers designed from the *P. sakaiensis* MHETase sequence. According to NCBI Nucleotide BLAST analysis, the G2MHETase sequence showed the closest relationship to *Delftia* sp., *Delftia tsuruhatensis*, *Xenophilus aerolatus*, and *P. gummiphilus* (Table 3, Supplementary 2), with identity scores of 98–99%. The sequence length was identical to that of the IsMHETase sequence (Supplementary 4). These results are consistent with the bacterial diversity profile obtained from the enriched Galuga sample, which contained *Delftia*, *D. tsuruhatensis*, *P. gummiphilus*, and *X. aerolatus*, although all were present in very low abundance (Supplementary 6). The deduced amino acid se-

TABLE 3 NCBI nucleotide BLAST result of G2MHETase sequence.

Description	Scientific name	Accession	Query cover (%)	Per. Ident (%)
Delftia sp. DS1230 chromosome, complete genome	*Delftia* sp. DS1230	CP157763.1	100	99.39
Delftia tsuruhatensis strain B7 chromosome, complete genome	*Delftia tsuruhatensis*	CP171815.1	100	99.23
Xenophilus aerolatus strain 56729801 chromosome, complete genome	*Xenophilus aerolatus*	CP166702.1	100	98.57
Piscinibacter gummiphilus strain SBD 7-3 chromosome, complete genome	*Piscinibacter gummiphilus*	CP136336.1	100	98.57

quence of G2MHETase was highly conserved, even across different genera and species (Figure 3), showing the highest identity (98.5%) with both *P. sakaiensis* and *Delftia* sp.

Compared to IsMHETase, G2MHETase differed at eight residues (Figure 3): residue 19 (Alanine → Valine), residue 126 (Phenylalanine → Serine), residue 131 (Serine → Glycine), residue 363 (Glycine → Glutamic Acid), residue 404 (Serine → Asparagine), residue 405 (Serine → Cysteine), residue 508 (Glycine → Serine), and residue 541 (Leucine → Glutamine). According to previous reports (Knott et al. 2020; Palm et al. 2019), seven residues form the MHETase active site (Figure 3). The key catalytic residues are Ser225, Asp492, and His528, which form the catalytic triad. Two cysteines (Cys224 and Cys529) form a disulfide bond, while Arg411 and Ser416 contribute to substrate binding. Based on sequence alignment, the differences between G2MHETase and IsMHETase were limited to a few residues located outside the active site. Therefore, G2MHETase is expected to share a similar structure and function with IsMHETase. This information suggests that G2MHETase represents a functional gene successfully detected from the enriched Galuga environmental sample using a PCR-based approach.

3.3. Bacterial diversity analyses

At first, we hypothesized that both PETase and MHETase homologs could be detected in the enriched environmental samples from Galuga and Cipeucang, as both sites are landfills contaminated with PET and other plastics. These two enzymes should be in the same sample. However, only a partial PETase homolog was recovered from the Cipeucang sample, while a full-length MHETase homolog was identified from the Galuga sample. Several factors may explain why the MHETase homolog sequence and the new PETase homolog sequence were obtained from different samples. First, PET hydrolases and MHETases do not necessarily coexist within the same bacterial cell. Second, microbial communities may differ in composition and gene content, resulting in the detection of PETase at one site and MHETase at another. This interpretation is supported by our bacterial diversity data at the species level (Supplementary 5 and 6). Another possible explanation is the technical aspect: the DNA extracted from the environmental samples may have been highly fragmented rather than intact genomic DNA, thereby reducing PCR efficiency (Alaeddini et al. 2010; Kuo and Ochman 2010). Finally, PCR-based detection only identifies homologous proteins, unlike functional screening, which would detect active enzymes regardless of sequence similarity, PCR-based detection only identifies homologous proteins. Therefore, there is a possibility that enzymes (PETase or MHETase) with very different structures remain undetected (Sun 2024).

The bacterial community profiles of the two sites differed markedly (Supplementary 5, 6). The Galuga sample was richer in terms of species diversity, containing more than 1,100 bacterial species, compared to only about 760 species in the Cipeucang sample (Supplementary 5, 6). Moreover, although both samples harbored genera or species that could potentially encode PETase and MHETase homologs, *Ideonella sakaiensis* was not detected in either enriched sample.

