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Functional specialization and enzymatic mechanisms of poly (3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBH) degradation in anaerobic digesters: Insights from shotgun metagenomics and molecular modeling

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ABSTRACT

This study investigates the anaerobic degradation of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBH) using shotgun metagenomics and molecular docking to analyze temporal shifts in microbial communities and key enzymes under batch and semi-batch conditions. Comparative analysis of the microbial communities revealed a decline in generalist taxa and an increased contribution of Bacteroidota, Chloroflexota, and methanogenic Euryarchaeota. KEGG-annotations suggested that modules affiliated with depolymerases, esterases, β-oxidation and methanogenic pathways would be co-activated. Furthermore, PlasticDB-based computational analysis evidenced a stepwise enrichment of PHB- and PHA-related enzymes, which confirmed the substrate-mediated microbial specialization. A prominent metagenomic depolymerase (R1_379815) showed well-conserved catalytic residues (Ser134, His284, Asp211) and a substrate-binding affinity comparable to the native counterpart 9BYU, confirming its substrate preference and functional identity with previously reported PHB depolymerases. Collectively, this integrative metagenomic and computational approach provides mechanistic insights into PHBH biodegradation under anaerobic conditions, aiding in the identification of potential target enzymes for enhancing plastic degradability and methane recovery in anaerobic digestion systems. These findings contribute to the advancement of sustainable bioplastic waste management through process-level and enzymatic optimization.

1. Introduction

The urgent issue of the world-wide accumulation of plastic waste is one of the critical environmental challenges of our age. With serious environmental and economic repercussions of persistent petroleum-based plastics nowadays, the need and use of biodegradable plastics have gained increasing attention, among which are the polyhydroxyalkanoates (PHAs) (Mubayi et al., 2024). Of these, poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBH) is receiving attention because it has good mechanical properties, biodegradability, and potential for applications in several sectors such as packaging areas (Eraslan et al., 2022). With global bioplastic production capacity projected to increase from 2.47 million tons in 2024 to 5.73 million tons by 2029 (*European Bioplastics*, 2024), there is a need for better waste management solutions. Within these considerations, anaerobic digestion (AD) is a well-established technology and process for organic waste treatment and bioenergy production, which can be a sustainable waste management option for some non-recyclable biodegradable bioplastics (Vardar et al., 2022). AD efficiency is mainly influenced by microbial community structure, enzyme activities, as well as the operating conditions of the digester (Abraham et al., 2021). The detailed knowledge of the microbial dynamics in AD systems is crucial for the efficient degradation of complex substrates. Despite recent advances, the microbial-mediated anaerobic digestion of PHBH remains only partially

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understood.

In recent decades, the integration of high-throughput sequencing and bioinformatics has revolutionized microbial ecology research in anaerobic digesters. Metagenomic tools have been applied to characterize the structure of microbial communities, their functional potential, and metabolic interactions contributing to an understanding on individual roles of microbial taxa in polymer degradation (Pilarska et al., 2023; Yokoyama et al., 2023). These techniques have been employed to describe the differences in the anaerobic microbial communities at different operating conditions and substrate (Jahanshahi et al., 2023). Methanogenic studies are recently emerging and reveal the major players in the AD systems. Moreover, the metagenomic approach was employed to evaluate the effects of different operative parameters on the microbial dynamics of AD of several bioplastics (Cazaudehore et al., 2022, 2023). Most investigations have relied on amplicon sequencing approaches that predominantly reveal taxonomic composition; however, with the emergence of shotgun metagenomics, it is now possible to unravel the functional potential of microbial communities in AD systems (Salam, 2024).

In contrast to marker gene-based approaches targeting specific genes of interest, shotgun metagenomics enables the reconstruction of individual genomes from complex microbial communities and provides insights into both their taxonomic affiliation and potential metabolic functions (Shafana Farveen and Narayanan, 2024). This technique has been used to investigate microbial responses to plastic waste in several studies. Similarly, Pinnell and Turner (2019) utilized a genome-centric metagenomics approach to examine how microbial communities evolve over time in response to bioplastics, underscoring the microbiome's capacity for adaptation. This study also revealed that the consortia contained genes integral to both bioplastic degradation and sulphate reduction. Further, Saleem et al. (2023) characterized plastisphere microbiomes from waste sites, revealing microbial potential for plastic degradation and enzyme production. More recently, metagenome mining has uncovered previously uncharacterized enzymes involved in plastic degradation, underscoring the continuous advancement of our understanding of microbial plastic degradation pathways (Frey et al., 2024). Although shotgun metagenomics has been more valuable to unveil the genome-centric information of a microbial niche, it has been less employed for the investigation of degradation trend and the microbial response which is specific to a type of plastic. Within this context, Pinto et al. (2022) provided valuable insights by examining low-density polyethylene degraders and their adaptive responses over a two-year period.

With the growing adoption of bioplastics, understanding the anaerobic degradation mechanisms of bioplastics like PHBH has become increasingly important. Existing literature has primarily focused on methane yield evaluation and microbial community profiling during the AD of bioplastics, whereas the molecular-level metabolic pathways and enzymatic mechanisms driving the PHBH degradation remain largely unexplored. Moreover, the insights into microbial succession and functional pathway shifts under different operational parameters are limited, thereby constraining our understanding of process optimization for large-scale bioplastic integration within wastewater treatment systems. Our previous research on batch and semi-batch AD of PHBH provided critical insights into methane yield under different operational conditions such as batch and semi-batch modes (Shafana Farveen et al., 2025). Addressing this gap, the present study integrates a time-series shotgun metagenomic sequencing with molecular docking analysis offering a molecular-level insight to our understanding of PHBH biodegradation in AD systems. Therefore, the primary objectives of this work were framed to (i) characterize microbial community dynamics and functional adaptations under batch and semi-batch operational modes; (ii) map the metabolic networks governing PHBH conversion to methane; and (iii) understand the enzymatic interaction with the substrate via computational analysis, to provide supportive evidence for enzyme–substrate interactions. Thus, the present study aims to contribute to aiding in identifying potential target enzymes for process enhancement, contributing to the optimization of bioplastic waste management strategies and ultimately supporting the transition toward more sustainable plastic waste disposal systems.

2. Materials and methods

2.1. Study design - AD systems

This metagenomic study was designed to complement our previous experimental investigation (Shafana Farveen et al., 2025) with the objective of elucidating the microbial mechanisms underlying the observed PHBH degradation. The previous work (Shafana Farveen et al., 2025) focused on process kinetics and polymer characterization, establishing a robust foundation for the molecular-level analysis. The present study employs a time-series sampling approach across two operational modes (batch and semi-batch) to capture the dynamic evolution of microbial communities in response to PHBH substrate availability and degradation progression. As reported in Shafana Farveen et al., 2025, the batch-mode operation were carried out at mesophilic conditions in nine (three blanks and six samples) 2.1 L glass bottles with a working volume of 0.5 L, each containing anaerobic sewage sludge (TS = 19.26 g/kg; VS = 11.85 g/kg; pH 7.97 ± 0.02), obtained from the domestic wastewater treatment plant, Valladolid, Spain. The bioplastic PHBH [(C4H6O2)m (C6H10O2)n], with <10 mol% of 3-Hydroxyhexanoate, and the rest composed of 3-Hydroxybutyrate, comprising of 68.51 % carbon and 31.49 % oxygen as per Energy-Dispersive X-ray Spectroscopy (EDAX) was used as the substrate. 2.96 g of the substrate PHBH, with 0.9 mm thickness, was added into the digesters at an F/M ratio of 0.5 (VS basis). Upon operating it for 50 days, the batch system achieved 550.5 ± 78.8 NmL CH_4/g VS added. In this 50-day batch-mode operation, day 25 and day 50 were identified as key milestones representing exponential and stationary phases, respectively. And thus, triplicate digesters were sacrificed at these time-points to capture the microbial community transition. The semi-batch system in the same study was operated in a 3.1 L reactor with a working volume of 2 L for 112 days with continuous stirring at 200 rpm. 1 g of the substrate PHBH was added to the reactor on a daily basis at an organic loading rate (OLR) of 0.5 g VS/L-d until day 99. This continuous addition of PHBH yielded 562.34 ± 44.9 NmL CH_4/g VS added. This semi-batch mode operation of the reactor was designed to evaluate microbial adaptation and functional specialization under sustained substrate pressure, representing more realistic waste management scenarios with continuous bioplastic input. In addition to methane yield, the physicochemical properties of the subjected PHBH was characterized employing SEM, FTIR, and XRD, which collectively demonstrated surface erosion, chemical modifications of its structure. The experimental setup is shown in the Supplementary material and further operational details of both AD systems are provided in Shafana Farveen et al. (2025).

