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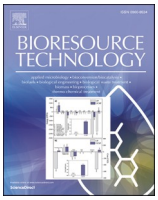

Reliable methodology to determine biotransformation of PBAT in anaerobic conditions

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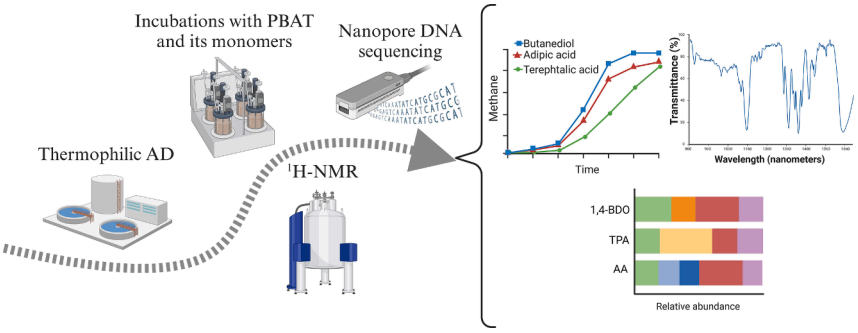
HIGHLIGHTS

- PBAT-derived 1,4-butanediol was quickly degraded.
- PBAT derived monomers adipic acid and terephthalic acid accumulated during AD.
- Microbial hydrolysis of 2 g powdered PBAT·L^{-1} was confirmed during thermophilic AD.
- ^{13}C-BDO was biotransformed into succinic acid.
- ^{1}H NMR quantification is a reliable methodology for hydrolysis assessment.

GRAPHICAL ABSTRACT



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ABSTRACT

Biodegradable plastics can enhance food waste utilization in anaerobic digestion (AD) units, but their fate under thermophilic conditions remains unclear. Previous studies using methane production, calorimetry, or spectroscopic analyses often report inconsistent results. This study tracks the biotransformation of polybutylene adipate co-terephthalate (PBAT) in thermophilic AD (55 °C) using ^{1}H NMR to quantify monomers. While 1,4-butanediol degraded quickly, adipic (AA) and terephthalic acid (TPA) accumulated over time. Monomer analysis estimated PBAT biotransformation at 11.1 ± 1.9 % (TPA) and 10.1 ± 2.3 % (AA). The core microbial community remained stable, indicating intrinsic hydrolytic capacities, which were stable despite TPA and AA accumulation. This workflow provides a robust methodology to evaluate the biotransformation of plastics.

1. Introduction

Plastic pollution is a significant environmental challenge, consuming 5–7 % of global oil supply and releasing over 800 million tons of CO_2 annually, approximately 2 % of global CO_2 emissions (Rosenboom et al., 2022). Replacing conventional plastics with biodegradable alternatives, such as poly(butylene adipate-*co*-terephthalate) (PBAT), is gaining attention for applications in packaging, compostable bags, and agricultural mulch (Ferreira et al., 2019; Jian et al., 2020). However, the fate of PBAT in anaerobic digesters (ADs) for waste management and

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renewable energy production is poorly understood.

The increasing use of Biodegradable Plastics (BPs) has given rise to a new waste processing methods. Landfills, accounting for 79 % of plastic disposal (Geyer et al., 2017), are being phased out in favour of circular economy approaches like chemical recycling (Coates et al., 2020; Jaime-Azuara et al., 2023). In contrast, biological-based processes offer significant advantages, such as cost-effectiveness and the production of innocuous products. Composting is widely used, but challenges like CO_2 and microplastic emissions, large area and aeration requirements, and long processing times persist (Zumstein et al., 2018; Liu et al., 2023). PBAT biodegradability has been demonstrated in soil and composting communities. In aerobic environments, depolymerization or hydrolysis occurs through extracellular enzymes such as cutinase-like serine hydrolases, mostly originating from terrestrial *Actinomycetes*, fungi, and various microbial species like *Alcaligenes faecalis, Comomonas acidivorans,* and *Pestalotiopsis microspora* (Šerá et al., 2020; Trivedi et al., 2016). Most PBAT-degrading microorganisms discovered to date cannot use the monomers as a carbon source and therefore are not able to mineralize the polymer into biomass and CO_2. However, other microbes can metabolize the released monomers, such as adipic acid (AA), 1, 4-butanediol (BDO), and terephthalic acid (TPA), as observed in mature compost (Yoshie et al., 2002). The application of ^{13}C-labeled compounds has unequivocally demonstrated the use of PBAT carbon to gain energy and form biomass in soil (Zumstein et al., 2018) and by the microbiome of an anaerobic digester (AD) (Poulsen et al., 2023).