Although PETase and MHETase homologs have been identified in diverse bacterial taxa and metagenomes worldwide (Danso et al. 2018; Meyer-Cifuentes et al. 2020), *P. sakaiensis* itself has been difficult to isolate outside its original discovery site in Japan. The bacterium was first isolated from PET-contaminated sediment near a plastic recycling facility in Sakai, Japan (Yoshida et al. 2016), and to date, no reports have confirmed its successful isolation from other habitats. This suggests that *P. sakaiensis* may be rare or ecologically restricted, with its specialized PET degrading capability instead disseminated through horizontal gene transfer to other genera such as *Delftia*, other *Piscinibacter*, and *Xenophilus* (Bollinger et al. 2020; Danso et al. 2019). Consequently, while *P. sakaiensis* has not been detected in our enriched samples, the presence of PETase and MHETase homolog sequences in other bacterial lineages may exist.

In this study, MHETase was more readily detected than PETase, even with specific primers, suggesting that MHETase is more widely distributed and conserved in nature. While *P. sakaiensis* remains the only bacterium known to carry both PETase and MHETase in the same genome, enabling complete PET metabolism (Yoshida et al. 2016), our study indicates that rubber-degrading *P. gummiphilus* may also possess both enzymes (Birke et al. 2018; Imai et al. 2013; Kasai et al. 2017). By contrast, many bacteria encode PETase homolog enzymes without a corresponding MHETase, limiting them to partial PET degradation unless supported by microbial consor-