2.2. Sample collection

Sample collection timepoints were strategically selected to correspond with key degradation phases: In the batch system, triplicate samples (5 mL) were collected on day 0 (inoculum/sludge-only control), day 25 (during the exponential phase of methane production), and day 50 (the stationary phase of methane production). three independent digesters as biological replicates for each phase, to capture the correlation of community dynamics at the key phases of methane production. For the semi-batch mode, samples were collected during the pseudo-steady state (PSS) to characterize stable operational conditions. Triplicate samples (5 mL) were collected from the same reactor at three different time points (days 43, 64, and 92), with each timepoint treated as a replicate of the PSS phase. These temporal replicates captured the microbial community structure during sustained, stable performance. These sampling points were selected to provide a comprehensive

assessment of microbial succession and functional adaptations during PHBH biodegradation.

2.3. DNA extraction and sequencing

Total genomic DNA was extracted from the anaerobic digester samples using the FastDNA™ SPIN Kit for Soil, following the manufacturer's instructions and quantified using Qubit™ dsDNA HS assay kits. The genomic DNA sample was fragmented into short fragments. These DNA fragments were then end-repaired, A-tailed and ligated with sequencing adapters. Following PCR amplification, purification was conducted through the AMPure XP system for DNA cleanup. The resulting library was assessed on the Agilent Fragment Analyzer System. Shotgun metagenomic sequencing was performed using the Illumina NovaSeq X Plus platform (Novogene, Europe), generating 150 bp paired-end reads. The raw sequence data has been deposited in the NCBI GenBank's Sequence Read Archive (SRA) under BioProject accession number PRJNA1269072.

2.4. Bioinformatic analysis

Raw sequencing data were pre-processed using Fastp (https://github.com/OpenGene/fastp) to remove adapter sequences, trim low-quality bases, and filter out poor-quality reads. The resulting high-quality reads were assembled using MEGAHIT v1.2.9 with default parameters (Li et al., 2015). MetaGeneMark (http://topaz.gatech.edu/GeneMark/) was used to predict open reading frames (ORFs) for contigs (≥500), filtering out smaller contigs (Mende et al., 2012; Sunagawa et al., 2015). CD-HIT software was used to eliminate redundancy in obtained ORFs, using a stringent criterion of 95 % identity (Fu et al., 2012). For taxonomic profiling, unigene sequences were aligned against the Micro_NR database (NCBI; https://www.ncbi.nlm.nih.gov/) using DIAMOND v2.1.6 (Karlsson et al., 2013) with the BLASTp algorithm. Alignments were filtered with a minimum percentage identity of 70 % and an e-value cutoff of 1e–5. For each query, 50 hits with e-values ≤ minimum e-value × 10 were retained, and taxonomic assignment was performed using the Lowest Common Ancestor (LCA) algorithm in MEGAN (Huson et al., 2011), which identifies the most specific shared taxonomic level among high-quality matches and the top 35 genera were visualized. Similarly, gene functional annotation were performed by blastp search (identity percentage ≥ 70 %; e-value <1e-5) with the KEGG database (https://www.kegg.jp/kegg/) and targeted functional annotation of plastic biodegradation-related genes was performed using the PlasticDB web-annotation tool (https://plasticdb.org/), applying similar parameters to identify putative plastic-degrading proteins and enzymes. Additionally, integration of KEGG-based functional annotation with taxonomic classification was performed at contig-level, to resolve the microbial origin of the observed functional patterns. For visualization, heatmaps were generated using the tidyverse (v2.0.0) and ggplot2 (v4.0.0) packages in R (v4.3.3) based on fold-change values of taxonomic and functional abundances across time points. Abundance clustering heatmaps were constructed at the taxonomic, KEGG Level-2 functional, and enzyme levels to illustrate comparative trends. Statistical analyses were performed using the vegan package (v2.7-1), including Principal Coordinates Analysis (PCoA) based on Bray-Curtis dissimilarity to assess community structure, and PERMANOVA to test for significant differences across time points. The abundance of key enzymes related to PHBH breakdown and methane production was utilized to elucidate the PHBH degradation pathway. Metabolic pathways were reconstructed using the KEGG database (https://www.genome.jp/kegg/mapper/) and the MetaCyc Metabolic Pathway Database (https://metacyc.org/).

2.5. Enzymatic interaction analysis

To elucidate the enzymatic interactions with PHBH, molecular docking analyses were performed using putative plastic-degrading enzymes obtained from the metagenomic dataset. The enzyme sequences were filtered based on a minimum identity threshold ≥60 % to experimentally characterized enzymes, to retain homologs exhibiting conserved structural core (Tian and Skolnick, 2003). While the identity threshold served solely as a preliminary screening criterion, subsequently, the sequences were examined for the presence of intact start and stop codons to ensure structural integrity. The homology modeling of this filtered target enzymes was conducted using SWISS-MODEL, generating three-dimensional structural models for subsequent docking studies (Waterhouse et al., 2018). Further, the reliability of the generated 3D models were validated using multiple assessment tools, including Verify3D (Bowie et al., 1991), ERRAT (Colovos and Yeates, 1993), ProSA (Wiederstein and Sippl, 2007), and Ramachandran plot analysis (Mayank Rathore, 2025). These evaluations provided insights into stereochemical quality, non-bonded atomic interactions, and overall 3D-1D profile consistency. Substrate-binding residues were identified using COACH, a meta-server that integrates outputs from TM-SITE, COFACTOR, FINDSITE, S-SITE, and ConCavity, further refined via Support Vector Machine (SVM)-based algorithms to pinpoint functional domains and potential ligand-binding sites (Yang et al., 2013). Furthermore, to proceed with molecular docking analysis, experimentally validated plastic-degrading enzymes were included as positive controls to benchmark binding interactions and affinity profiles, and multiple sequence alignment was conducted between the target enzyme and the control enzyme using ClustalW to identify conserved regions and assess similarity with the target structure (https://www.genome.jp/tools-bin/clustalw). Molecular docking simulations were carried out using AutoDock 4.2 to evaluate the binding interactions between enzymes and PHBH. Prior to docking, receptor preparation included the removal of water molecules, the addition of polar hydrogens, and the assignment of Gasteiger partial charges (Morris et al., 1998). Docking protocols employed the Lamarckian Genetic Algorithm with 10 independent runs per ligand, each comprising a maximum of 2,500,000 energy evaluations. Binding affinities (kcal/mol) were used to evaluate the strength and stability of enzyme-substrate interactions. The resulting docked complexes were visualized and analyzed using BIOVIA Discovery Studio Client 2020 and PyMOL to assess conformational arrangements and interaction interfaces.

3. Results and discussion

3.1. Overview of metagenomic sequencing and quality assessment

Shotgun metagenomic sequencing was conducted on triplicate samples obtained from batch and semi-batch AD systems, generating a total of 83.96 GB (nine samples, triplicates at each time point) and 37.89 GB (triplicates) of raw data respectively. These sequencing depths were within the expected range for metagenomic studies of AD microbiomes, ensuring sufficient coverage for downstream taxonomic and functional profiling (Campanaro et al., 2020; Vanwonterghem et al., 2016). Quality control steps removed adapters, low-quality bases, and ambiguous reads, retaining high-quality reads with an average Phred quality score (≥Q30) of 92.97 % for the batch system and 92.02 % for the semi-batch system. Additional QC metrics are provided in the Supplementary material. Subsequent de novo assembly of the filtered high-quality reads generated an average of 54.33 million base pairs (bp) with 388,799 contigs and an N50 of 1687 in the batch system, and 52.83 million bp with 376,457 contigs and an N50 of 1978 in the semi-batch system. In addition, a total of 2.14 million and 1.84 million non-redundant ORFs were predicted from the batch and semi-batch assemblies, respectively. The detailed assembly statistics are provided in the Supplementary material.