AD represents a promising strategy for plastic management, offering advantages such as speed, lower CO_2 emissions, cost-effectiveness, and eco-friendliness compared to composting (Abraham et al., 2021; Cazaudahore et al., 2022). Additionally, AD produces methane for renewable energy production. According to Lin et al, 2018, generally, and without taking transportation into account, AD has the potential to save 200–1200 kg CO^2-eq·MT^{-1} waste compared to composting. The main advantage of AD regarding greenhouse gases reduction relies on its replacement of fossil fuel-based energy with biogas. Notably, it provides a disposal option for food waste contaminated with biodegradable plastics, such as packaging, which can be directly fed into anaerobic digesters. This process allows for simultaneous harvesting of energy and effective disposal of plastics, making it compatible with the use of bioplastic bags for organic waste collection. Plastics can also be co-digested with other materials such as agricultural residues or wastewater sludge, and after AD, the solid residues can be used as organic fertilizers for agriculture or gardening. In a scenario where BPs are widely used in daily life applications, they are expected to reach urban wastewater treatment plants (WWTPs) in progressively higher concentrations (Sun et al., 2019). However, the presence of impurities, additives, or non-biodegradable monomers can negatively affect the process, requiring individual evaluation (Jian et al., 2020; Yiwen et al., 2024).

Generally, there is a lack of understanding on the fate of BPs and its monomers in AD. Previous studies indicated rapid biodegradation under both mesophilic and thermophilic anaerobic digestion of poly(3-hydroxybutyrate) and thermoplastic starch, while PLA and poly(Ɛ-caprolactone) (PCL) rendered low methane production in both conditions. PBAT and polybutylene succinate (PBS) have shown negligible or no biodegradation potential (Peng et al, 2024), while the biodegradability of PLA and PBAT was greater in thermophilic AD, although PBAT were more resistant (Cazaudehore et al, 2023). Carbon assimilation derived from PBAT was previously demonstrated during thermophilic AD (Poulsen et al., 2023).

Assessing plastic biodegradation during AD remains challenging. Traditional methods such as morphology analysis, methane production, Fourier transform infrared spectroscopy (FTIR) and RAMAN spectroscopy have limitations in reproducibility and resolution, especially for detecting partial degradation (*e.g.*, Thew et al., 2024; Cazaudehore et al., 2022).

In recent years, nuclear magnetic resonance spectroscopy detection (q-NMR) has been explored for the quantification of plastics and their derived monomers, offering advantages such as minimal sample preparation and the potential to identify and quantify multiple compounds in a single analysis. Quantitative NMR can be performed by supplementing a known amount of internal standard to each sample or by directly quantifying against external standards. Notably, it does not require standard curves, as the response factor per hydrogen atom remains consistent across different compounds. A previous study developed a method for extracting and quantifying PBAT from agricultural mulch degradation in soils using Soxhlet extraction or accelerated solvent extraction (ASE) combined with quantitative ^{1}H NMR analysis (Nelson et al., 2020). More recently, a quantitative^{1}H NMR method for analysing depolymerized products from polyethylene terephthalate (PET) in aqueous solutions was described (Jaime-Azuara et al., 2023).

We hypothesize that it is feasible to quantify the biodegradation of plastics by assessing the concentration of their derived monomers. In this study, we employed a combination of batch incubations using anaerobic digestion sludge from a full-scale urban WWTP, along with PBAT. The analytical techniques utilized included gas and liquid chromatography, q-^{1}H NMR analyses, FTIR, and bacterial and long-read DNA sequencing. This study focused on two objectives: evaluating the feasibility of the proposed methodology for quantifying plastic hydrolysis during anaerobic digestion and assessing the hydrolysis of PBAT during thermophilic AD.

2. Materials and methods

2.1. Plastic and monomers

Amorphous and cryo-milled PBAT (Ø <50 μm as verified through light microscopy) was obtained from AIMPLAS Technological Center (Valencia, Spain). Non-labelled monomers, including adipic acid, 1,4-butanediol, and terephthalic acid, were purchased from Sigma Aldrich. The ^{13}C-labelled monomers $^{13}C_6$-adipic acid and 1,4-$^{13}C_4$-butanediol were purchased from Merck, ensuring a carbon isotopic purity exceeding 99 %. To obtain $^{13}C_8$-terephthalate, hydrolysis of dimethyl ($^{13}C_8$-terephthalate) purchased from Merck was performed. The synthesis procedure adhered to the previously described approach (Poulsen et al., 2023; Zumstein et al., 2018). The final product's integrity was confirmed through ^{1}H- and ^{13}C NMR analyses.

2.2. Inoculum and incubations

Sludge from the full-scale thermophilic anaerobic reactor from Aalborg West Wastewater treatment plant (Aalborg, Denmark) was utilized in all experiments presented in this study. Fresh biomass was collected before initiating each experiment, with all samplings and incubations conducted between May and October 2023. Physical-chemical analyses characterized the sludge during each sampling, and corresponding data are presented in the supplementary material. All batch incubations included in this study took place in glass serum bottles sealed with thick rubber stoppers and aluminium crimps. The headspace and liquid phases were purged with nitrogen to maintain strictly anoxic conditions during sampling and handlings at 55 °C, in dark conditions, with continuous stirring at 150–200 rpm (conditions scalable at industrial pilot plants).