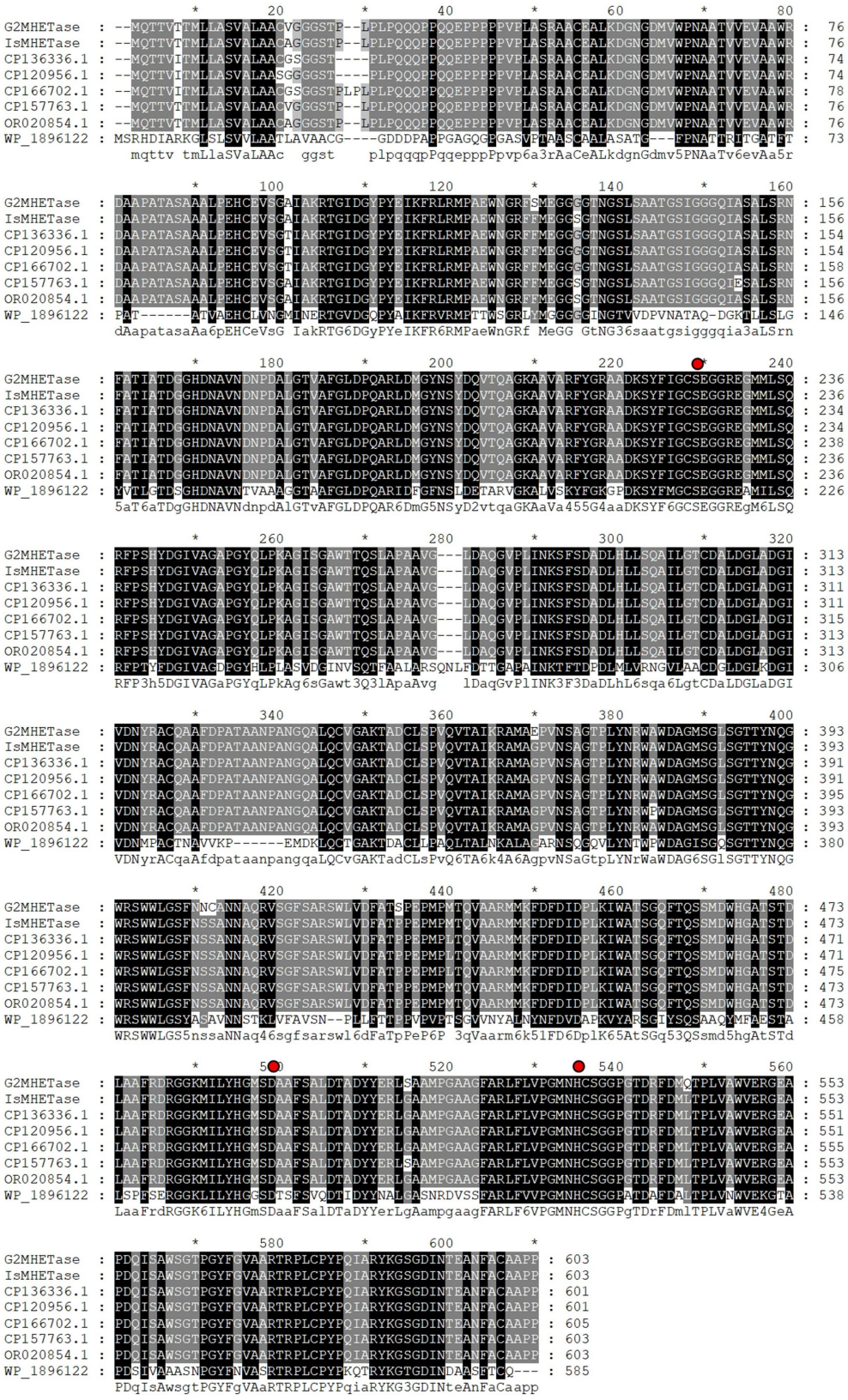

FIGURE 3 Multiple sequence alignment of amino acid sequences of MHETase homolog enzyme detected in soil sample of Galuga (G2MHETase) in comparison to MHETase from *I. sakaiensis* (IsMHETase, 98.5%), *Delftia* sp. (CP157763.1, 98.5%), *Xenophilus aerolatus* (CP166702.1, 97.5%), *Piscinibacter gummiphilus* strain SBD 7-3 (CP136336.1, 97.5%), *Delftia tsuruhatensis* strain Ery-6A (CP120956.1, 97.5%), synthetic construct (OR020854.1, 98.5%), and tannase/feruloyl esterase family α/β-hydrolase of *Pigmentiphaga litoralis* (WP_189612281.1 , 46.7%). Active sites are indicated by red filled circle (●). The catalytics motifs or catalytic triad Ser225 –His528 –Asp492 of IsMHETase was conserved.

tia (Danso et al. 2019). Recent metagenomic surveys further support this view, showing that PETase homologs are taxonomically widespread yet unevenly distributed across marine ecosystems (Alam et al. 2025; García-Meseguer et al. 2023), whereas MHETase homolog enzymes appear broadly conserved across microbial communities and trace their evolutionary origins to ferulic acid esterases (Watts et al. 2024). Together, these findings highlight the broader ecological conservation of MHETase compared to PETase.

Future studies could strengthen the connection between enrichment methods and culture-independent techniques such as metagenomic or transcriptional analysis. Complementary genome-level approaches would offer a broader perspective on the distribution and potential activity of PET-associated hydrolases under conditions closer to their natural environment. While enrichment culture alters community composition, it remains a practical approach for increasing the abundance of target organisms and improving detection of functional genes that might otherwise remain hidden. In this study, we examined bacterial diversity through nanopore sequencing of 16S rDNA from enriched samples. This allowed us to evaluate the relationship between the detected DNA fragments and the microbial community composition.

4. Conclusions

This study demonstrates the feasibility of detecting PETase homologous DNA from plastic-contaminated soils using a PCR-based approach. A full length MHETase homolog was recovered from Galuga and showed high sequence similarity to *P. sakaiensis* and *Delftia* sp., whereas the PETase signal from Cipeucang was limited to a partial fragment closely related to *P. sakaiensis* and *P. gummiphilus*. Both sequences contained conserved catalytic motifs characteristic of known PET hydrolases, indicating that related hydrolase genes are present in plastic impacted terrestrial environments. Bacterial diversity profiling further revealed site specific community patterns consistent with these detections. The results also suggest that MHETase homologs may be more widespread and conserved than PETase, and they underscore the continuing difficulty of detecting *I. sakaiensis* in environmental samples. Because the analysis relies on sequence similarity and enrichment cultures, the results should not be interpreted as direct evidence of active PET biodegradation *in situ*. Confirmation through recovery of complete gene sequences, heterologous expression, and enzymatic assays will be necessary. Taken together, these findings support enrichment assisted molecular screening as a useful first step for identifying candidate plastic degrading enzymes from environmental samples, while highlighting the need for functional and ecological validation to determine their environmental relevance and biotechnological potential.

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Authors' contributions

IH, NN designed the study. IH supervised all the experiments. SRW, GCS, MU carried out the laboratory work. SRW, GCS, JPT, MU analyzed the data. IH. NN reviewed and eited the manuscript. SRW, GCS, JPT wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

We declare that there is no conflict of interest.

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