3.2. Microbial community analysis

A comprehensive microbial community profiling at both phylum and genus taxonomic levels was conducted to elucidate the microbial dynamics and identify the predominant microorganisms driving PHBH biodegradation in both batch and semi-batch AD systems. Notable microbial shifts occurred between initial inoculation and operational phases in both systems, correlating with the observed PHBH degradation patterns and methane production phases observed in our previous study (Shafana Farveen et al., 2025). The transition between phases demonstrated differences in key genera involved in hydrolytic activity, syntrophic metabolism and methanogenesis, showing community restructuring in response to PHBH substrate.

3.2.1. Microbial adaptation: batch-mode

The initial inoculum exhibited remarkably high taxonomic richness and evenness (Shannon diversity index: 4.102 ± 0.02), indicating a robust and diverse microbial consortium. As PHBH degradation progressed in the batch system, a significant decline was observed in alpha diversity metrics, with Shannon indices decreasing to 3.935 ± 0.01 during the exponential methane production phase (day 25), when active PHBH was observed, and 52.85 ± 4.90 % weight loss was reported (Shafana Farveen et al., 2025), and further down to 3.871 ± 0.01 at the stationary phase (day 50) indicating community specialization. A Kruskal-Wallis test confirmed significant differences among phases ($p < 0.05$; $p = 0.0273$), with post hoc Wilcoxon tests revealing significant pairwise differences between Day 0–25 ($p < 0.05$; $p = 0.010$), Day 0–50 ($p < 0.001$; $p = 0.0003$), and Day 25–50 ($p < 0.05$; $p = 0.010$), with an effect size ($r = 0.802$; $r > 0.5$), indicating strong group separation and clear community restructuring. This diversity reduction aligns with findings from Bandini et al. (2022), who demonstrated that specialized microbial communities adapt during polymer degradation in closed systems. Collectively, these results demonstrate a progressive loss of microbial diversity during PHBH degradation, consistent with the ecological succession of specialized degraders in closed anaerobic systems (Bandini et al., 2022). Further, beta diversity analysis using principal component analysis (PCA) based on Bray-Curtis distances revealed clear distinctions between initial and later microbial communities, driven by PHBH substrate availability. The comprehensive alpha and beta diversity metrics are provided in the Supplementary file.

In terms of microbial community, the phyla Bacteroidota, Chloroflexota, and Euryarchaeota were present throughout the digestion process. At day 0 (sludge-only control/inoculum), Bacteroidota exhibited the highest abundance (14.87 %), which remained stable during active degradation (day 25; 15.60 %) but slightly declined by day 50 (13.35 %). This pattern aligns with recent findings by Ottoni et al., 2022, who reported similar Bacteroidota dominance during the hydrolysis of agro-industrial waste in anaerobic environments and in particular, an increase in their abundance in the presence of polycyclic aromatic hydrocarbons (Martinez-Varela et al., 2023). The relative abundance of Chloroflexota increased substantially from 7.86 % to 12.69 % by day 50, highlighting its enhanced contribution as digestion progressed, likely due to its facultative anaerobic metabolism that offers a competitive advantage under fluctuating oxic–anoxic conditions (Freches and Fradinho, 2024; Petriglieri et al., 2023). *Euryarchaeota*, predominantly comprising methanogenic archaea, exhibited significant proliferation from 4.21 % at day 0 to 9.20 % by day 50, underscoring their critical role in methane production during the terminal stages of AD and aligning with similar enrichment trends reported by Loughrin et al. (2023). In contrast, some phyla, such as Candidatus Cloacimonetes, demonstrated declining trends, dropping from 4.97 % to 3.77 % at day 50, suggesting a shift from hydrolytic processes as methanogenesis became dominant, similarly, Bacillota and Acidobacteriota exhibited moderate reductions, likely reflecting competitive microbial interactions and substrate-specific selection (Bandini et al., 2022; Westerholm and Schnürer, 2019).

At the genus level, the acetoclastic methanogen *Methanothrix* displayed remarkable enrichment from 1.25 % to 5.01 % by day 50 (Fig. 1A). Syntrophic bacteria including *Anaerolinea*, *Levilinea*, and *Syntrophus* became increasingly dominant. The hydrogenotrophic methanogen *Methanoculleus* exhibited a minimal increase from 0.38 % to 0.52 % over the same period, suggesting that acetoclastic methanogenesis dominated the anaerobic sludge (Gao et al., 2023; Zhou et al., 2023). Conversely, genera such as *Candidatus Cloacimonas*, and *Candidatus Microthrix* showed a declining trend, likely reflecting their predominant roles in the initial hydrolytic and fermentative stages, which become less critical as digestion progresses toward methanogenesis.

3.2.2. Microbial adaptation: semi-batch mode

In the semi-batch operation with continuous PHBH supplementation, more pronounced community restructuring was observed, with Shannon diversity indices significantly decreasing from 4.102 ± 0.02 to 3.445 ± 0.04 ($p < 0.001$), reflecting the sustained selective pressure from continuous substrate loading (0.5 g VS/L-d for 99 days) that maintained stable methane yields of 562.34 ± 44.9 NmL CH_4/g VS added (Shafana Farveen et al., 2025). These findings are consistent with recent metagenomic studies showing that continuous substrate availability in AD systems reduces functional redundancy and promotes metabolic specialization, leading to decreased alpha diversity over time (Ostos et al., 2024). In this context, operational parameters such as feeding regime, temperature, and food-to-inoculum (F/I) ratio also play a role as crucial modulators that fundamentally shape the microbial community structure (Bandini et al., 2022; Cazaudehore et al., 2022; Liu et al., 2009).

While the batch system provided comprehensive insights into the microbial succession between the PHBH degradation phase, the microbiome profiling conducted during pseudo-steady-state (PSS) conditions in the semi-batch mode offers valuable information regarding the consistent phyla that served as key contributors to sustained methane production. In the semi-batch system, dramatic community restructuring was observed, likely reflecting adaptive responses to continuous substrate availability. Although the phyla, Bacteroidota (14.91 %) and Chloroflexota (7.92 %) demonstrated high abundance, the phylum Bacillota emerged as the dominant phylum, increasing substantially from an initial abundance of 3.98 % to 24.33 %, signifying functional adaptation to semi-continuous feeding conditions. This phylum Bacillota typically encompasses fermentative and syntrophic bacteria essential for intermediary metabolic processes in AD systems (Harirchi et al., 2022; Loughrin et al., 2023). Concurrently, Euryarchaeota exhibited a considerable increase from 4.34 % to 9.68 %. In contrast, Thermodesulfobacteriota demonstrated a marked decline from 5.67 % to 1.52 %, suggesting competitive exclusion or substrate-specific limitations (Bandini et al., 2022).

At the genus level, the relative abundance of *Methanothrix*, an acetoclastic methanogen, surged from an initial 1.34 % to 6.66 %, along with other methanogens such as *Methanoculleus*, a hydrogenotrophic methanogen, reinforcing their role as the dominant methane-producing community (Fig. 1B). This substantial increase likely reflects continuous acetate availability and highlights adaptability and metabolic efficiency under steady operational conditions (Gao et al., 2023). Additionally, the genus *Thermanaerosceptrum* exhibited a remarkable increase from a negligible 0.005 % to 3.46 %, suggesting notable adaptation to the operational environment. Further, genera such as *Syntrophosphaera* and *Anaerolinea* also displayed considerable increases (0.13 % to 1.03 % and 0.99 % to 1.45 %, respectively), emphasizing their involvement in syntrophic processes, particularly in fatty acid and alcohol degradation, which is crucial for maintaining continuous methane production during PHBH degradation (Campanaro et al., 2020; Lee et al., 2017). The concurrent enrichment of both acetoclastic and hydrogenotrophic methanogens, alongside syntrophic fatty acid oxidizers, under continuous substrate influx supports a dual methanogenesis pathway, aligning with the expected degradation pathway of PHBH, which involves ester