2.3. Batch incubations with powdered PBAT

Batch reactors, set up in 250 mL bottles, were inoculated with 150 mL of sludge. Incubations included 1 g·L^{-1} glucose, maintaining a pH of 8.31 ± 0.01. pH adjustment or buffering unnecessary due to the alkalinity of the sludge (7.9 ± 0.1 g $CaCO_3$·L^{-1}). Experimental setup involved sludge fed with glucose, controls, and sludge fed with glucose and 2 g PBAT·L^{-1}, both were run in triplicates. Methane production was continuously monitored with an AMPTS II (Bioprocess Control) equipment following a CO_2 trap (3 M NaOH). Biomass was sampled every 48 h

for 18 days, and analysed for pH, total and soluble chemical oxygen demand (CODt, CODs), NMR, and microbial composition. After each sampling, bioreactors were replenished with glucose, and bulk volume was restored by g supplementing with fresh sludge. COD ($g{\cdot}L^{-1}$) was determined spectroscopically (LCK014, Hach). Total volatile solids (TVS) were determined with 1 mL aliquots using a thermogravimetric balance (HB43-S, Mettler Toledo). Biodegradability was calculated based on the COD of the PBAT used and its PBAT-derived monomers, considering the monomer ratio determined by NMR analyses in this study.

2.4. Batch incubations with ^{13}C labeled 1,4-butanediol

Six sacrificial batch reactors were prepared in 60 mL serum bottles, each containing 10 mL of sludge from the previous incubations with 2 g $PBAT{\cdot}L^{-1}$. Six replicates were supplemented with 5 mg of ^{13}C-butanediol and spiked with glucose (1 $g{\cdot}L^{-1}$) at the start of the experiment. No pH adjustment or bicarbonate was included. Incubations were terminated by freezing the serum bottles at different time points, ranging from zero to 48 h, for NMR analyses. An additional control reactor, contained the same inoculum without any monomer addition, was sampled at time zero.

2.5. Batch incubations with non-labeled monomers

Twelve batch reactors, set up in 125 mL glass bottles, were inoculated with 100 mL of sludge. Incubations were conducted without extra carbon sources or pH buffers. Four groups of reactors were established: i) sludge (controls), ii) sludge and adipic acid (0.05 $g{\cdot}L^{-1}$), iii) sludge and terephthalic acid (0.05 $g{\cdot}L^{-1}$), and iv) sludge and 1,4-butanediol (0.05 $g{\cdot}L^{-1}$). Each treatment (concentration) consisted of triplicate reactors, and the incubation lasted for 7 days. Daily biogas production was determined for each reactor using a manometer (Model PSI-5, Centrepoint Electronics), and biogas composition was analysed on a gas chromatograph (HP 5890 Series II). All handling and sampling were conducted using strict anaerobic techniques. COD, TS, and TVS were determined on the reactors at days 0 and 7 following standard methods (APHA, 2017).

Monomer concentrations were determined at time zero and day 7 by high-performance liquid chromatography. Adipic acid and 1,4-butanediol (BDO) were analyzed using an HPLC-1260 Infinity II equipped with a Rezex ROA organic acid analysis column (150 × 7.8 mm, Phenomenex) and an Agilent 1260 differential refractometer detector. The column temperature was 55 °C and the eluent was 5 mM H_2SO_4 at a flow rate of 0.5 $mL{\cdot}min^{-1}$. Terephthalic acid was analyzed using an X-LC™ Jasco (Hachioji) with a C_{18} Gemini® column (3 μm, 110 Å, 4.6 × 150 mm, Phenomenex) and a diode array detector at 240 nm. The column temperature was set to 30 °C, and the eluent consisted of acetonitrile: H_2O 30:70 (0.1 % Formic acid), with a flow rate of 0.7 $mL{\cdot}min^{-1}$.

The mathematical adjustment and the kinetic parameters for the batch anaerobic digestion from the experimental data obtained were determined through a non-linear regression using the software SigmaPlot (version 15.0). A first-order kinetic model for the different substrates was used, according to the following expressions (Eqs. (1) and (2)):

$$G = G_{max}{\cdot}\left(1 - e^{-k{\cdot}t}\right) \quad (1)$$

$$R_m = G_{max} \times k \quad (2)$$

where G (mL CH_4) is the cumulative specific methane production, G_{max} (mL CH_4) is the ultimate specific methane production, k (day^{-1}) is the specific rate constant or apparent kinetic constant, t (day) is the time, and R_m (mL $CH_4 \cdot day^{-1}$) is the methane production rate.

2.6. Sample preparation

Every 48 h, 3 mL samples were collected from each reactor (section 2.3) and frozen at −20 °C for a minimum of 24 h. After thawing at room temperature and centrifugation at 4830 × g for 10 min at 4 °C, 500 μL of the supernatant was transferred to Bruker® 5 mm NMR tubes, mixed with 25 μL of 1 mM DSS in D_2O. As a positive control, powdered PBAT was chemically hydrolyzed in 1 M KOH, at 90 °C for 8 h. For a killed control, the sludge was autoclaved and further supplemented with 4 g powdered PBAT, and incubated at 55 °C, 100 rpm for 72 h. The digested PBAT was collected at the end of the incubations and dissolved in deuterated chloroform supplemented with TMS.