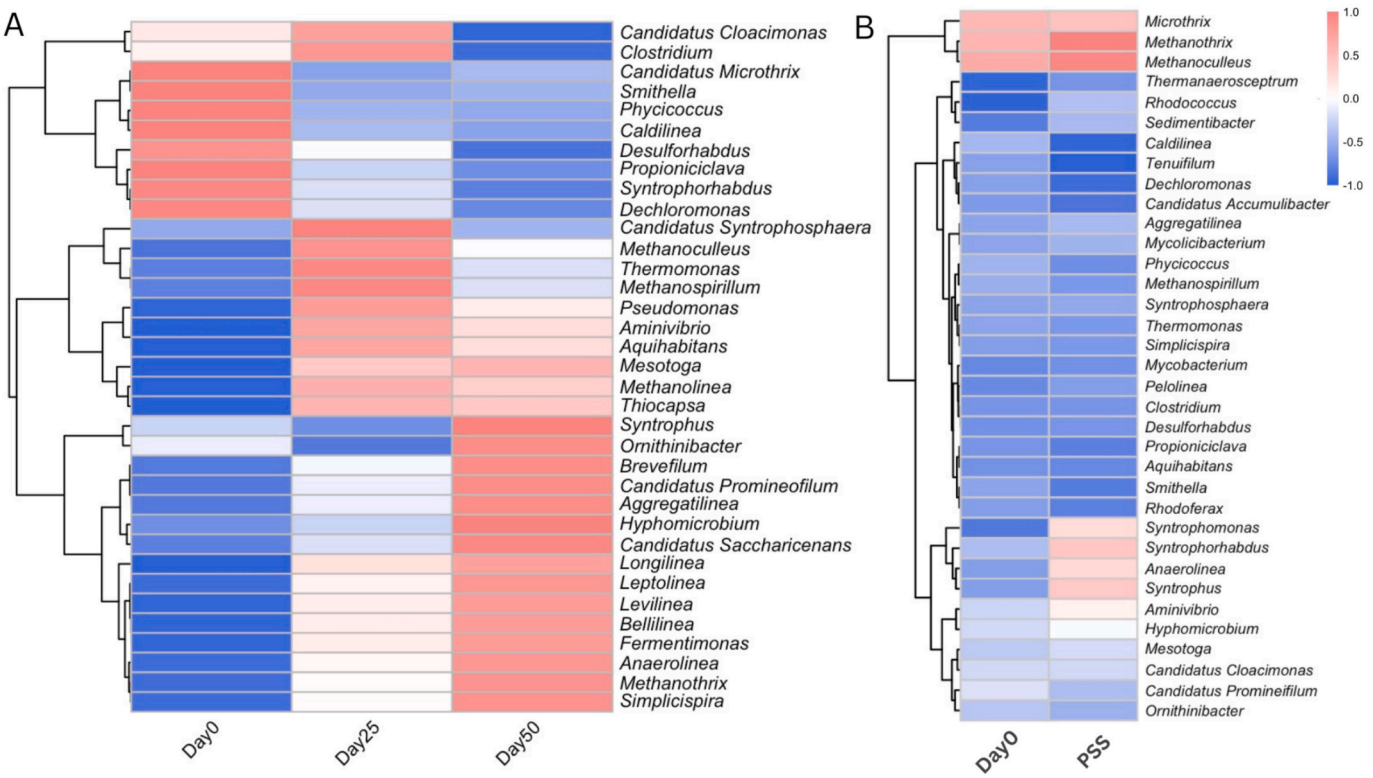

Fig. 1. Standardized genus-level abundance profiles showing relative abundances across (A) batch and (B) semi-batch modes.

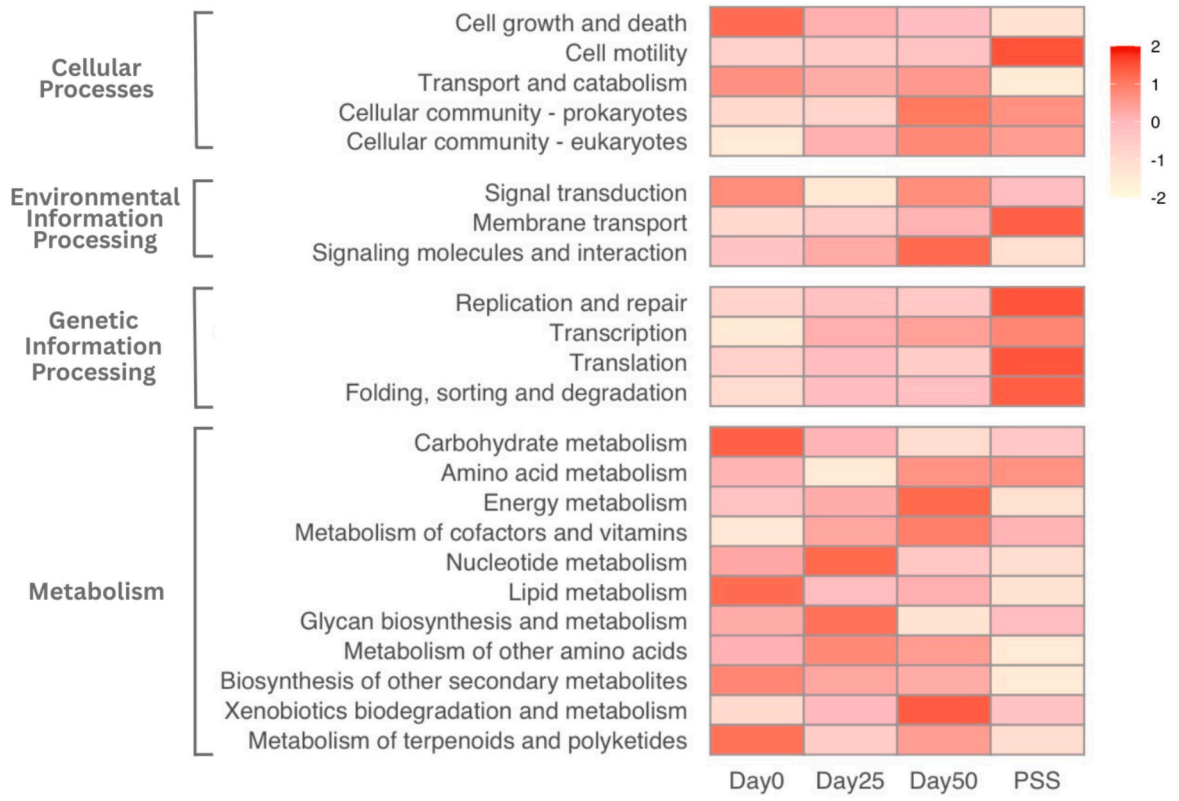

Fig. 2. KEGG level-2 functional annotation of microbial communities involved in PHBH anaerobic digestion across different operational modes and times: Day 0 (initial inoculum), Day 25 and Day 50 (batch mode), and PSS (pseudo-steady state during semi-batch feeding).

bond cleavage, β-oxidation, and subsequent methanogenesis (Demirel and Scherer, 2008; Venkiteshwaran et al., 2015). Overall, despite similar methane yields between the two systems (550.5 ± 78.79 vs. 562.34 ± 44.97 NmL CH_4/g VS), distinct taxonomic specialization patterns emerged, highlighting the influence of operational mode on microbial community structuring during PHBH degradation.

3.3. Microbial metabolic profiling

3.3.1. KEGG-based functional annotation

The metabolic potential of microbial communities involved in AD of PHBH was investigated through KEGG-based functional annotation across four different conditions: day 0 (inoculum), day 25 (exponential phase of batch digestion), day 50 (stationary phase of batch digestion), and PSS (pseudo-steady state during semi-batch feeding). Further, to resolve the microbial origin of the observed functional trends, contig-based taxonomic assignment was employed. While metagenomic analysis reveals the functional potential of microbial communities rather than direct gene expression, tracking the abundance of annotated protein sequences over time provides insights into metabolic and ecological shifts within the community (Gao et al., 2024). The key metabolic pathways, their relevance to PHBH degradation, and their temporal shifts across batch and semi-batch system reflect their functional responses in the different operating systems (Fig. 2). Further, a detailed carbon flux mapping integrated with enzyme abundance aided in deriving the fate of PHBH-driven carbon and inferring the enzymatic drivers of PHBH-focused methanogenesis is depicted in Fig. 3.

At day 0 (initial inoculum), metagenomic profiling revealed the baseline metabolic capacity of the inoculum prior to PHBH addition (Figs. 2 and 3). Baseline profiling reflected the inherent metabolic potential of the inoculum, shaped by its prior exposure to domestic sewage sludge in the anaerobic digester. Upon PHBH addition, a marked activation of hydrolases and xenobiotic degradation pathways was observed between days 25 and 50, with sustained expression into the PSS. The structural similarity between PHBH and certain aliphatic polyesters likely induced the broad-specificity esterases (EC 3.1.1.75) and carboxylesterases (K03928), which catalyze the initial depolymerization (Santos-Beneit et al., 2023). Members of the phylum *Pseudomonadota*, particularly, *Hyphomicrobium*, *Acidovorax* and *Ottowia* dominated this hydrolytic phase, reflecting their role in early polymer depolymerization.

To sustain this effective activity of these extracellular enzymes, the microbial community maintained the production of extracellular polymeric substances (EPS), as evidenced by the stable expression of glycan metabolism pathways across all time points (Fig. 2). Key KO markers such as K01784 (UDP-glucose 4-epimerase) and K03821 (exopolysaccharide export protein) indicated continuous EPS secretion, promoting biofilm formation that enhanced microbial adhesion to PHBH surfaces and enabled the surface-level hydrolysis documented through SEM analysis (Shafana Farveen et al., 2025; Morohoshi et al., 2018). These secretions enhance microbial adhesion and expand the interfacial area for enzymatic action, facilitating surface-level hydrolysis of the hydrophobic polymer (Ma et al., 2019; Wang et al., 2023). Together, these findings support a surface-mediated degradation strategy, which directly correlates with the experimentally observed degradation mechanism, where coordinated EPS production and enzyme activity improved substrate accessibility (Shafana Farveen et al., 2025). This surface-mediated strategy is further supported by the increased abundance of the extracellular PHB depolymerase (EC 3.1.1.1) and esterase (EC 3.1.1.75) (Fig. 3), which directly catalyzes the ester bond cleavage of the PHBH, as observed through FTIR spectroscopy (Shafana Farveen et al., 2025), converting PHBH into 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) monomers and initiating β-oxidation of hydroxyalkanoate side chains (Sugiyama et al., 2004; Tang et al., 2022).

As extracellular hydrolysis products accumulated, to effectively utilize them, the microbial community exhibited a concurrent dominance of membrane-associated transport systems, notably ATP-binding cassette (ABC) transporters and phosphotransferase systems (PTS), which are involved in the import of monomeric and oligomeric degradation intermediates (Fig. 2). Further, membrane transport primarily involves ABC transporters and PTS, and signal transduction pathways involving quorum sensing play a pivotal role during the early and transitional phases of PHBH degradation (Fig. 2) (Aziz, 2021; Davidson et al., 2008). This co-enrichment of extracellular enzymes and transport modules, alongside regulatory modules, reflects an integrated metabolic strategy, indicating active uptake of hydrolysis products while coordinating microbial communication and biofilm formation during initial colonization (Markowska et al., 2024; Morohoshi et al., 2018). Notably, the decline of these pathways at PSS indicates reduced transport needs and regulatory signaling as syntrophic stability is established. This pattern was further supported by cellular process pathways, where genes associated with quorum sensing, biofilm formation, and motility

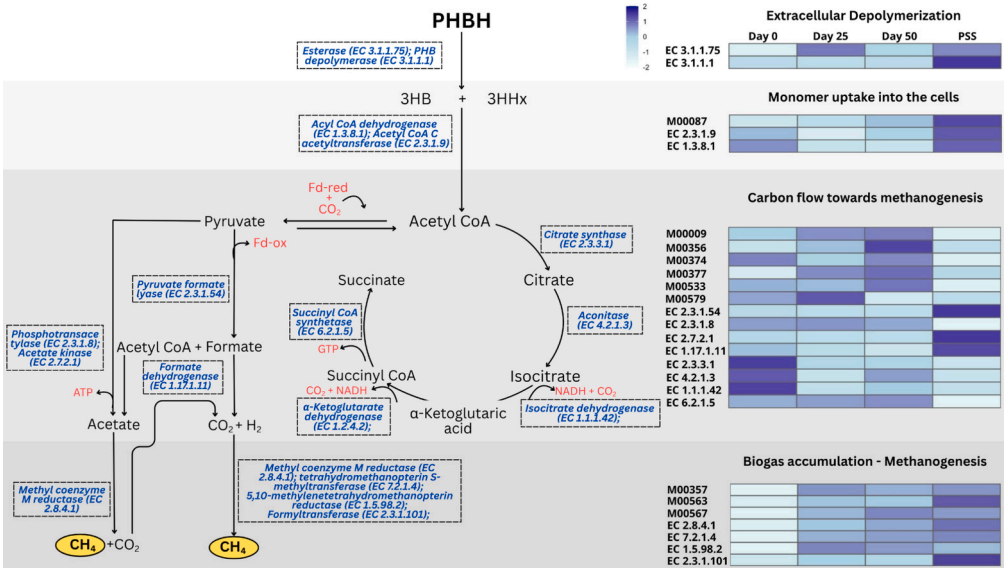

Fig. 3. Schematic representation of the metabolic pathway from PHBH depolymerization to methane formation with corresponding enzyme and module abundances across operational modes: Day 0 (initial inoculum), Day 25 and Day 50 (batch mode) and PSS (semi-batch mode).

showed elevated expression during days 25 and 50, and reached stabilization by PSS. This reinforced earlier observations of microbial coordination, surface c olonization, and community adaptation.

As digestion progressed, a metabolic shift was evident, with an enhanced abundance of modules related to energy metabolism, carbohydrate metabolism, and amino acid biosynthesis (Fig. 2). Enzymes such as acyl-CoA dehydrogenase (EC 1.3.8.1) and acetyl-CoA acetyltransferase (EC 2.3.1.9) demonstrated increased abundance, indicating a sustained flux through β-oxidation pathway (M00087) and active conversion of intermediates such as 3HB/3HHx into acetyl-CoA (Fig. 3). These activities were mainly contributed by Bacillota, Actinomycetota, and Chloroflexota, represented by *Clostridium*, *Thermanaerosceptrum*, *Microthrix*, and *Aquihabitans*, indicating their role in anaerobic oxidation of PHBH-derived intermediates. Furthermore, the microbial communities also appear to contribute to maintaining the cofactor homeostasis as a strategy to support the activity of key enzymes during substrate transformation and to stabilize redox balance during the β-oxidation of hydroxyalkanoate monomers (Yadav et al., 2022). The NADH dehydrogenase (K00134), whose enrichment indicates efficient NAD^+ regeneration and proton translocation essential for ATP synthesis, both essential for methanogenesis. Together, these enzymes maintain redox balance, energy generation, and cofactor availability in PHBH-degrading anaerobic consortia (Mendoza et al., 2023). These functional adaptations were further reflected in the dynamics of energy metabolism pathways, which peaked during days 25 and 50 (Fig. 2). This likely corresponds to heightened microbial turnover, increased enzymatic demand, and intensive interspecies metabolite exchange (Clagnan et al., 2023; Yang et al., 2024). In the case of PSS, the stabilization of energy metabolism pathways suggests a transition from growth-associated energy demands to maintenance-driven processes. This metabolic downshifting is consistent with the establishment of syntrophic interactions and interspecies electron transfer mechanisms necessary for stable methanogenesis (Fig. 2) (Krohn et al., 2022). By PSS, these pathways began to stabilize, consistent with reduced growth activity and metabolic stabilization. The concurrent increase in replication and repair modules at PSS indicates a community-level adaptation to oxidative and proteotoxic stress during prolonged PHBH degradation (Fig. 2) (Harirchi et al., 2022; Ma et al., 2021).