Samples from the ^{13}C labeled incubations were frozen for a minimum of 24 h, centrifuged at 4830 × g, and the pellet was washed twice with demineralized water. The washing water was combined with the supernatant. Samples were then freeze-dried for 48 h and resuspended in 700 μL of 1 mM DSS in D_2O, 2 mM NaN_3.

2.7. Characterization

NMR spectra were recorded on a BRUKER AVIII-600 MHz spectrometer equipped with a cryogenically cooled CPP-TCI 5 mm probe. Topspin 3.6.4 (Bruker) was used for processing, and quantification was performed with the SMA (Simple Mixture Analysis) tool from MNova 14.2.3 (Mestrelab Research S.L.). Quantitative ^1H NMR spectra were recorded with a standard 1D pulse sequence with an acquisition time of 2.73 s (64 k complex data points, spectral width of 20 ppm). The relaxation delay was set to 25.5 s. During the last 5 s of the relaxation delay, a weak continuous-wave pulse of $\gamma B1/2\pi = 70$ Hz was applied for water suppression.

For the identification of the breakdown product, ^{13}C, [^1H,^{13}C]-HSQC and (1,1)-ADEQUATE spectra were recorded.

^1H NMR and [^1H,^{13}C]-HSQC spectra of commercially obtained standards of AA, BDO and TPA were recorded in 95 % H_2O/5% D_2O at a pH of 9. Resonance assignment in product mixtures was performed by comparing chemical shifts and spectra superposition.

The percentage of PBAT hydrolysed was calculated based on the concentrations of TPA and AA detected in the supernatant of the powdered PBAT incubations at day 15 through the following equations:

$$[mM_m]_0 = \frac{C_{PBAT} \times F_m}{MW_m} \quad (3)$$

$$\%Hidrolisis = \frac{[mM_m]_t}{[mM_m]_0} \times 100 \quad (4)$$

Where $[mM_m]_0$ is the initial molar concentration of each monomer, C_{PBAT} is the concentration of PBAT, F_m is the mass fraction of each monomer, MW_m is the mass molar of each monomer, and $[mM_m]_t$ is the final molar concentration of each monomer.

Powdered PBAT collected after the 17-day incubations were analyzed by FTIR and compared against the raw non-treated powdered PBAT. FTIR spectra were recorded in a N_2 atmosphere using a a Tensor 27 FTIR spectrophotometer (Bruker) equipped with a deuterated triglycine sulfate Mid-infrared detector and a Platinum ATR total reflectance (ATR) cell (Bruker). ATR spectra were recorded from 4000–1000 cm^{-1} using a nominal resolution of 2 cm^{-1} and 64 accumulations. Resulting spectra were baseline corrected and interfering signals from H_2O and CO_2 were removed using the atmospheric compensation filter in the OPUS 7.5 system (Bruker). The key features of the spectra confirmed PBAT through the strong peak around 1715 cm^{-1} confirms the presence of ester carbonyl groups, peaks in the 1400–1600 cm^{-1} range confirm the aromatic nature of part of the polymer backbone due to the terephthalate units. The presence of peaks in the 1150–1300 cm^{-1} and 2850–2950 cm^{-1} ranges indicates the C—O stretching vibrations in the ester bonds and the methylene and methyl groups from the butylene

and adipate segments. Spectra from the materials can be found in the Supplementary.

2.8. Amplicon sequencing and Bioinformatics

Total DNA extraction was performed using the DNeasy® PowerLyzer Powersoil kit (QIAGEN) following the manufacturer's specifications. The concentrations of DNA were quantified using the Qubit™ 1x dsDNA HS Assay Kit (Invitrogen, USA) on a Qubit 4 fluorometer (Invitrogen).

For the assessment of bacterial community composition, the V1-8 region of the 16S rRNA gene was amplified using primers from Klindworth et al. (2013). Methanogenic archaeal communities were evaluated using the functional mcrA gene (Luton et al., 2002), and amplifications were conducted according to Poulsen et al. (2022). Barcoding and equimolar pooling of PCR products were carried out, and the resulting library barcoding was prepared for sequencing using ligation sequencing kit SQK-LSK114 and barcoding expansion EXP-PBC096, following the manufacturer's recommendations and protocol version PBC_9182_V114_REVG_07MAR2023.

Approximately 50 fmol of the prepared library was loaded onto a FLO-PRO114M (Oxford Nanopore Technologies), and the library was sequenced for 4 hrs with live-basecalling enabled in high-accuracy mode. Data processing and OTU-tables were conducted using the ONT OTU table workflow (https://github.com/MathiasEskildsen/ONT_OTU_Table). Visualization of microbial composition was performed using the R-package Ampvis2 (https://github.com/kasperskytte/ampvis2/).

3. Results and discussion

3.1. Degradability of PBAT monomers

To validate the hypothesis regarding the potential tracking of plastic biodegradation through quantification of derived monomers, we initiated an assessment of the degradability of the three individual PBAT monomers. The compounds were individually amended at 0.05 $g{\cdot}L^{-1}$ in microcosms inoculated with fresh sludge and were incubated under anaerobic and thermophilic conditions at 55 °C. The pH was 7.8 ± 0.1, and the concentrations were 6.7 ± 0.1 g $TS{\cdot}L^{-1}$, 4.3 ± 0.1 g $TVS{\cdot}L^{-1}$, 8.1 ± 0.1 g $CODt{\cdot}L^{-1}$, and 0.5 ± 0.1 g $CODs{\cdot}L^{-1}$ at the start of the incubations.

After the 7 days test period, 1,4-BDO was nearly depleted (95.8 % ±7.3), as determined by HPLC. Consequently, methane production at day 7 was higher in the microcosms with 1,4-BDO compared to the control. The complete conversion of BDP into methane resulted in 100 % biodegradability. This aligns with earlier findings (Yiwen et al., 2024), in which they observed substantial biodegradation of 1,2-BDO by methanogenic sludge in reactors handling PBAT synthesis wastewater. Additionally, the incorporation of ^{13}C-labelled 1,4-BDO into specific proteins has previous been detected, in sludge from the same WWTP, through stable isotope probing (SIP) proteomics (Poulsen et al., 2023), further supporting these findings. Approximately 24.8 % ± 0.1 of the amended 1,4-BDO was transformed into CH_4.

In contrast, adipic acid (AA) exhibited biodegradation below the quantification limit (0.001 $g{\cdot}L^{-1}$). Regarding terephthalic acid (TPA), its quantification was achieved through NMR and revealed its stability in the tested conditions. Therefore, we suggest the use of AA and TPA to monitor the biotransformation of raw PBAT during AD.

In previous studies performed in soil environments monomers preference has been shown to be affected by redox conditions, strain-dependencies or different enzymatic capabilities. As an example, an aerobic isolated *Bacillus* strain degraded TP by 18 %, AA by 50 %, and BD by 11 % (AA > TP > BD). In contrast, *B. licheniformis* from the same genus biodegraded TP by 9 % and AA by 20 % while it did not degrade BD during the same time interval (Morro et al, 2019). A more in-depth analysis of the microorganisms present in the sludge and more specifically, their enzymatic capacities could enlighten the reasons for the differential use of PBAT-derived monomers in the present conditions.

To elucidate the fate of the reminding 1,4-BDO, a series of incubations was conducted using the ^{13}C-labeled monomer, and the supernatant was subsequently analysed through ^{13}C NMR. The initial pH of the bulk was 7.65 ± 0.1. At time zero of the incubations, 45 g $SSV{\cdot}L^{-1}$ and 27.8 g $CODt{\cdot}L^{-1}$ were determined. Only one transformation product, incorporating the ^{13}C label, was identified (see Fig. 2). The chemical shifts and observed NMR peaks were consistent with succinic acid. Succinic acid has previously been reported as a transformation product of 1,4-BDO through *Pseudomonas putida* metabolism (Li et al., 2020).

3.2. Batch incubations with powdered PBAT

A batch experiment was conducted, employing fresh inoculum and powdered PBAT (2 $g{\cdot}L^{-1}$), to evaluate the possibility of monitoring its hydrolysis by ^{1}H NMR over a 15-day incubation period. The thermophilic anaerobic digester sludge used as inoculum had an initial pH of 8.2 ± 0.1, 28.8 ± 0.1 g $CODt{\cdot}L^{-1}$, and 37 ± 1 g of $TVS{\cdot}L^{-1}$. Throughout the experiment, the bulk pH experienced a slight acidification, reaching a final average value of 7.96 ± 0.01 (Fig. S1A).

The CODs showed an initial decrease from 3.01 ± 0.03 $g{\cdot}L^{-1}$ to 2.12 ± 0.12 on the first 2 days of incubation. Subsequently, it stabilized at 2.10 ± 0.16 in the control group and 2.45 ± 0.3 in the bioreactors fed with PBAT (Fig. S1B). Methane production remained consistent during the operation, facilitated by the continuous supply of 1 $g{\cdot}L^{-1}$ glucose every 48 h (Fig. 1). However, beyond day 12, methane production in the PBAT-containing microcosms was slightly lower than in the controls (although the difference was statistically not significant). This small decrease could be attributed to the rising concentrations of additives, or plasticizers, such as acetyl tributyl citrate (ATBC), epoxy based Joncryl compatibilizer, or phthalic anhydride, among others, commonly present in PBAT formulations (Jian et al., 2020).

3.3. Monomers quantification

Quantitative ^{1}H NMR spectroscopy was employed to assess PBAT hydrolysis by quantifying its derived monomers. This technique offers advantages such as minimal sample preparation, non-destructive analysis, and simultaneous identification and quantification of multiple compounds. Unlike methods requiring standard calibration curves, ^{1}H NMR utilizes the consistent response factor of hydrogen atoms across compounds for reliable quantification. Supernatant samples from the powdered PBAT incubations were analyzed to determine the feasibility of monitoring PBAT biodegradation through its monomers. Fig. 3 and S6 illustrate the evolution of AA and TPA monomers during incubations with 2 g powdered $PBAT{\cdot}L^{-1}$. Notably, 1,4-BDO was never detected, indicating its rapid and complete depletion by the AD microbiota. This finding aligns with individual monomer tests and with the previous reports (Poulsen et al., 2023) in which assimilation of ^{13}C derived from 1,4-BDO into biomass was detected within remarkably short time of 6 h.