As PHBH-derived carbon progressed toward terminal methanogenic routes, a consistent enrichment of enzymes mediating the conversion of acetyl-CoA to acetate was observed (Fig. 3), which is an essential precursor for acetoclastic methanogenesis (Ferry, 2011; Zheng et al., 2025). This enzymatic activity was predominantly associated with members of the phyla Bacillota and Pseudomonadota, with increased contributions from genera such as *Ornithinibacter*, *Sedimentibacter*, and *Syntrophomonas*. Specifically, phosphotransacetylase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1), integral components of the phosphate acetyltransferase–acetate kinase pathway (M00579), were prominently expressed in these taxa, indicating active flux through this pathway during anaerobic digestion. Given the inactivity of the classical NAD^+-dependent pyruvate dehydrogenase complex under strict anaerobic conditions, the decarboxylation of pyruvate was facilitated primarily by pyruvate formate lyase (EC 2.3.1.54) expressed mainly by genera *Clostridium*, *Sedimentibacter*, and *Cloacibacillus* within the Bacillota and Synergistota phyla (Crain and Broderick, 2014). This enzymatic process generated equimolar acetyl-CoA and formate, critical intermediates for downstream microbial metabolism. Supporting this, the consistent expression of key enzymes such as pyruvate formate lyase (EC 2.3.1.54) and formate dehydrogenase (EC 1.17.1.11) was identified, supported by the KO-level enrichment of K04069 (pyruvate formate lyase activating enzyme) and K21835 (secondary alcohol dehydrogenase), both involved in anaerobic carbon conversion and redox balance, showing increases of 66.5 % and 24.7 %, respectively. Additionally, the corresponding reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) (M00377) showed increased expression, representing a metabolic shift toward syntrophic fermentation. Formate generated through this route functions as a key electron carrier, further oxidized to H_2 and CO_2 by formate dehydrogenase (EC 1.17.1.11), contributing to redox balance and supplying reducing equivalents for methanogenesis (Xie et al., 2023). The formate dehydrogenase activity was closely associated with *Methanothrix* and *Methanoculleus* of the phylum Euryarchaeota, emphasizing the syntrophic interplay between bacteria and methanogenic archaea. This fermentation-derived intermediates, such as acetate, formate, H_2 and CO_2, served as key substrates for terminal methanogenesis, which proceeded via two distinct but functionally complementary pathways. These observations were reinforced by the strong dominance of the observed methanogenesis modules (Fig. 3), largely driven by members of the Archaean phylum Euryarchaeota, including genera *Methanothrix*, *Methanoculleus*, and *Methanobacterium*, suggesting a broad functional shift toward a syntrophic methanogenic consortium. The co-occurrence of dual methanogenesis routes, acetoclastic and hydrogenotrophic, implies functional redundancy and ecological stability that are typical of mature anaerobic digesters (Wintsche et al., 2018). This interpretation is quantitatively supported by a strong positive co-activation trend between methanogenesis modules, where a spearman correlation ($\rho = 1$, $p < 0.005$; $p = 0.00278$) confirmed a significant relationship between the abundances of modules M00357 (acetoclastic methanogenesis) and M00567 (hydrogenotrophic methanogenesis) at both Day 0 and PSS. This finding indicates that the two methanogenic routes were synchronously activated. This metabolic convergence, confirmed by the experimental carbon mass balance showing 84.3 % conversion to biogas, with the remaining 15.7 % distributed among soluble intermediates including volatile fatty acids (0.09 %), dissolved inorganic carbon (1.07 %), dissolved organic carbon (0.63 %), and approximately 10 % assimilated into newly formed microbial biomass, yielded an overall carbon recovery of 96.09 %, indicative of near-complete conversion of intermediates and a stable anaerobic digestion process (Shafana Farveen et al., 2025). This temporal restructuring was further evaluated through PCoA based on Bray-Curtis dissimilarity matrices. PCoA of KEGG Level 2 pathway profiles revealed clear phase-wise separation of samples, with statistical significance confirmed by PERMANOVA ($R^2 = 0.94669$, $p < 0.005$). Similarly, PCoA of KEGG module and EC annotations demonstrated distinct clustering across digestion phases ($R^2 = 0.94765$, $p < 0.005$), reinforcing the enzymatic and ecological transition during PHBH degradation. The corresponding PCoA plots are provided in Supplementary material.

3.3.2. *PlasticDB-based functional annotation*

To evaluate the enzymatic potential for plastic degradation under anaerobic conditions, metagenomic datasets were annotated against PlasticDB, a curated database of experimentally validated plastic-degrading enzymes. This targeted annotation revealed distinct patterns in both the abundance and similarity-based confidence of predicted functional homologs across different phases of anaerobic digestion, offering insight into the dynamic restructuring of microbial degradative capabilities in response to PHBH enrichment. This was supported by statistical analysis, wherein Bray-Curtis-based PCoA showed clear temporal separation (Supplementary material). PERMANOVA confirmed a significant impact of timepoint on enzyme profiles ($R^2 = 0.98$, $p < 0.005$). These trends provide insight into the dynamic restructuring of the microbial plastic degradative potential in response to PHBH enrichment.

As depicted in Fig. 4, PHB and PHA depolymerases displayed the most consistent abundance and high-confidence similarity throughout all time points. These polyester-specific depolymerases likely catalyze the initial hydrolysis of PHBH (Fig. 3), likely targeting the 3-hydroxybutyrate (3HB) backbone shared across these bioplastics (Paloyan et al., 2025). Their consistent abundance across all time points suggest that they form the enzymatic core of the anaerobic plastic-degrading consortium. Complementing these core depolymerases, PLA depolymerase was also detected at moderate abundance across all stages. This trend is consistent with the chemical compatibility between PLA and PHBH due

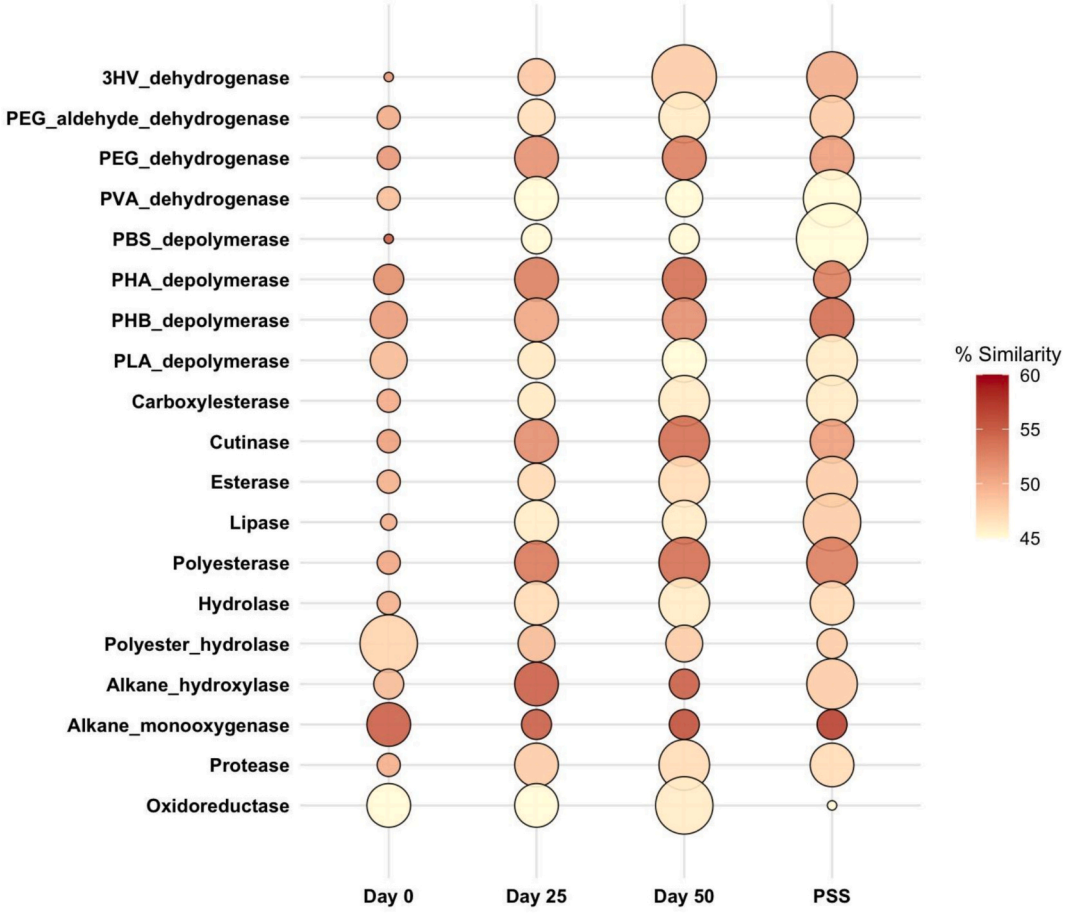

Fig. 4. Distribution of plastic-degrading enzyme homologs based on abundance and sequence similarity (≥70 %) across anaerobic digestion phases, where bubble size indicates relative abundance.