The molar ratio of the monomers in raw PBAT utilized in this study was determined by digestion with 1 M KOH at 90 °C, followed by analysis of the reaction mixture using ^{1}H NMR. The results demonstrated that TPA and AA occur in almost equimolar amounts (see supplementary material). The ratio of AA: TPA on the bioreactor's supernatant remained close to 1:1 during the incubation period, with the concentrations of both monomers increasing over time (Fig. 3, supplementary material). As hydrolysis is a surface-driven process, the availability of sites for PBAT attack increases over time, making it more accessible.

To investigate changes in the plastic material post-digestion, two approaches were employed. Firstly, an FTIR analysis of the powdered PBAT before and after 15 days of anaerobic digestion was conducted (see supplementary material), revealing no discernible chemical differences between the two materials (see supplementary material). Secondly, NMR of the raw PBAT and the remaining plastic collected at the experiment's conclusions (dissolved in chloroform) showed no

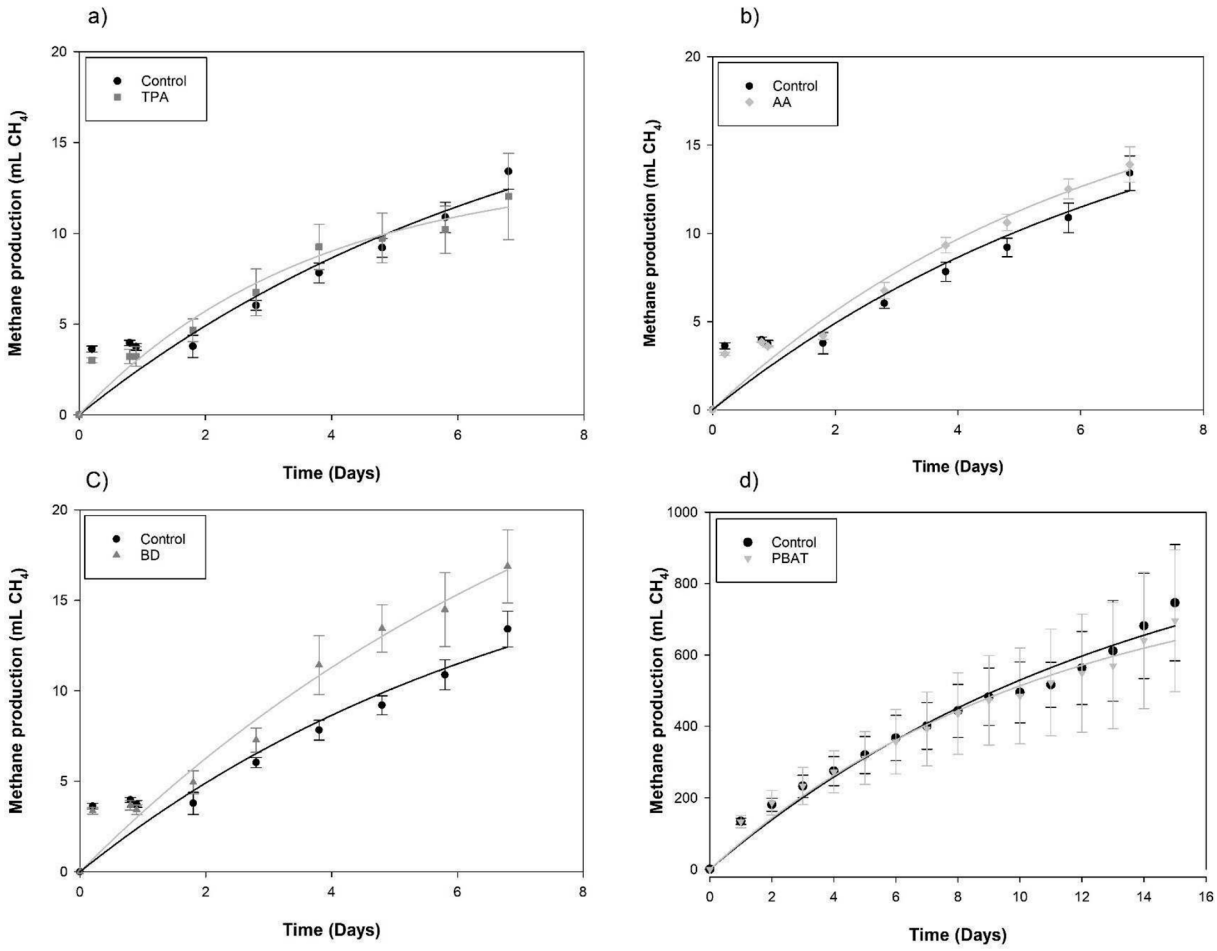

Fig. 1. Incubations with 0.05 g·L^{-1} of the individual PBAT monomers a) 1,4-butanediol (1,4-BDO), b) Terephthalic acid (TPA), c) adipic acid (AA) and d) raw PBAT. See Table S2 for rate constants.

distinctions between the two samples (see supplementary material).