to shared linear aliphatic ester linkages, enabling partial cross-substrate activity (Shalem et al., 2024; Thomas et al., 2022). Surrounding this functional core, a second tier of enzymes, such as the esterases and cutinases, demonstrated moderate and temporally stable abundance. These broad-spectrum hydrolases likely complement depolymerase activity by cleaving residual ester linkages in oligomeric intermediates, thereby facilitating complete depolymerization and monomer release. Their stable presence suggests a constitutive role in the downstream processing of partially degraded PHBH fragments (Abdelraheem et al., 2025; Sameshima-Yamashita et al., 2019). In contrast, hydrolases and proteases were among the most abundant enzyme categories but were predominantly matched with lower similarity scores (40–50 %), suggesting high sequence diversity. This trend may reflect the inclusion of generic hydrolase families that are widely distributed in microbial genomes, inflating the apparent abundance while lowering annotation confidence. These enzymes may be involved in secondary processes such as biofilm remodeling, peptide turnover, or co-substrate degradation (Sikora et al., 2019; Solanki et al., 2021). A third, peripheral enzymatic tier included redox-active enzymes such as PEG aldehyde dehydrogenase, alkane monooxygenase, and oxidoreductases. PEG aldehyde dehydrogenase was observed at low abundance but with consistently high similarity (≥60 %), indicating the presence of highly conserved but low-copy genes potentially involved in co-substrate processing (Kawai, 2002). Conversely, alkane monooxygenase and generic oxidoreductases were minimally detected, reflecting the limited relevance of oxidative pathways in the strictly anaerobic environment. These enzymes, typically implicated in aerobic polymer oxidation, are unlikely to contribute significantly under reducing conditions. Together, this structured enzymatic pattern reflects a community that is both functionally specialized and selectively enriched for enzymatic traits optimized for bioplastic breakdown under anaerobic conditions.

To contextualize the enzyme-level findings presented above, a complementary analysis was performed to assess the comprehensive plastic-degrading potential of the sludge. While Fig. 4 provided insights into the abundance of plastic-degrading-like enzymes, Fig. 5 expands this view by examining the number of hits associated with representative plastics based on sequence similarity to PlasticDB entries. An identity threshold (≥70 %) was applied to ensure high-confidence functional assignments. On day 0, the inoculum harboured enzymes whose homologs were reported to degrade a wide range of polymers, including PET, PEG, PLA, PHA, PHB, PU, and PBS. This enzymatic diversity reflects the complex metabolic potential typical of environmental wastewater consortia, which have evolved under continuous exposure to anthropogenic and xenobiotic compounds (Li et al., 2022). The wide range of plastic types potentially degradable by the inoculum correlates directly with the high abundance of generalist hydrolases and broad-specificity proteases identified in Fig. 4. This demonstrates that the presence of non-specific, versatile enzymes which could interact with diverse polymer substrates, makes the inoculum as a functionally versatile microbial community with wide degradative potential. However, as AD proceeded under PHBH-fed conditions, the enzymatic profile underwent marked specialization. From day 25 through day 50, and especially at the PSS, there was a progressive narrowing in the number of plastic types associated with annotated enzyme hits. This shift coincided with a pronounced enrichment in PHB, PHA, and PLA degraders, polymers that share structural features with PHBH, particularly in their ester-bonded aliphatic backbones (Naser et al., 2021). The narrowing of the functional range does not indicate a loss of degradative capability

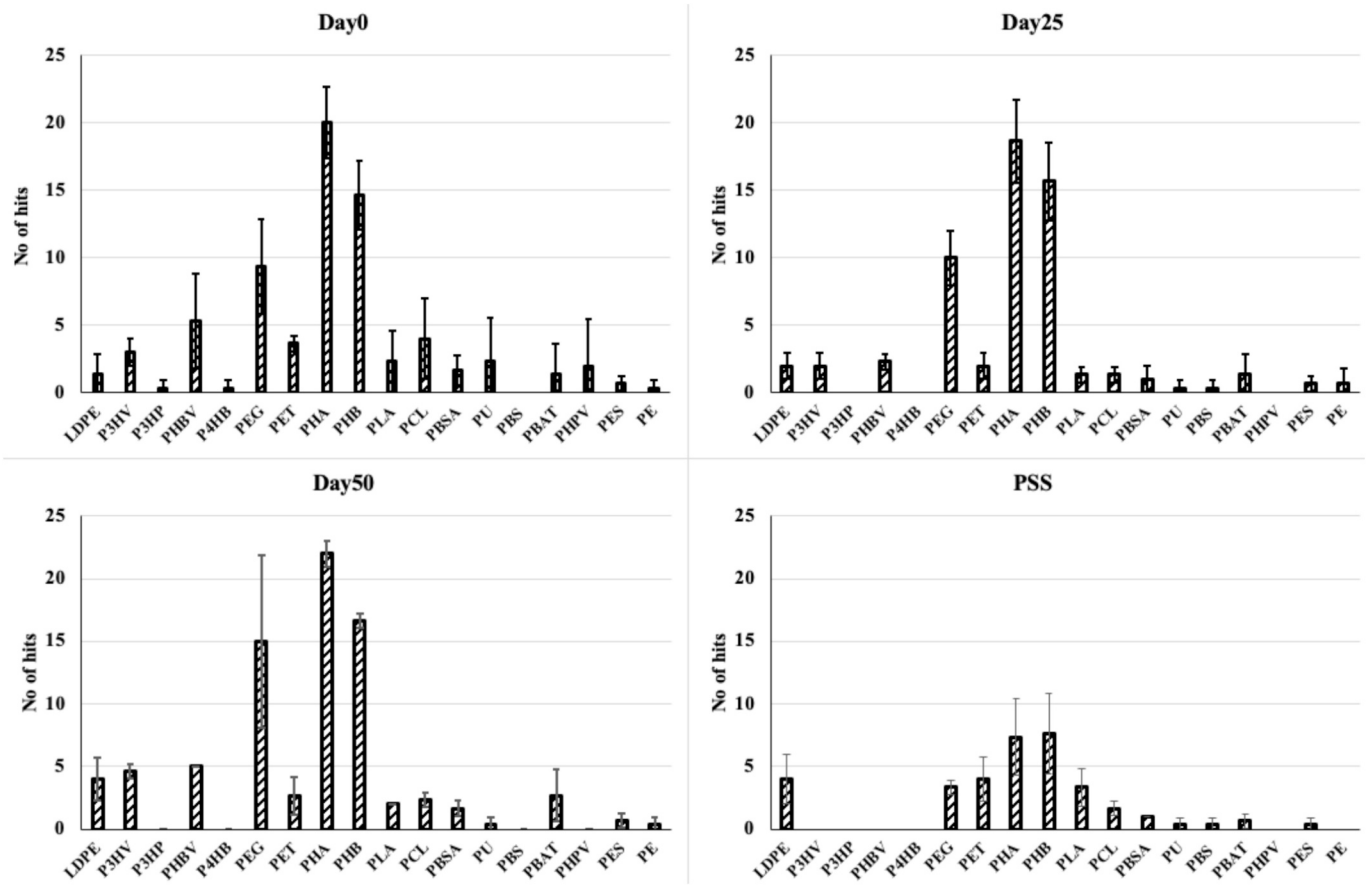

Fig. 5. Plastic-degrading enzymatic potential of sludge inferred from homology (≥70 % identity) to experimentally validated plastic-degrading enzymes. The results highlight a shift from broad-spectrum potential at day 0 to selective enrichment for PHBH-like bioplastics over time, reflecting substrate-driven functional specialization.

but rather reflects a substrate-driven specialization of the microbial consortia in response to sustained PHBH exposure (Bandini et al., 2022). The community became enriched in organisms harboring enzymes specifically relevant to PHBH degradation, as evidenced by the concurrent physical degradation observed (52.85 % weight loss, surface erosion) and successful methane production (550.5–562.3 NmL CH_4/g VS) documented in our experimental study (Shafana Farveen et al., 2025). Collectively, Figs. 4 and 5 demonstrate an observed shift in enzymatic composition and substrate specificity, which reflects a distinct ecological and functional transition from a broad-spectrum, environmentally sourced inoculum to a metabolically streamlined, syntrophically integrated consortium specialized for biopolyester degradation.

3.4. Molecular interaction analysis

To further elucidate the molecular interactions between PHBH and its degrading enzymes, molecular docking analysis was conducted. Enzyme candidates were selected based on consistent expression patterns and high-confidence annotations. As illustrated in Fig. 4, although hydrolases and proteases exhibited relatively high abundances, their associated confidence scores were low. Therefore, PHB depolymerase was selected for docking analysis. Among the various time points, the PSS phase was selected for sequence retrieval as it represented a stable microbial community characterized by prolonged operational stability (~60 days) and sustained methane production. This stability was deemed crucial for obtaining a robust and representative enzyme profile. Based on the screening criteria (≥ 60 % identity threshold, complete ORFs, removal of redundant entries), the sequence R1_379815, which shows 63.3 % identity to known and experimentally validated PHB depolymerases (From PlasticDB), was selected for interaction analysis. Although sequence identity alone cannot fully predict functional similarity, enzymes sharing 50–70 % identity typically poses conserved α/β-hydrolase fold and catalytic-site geometry, providing a structurally reliable basis for comparative docking, thereby elucidating enzyme–polymer binding interaction mechanisms (Tian and Skolnick, 2003). After sequence retrieval, homology modeling of R1_379815 was performed using SWISS-MODEL, employing the crystallographic structure of PHB depolymerase from *Lihuaxuella thermophila* (PDB ID: 9BYU; 1.75 Å) as the template, which shares 60.75 % sequence identity. The template structure 9BYU was retained as a docking control owing to its experimentally confirmed identity as a PHB depolymerase (Thomas et al., 2024). Validation of the modeled enzyme structure was performed using a range of computational tools, and the corresponding metrics are provided in the supplementary material. Multiple-sequence alignment revealed moderate conservation (60.75 %) between the metagenomic enzyme and 9BYU. Structural superposition yielded a root-mean-square deviation (RMSD) of ~2.636 Å, indicating a moderate degree of structural resemblance and supporting its suitability for comparative docking simulations.

Molecular interaction analyses were visualized using PyMOL and BIOVIA Discovery Studio. Residues contributing to ligand binding were mapped and compared across both sequences. Conserved and semi-conserved regions identified via ClustalW alignment were highlighted alongside COACH-predicted residues with high binding probabilities. Notably, active site residues overlapped between the control and modeled enzyme, indicating functional conservation despite sequence divergence (Fig. 6C). Docking simulations with the PHBH monomer

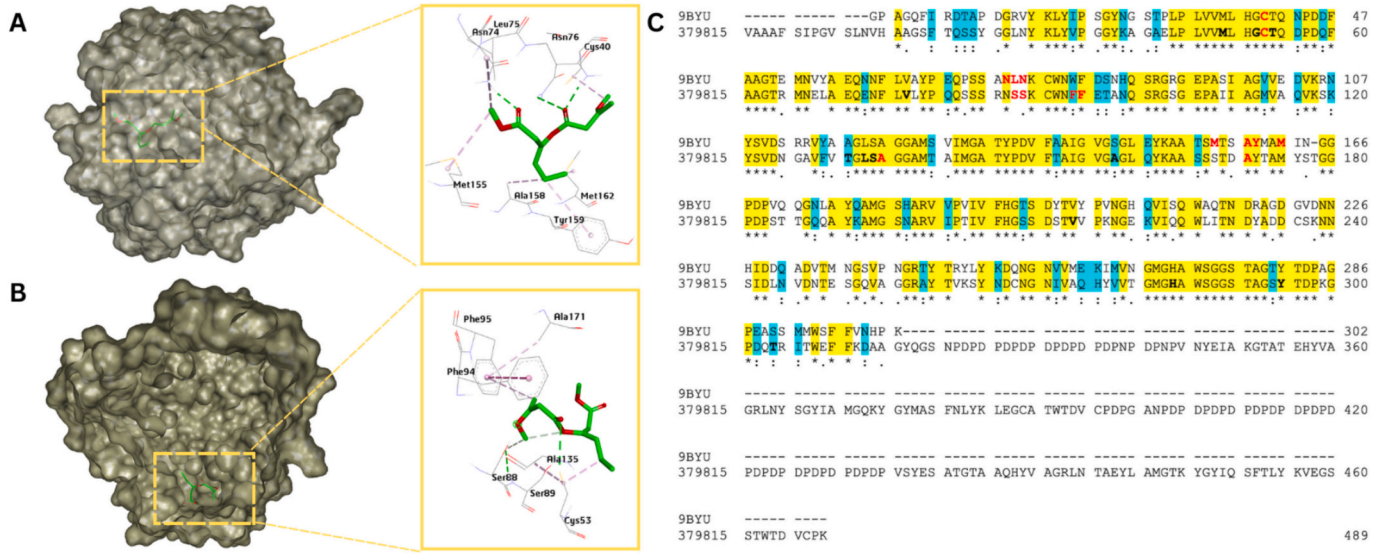

Fig. 6. Molecular docking analysis of PHB depolymerases with PHBH visualized using BIOVIA Discovery Studio (A) Control enzyme 9BYU; (B) Metagenome dataset derived enzyme R1_379815. In both green dashed lined indicate hydrogen bonds and pink dashed lines indicate π–alkyl or hydrophobic interactions (C) Sequence alignment showing predicted binding residues (bold), conserved residues (yellow), semi-conserved residues (blue), and interacting residues (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed comparable binding affinities for both the reference enzyme (–2.23 kcal/mol; Fig. 6A) and the metagenomic enzyme R1_379815 (–2.21 kcal/mol; Fig. 6B). Although these energies are relatively low, this is attributable to the use of a single PHBH ligand containing a single 3HB–3HH unit rather than the full polymeric chain with multiple units, which limits potential contact sites and underestimates the total interaction energy. Notably, with this modeled enzyme performing on par with 9BYU, a functionally validated PHB depolymerase (Thomas et al., 2022, 2024) aids in reliably capturing the binding geometry and analyse their interaction. The multiple sequence alignment (Fig. 6C) revealed that the active site residues involved in catalytic activity, including the classical Ser-His-Asp triad characteristic of PHB depolymerases (Kellici et al., 2017), were confirmed to be conserved in the modeled structure (R1_379815). In 9BYU, this triad is composed of Ser121-His270-Asp197 (Thomas et al., 2022), while in R1_379815, alignment revealed the corresponding residues as Ser134-His284-Asp211. Although these residues were not directly visualized in docking poses, their presence strongly suggests retained catalytic potential. This triad mediates hydrolysis by positioning the nucleophilic serine near the ester bond of the substrate, facilitating an attack on the carbonyl carbon (Rauwerdink and Kazlauskas, 2015). Such nucleophilic catalysis is stabilized by an oxyanion hole, represented by Cys40 in 9BYU, which is also conserved as Cys53 in the modeled enzyme, further supporting the functional fidelity of R1_379815 (Rauwerdink and Kazlauskas, 2015; Thomas et al., 2022). The modeled metagenomic enzyme exhibited a binding profile comparable to 9BYU. As observed in Fig. 6C, many of the interacting residues (highlighted in red in Fig. 6C) are conserved across both the enzymes. Key residues such as Cys53 and Ser88 formed hydrogen bonds with the ester carbonyl of PHBH, while residues like Ser89, Phe94, Phe95, Ala135, and Ala171 contributed through hydrophobic interactions, effectively positioning the substrate for nucleophilic attack. Overall, these results suggest that the metagenomic enzyme R1_379815 retains functional attributes characteristic of PHB depolymerases. This residue-level conservation, especially around the substrate-binding and catalytic regions, provides mechanistic insight into how these native key microbial enzymes interact with PHBH *in-situ*. These findings lay the foundation for further studies, including heterologous expression, kinetic characterization, and open avenues for enzyme engineering toward enhanced degradation of PHBH and related polyesters in environmental or industrial settings.

4. Conclusion

The integrated, molecular-level and computational analysis, elucidated the microbial and enzymatic mechanism of PHBH degradation under anaerobic conditions. By combining time-series metagenomic profiling with enzyme-level interaction analysis, this study establishes a clear link between PHBH degradation and methanogenesis pathways. Further, the selective enrichment of PHB/PHA-targeting enzymes under PHBH-fed conditions highlights substrate-driven microbial adaptation. Molecular docking additionally validated the functional potential of enzymes recovered from metagenomes, indicating their in-situ relevance. Together, these findings identify promising enzyme targets, that could be harnessed to accelerate bioplastic degradation and methane yield. They provide a foundation for the development of targeted enzymatic strategies through techniques such as site-directed mutagenesis and recombinant technologies, thereby facilitating enhanced bioplastic waste degradation under AD conditions.

CRediT authorship contribution statement

M. Shafana Farveen: Writing – original draft, Visualization, Methodology, Investigation. **Raul Muñoz:** Writing – review & editing, Resources, Methodology. **Rajnish Narayanan:** Writing – review & editing, Supervision. **Octavio García-Depraect:** Writing – review & editing, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2025.102424.

Data availability

Data will be made available on request.

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