No abiotic degradation is anticipated under the incubation conditions applied in this study, as PBAT exhibits resistance to temperatures of 350 °C (Jian et al., 2020), and the operational pH was consistently maintained within a circumneutral range. To further explore the potential of biological activity in the observed degradation, an additional set of bioreactors of PBAT was set up comprising: i) autoclaved sludge without PBAT, ii) autoclaved sludge with 2 g PBAT·L^{-1}, iii) active sludge without PBAT, and iv) active sludge with 2 g PBAT·L^{-1}. This set underwent incubation under the same conditions as the rest of the experiments (55 °C and 150 rpm) for a duration of 7 days. Fig. 4 illustrates the ^{1}H NMR spectra of the supernatants from this experiment.

In the bioreactors without PBAT, both autoclaved and active, none of the monomers were detected. In the active sludge with PBAT, both AA and TPA were identified, aligning with the findings of the initial experiment, while 1,4-BDO was not detected. Notably, the autoclaved sludge exhibited hydrolytic activity, with all three monomers detected in its supernatant, albeit at lower levels than in active sludge. This phenomenon could be attributed to the presence of biological or enzymatic activity resisting the autoclaving treatment, highlighting the potential role of living organisms in the observed hydrolysis. It would therefore be of interest to explore the performance of these hyperthermophile enzyme extracts for valuable biotechnological processes occurring at high temperatures. Some potential applications would be starch processing or the hydrolysis of long chains of cellulose polymers into smaller fermentable monosaccharides that can be used as feedstocks to produce biofuels and biorenewable chemicals.

The percentage of PBAT hydrolysed in both incubations with 2 g PBAT·L^{-1} was calculated based on the concentrations of TPA and AA detected. Specifically, 11.1 ± 1.9 % was determined based on TPA and 10.1 ± 2.3 % based on AA. In total, we attribute the hydrolysis of approximately 10 % of the added PBAT during the 15 days of thermophilic anaerobic digestion to microbiologically mediated reactions.

To date, only three published studies have investigated the thermophilic degradation of PBAT (Svoboda et al., 2018; Cazaudahore et al., 2023; Poulsen et al., 2023). One study reported a PBAT biodegradation level of 8.3 % over 126 days at 55 °C, accompanied by a significant reduction in PBAT molecular weight from 93,000 to 9,430 g·mol^{-1} as determined by differential scanning calorimetry (Svoboda et al., 2018). Another study found no biodegradation of PBAT under thermophilic conditions within 100 days, based on biochemical methane potential (BMP) test and Fourier-transformed infrared spectroscopy (FTIR) (Cazaudehore et al., 2023). However, they observed that 13.4 % ± 0.4 of PBAT was converted to methane over 500 days in mesophilic conditions, which is comparable to findings in the present study. A previous study demonstrated the assimilation of ^{13}C from two of the three PBAT monomers into thermophilic AD sludge proteins using metagenomics and SIP-proteomics, although quantification was not assessed (Poulsen et al., 2023). The differences on the biotransformation efficiencies on the different studies might derived from the different analytical methodologies applied. Also, the inoculum characteristics might have a relevant role. As for instance the inoculum used in the thermophilic experiments in Svoboda et al, 2018 derived from a mesophilic AD from a municipal WWTP, and therefore it was acclimated to thermophilic conditions for a shorter time than the one used in the present study.

The percentage obtained in this study, considering the experimental

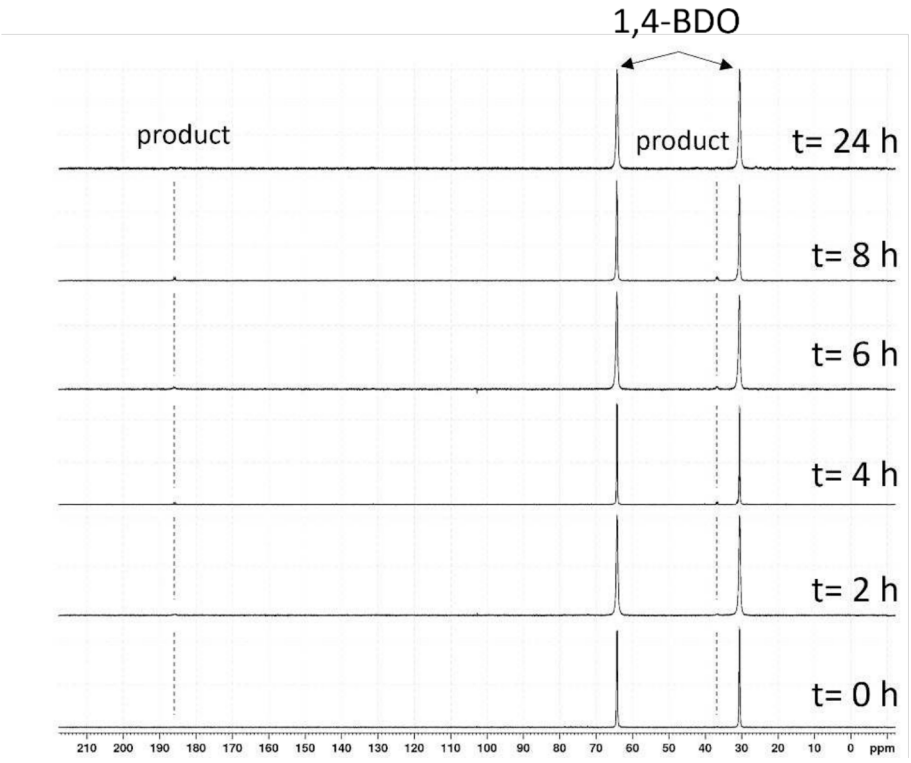

Fig. 2. ^{13}C NMR spectra of sludge incubated with U-^{13}C-1,4-butanediol at different times. 1,4-BDO yields two strong peaks at 64.25 and 30.56 ppm. At t = 2 h, two additional peaks appear at 185.86 and 36.7 ppm. They grow until t = 8h but are not visible at t = 24 h.

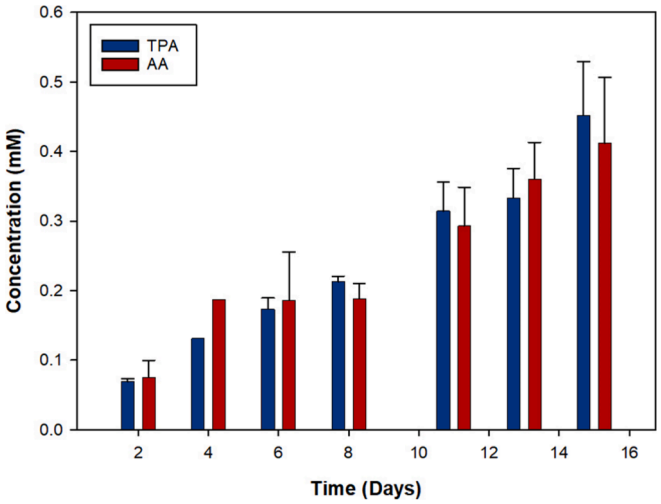

Fig. 3. The concentration of PBAT monomers TPA (terephthalic acid) and AA (adipic acid) detected in the supernatant of batch incubations with 2 g PBAT·L^{-1} over the time, as quantified by q-^{1}H NMR. The third monomer 1–4-butanediol was never detected. The raw data is presented in Fig. S6.

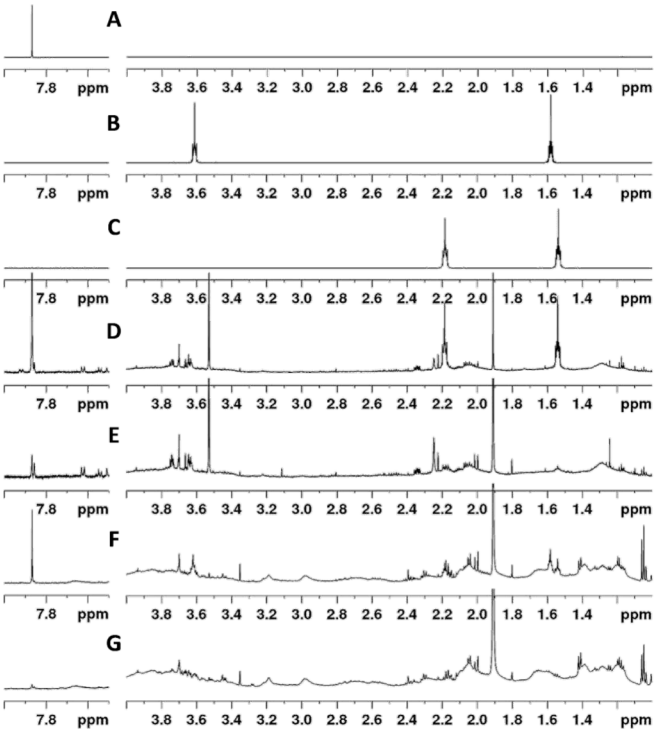

Fig. 4. Biotic vs. abiotic degradation of PBAT. A: TPA in D_2O, pH 9. B: 1,4-BDO in D_2O. C: AA in D_2O, pH 9. D: supernatant from live sludge, incubated with PBAT for 12 days. E: supernatant from live sludge incubated without PBAT (control) after 12 days. F: supernatant from autoclaved sludge, incubated with PBAT for 12 days. G: supernatant from autoclaved sludge, incubated without PBAT (control) after 12 days. All spectra were referenced to internal DSS-d_6.

time, suggests a more optimistic scenario for anaerobic digestion of PBAT compared to previous reports, given the relatively short experimental duration. Generally, research studies on the biodegradation of bioplastics under anaerobic conditions show lower values than those obtained from aerobic conditions. Petroleum-based BPs such as PBAT also exhibits lower levels of biodegradation efficiency under anaerobic conditions than bio-based alternatives (Cazaudehore et al., 2023). Thermophilic conditions are expected to enhance degradation rates, supported by increased solubility (Cazaudehore et al., 2022; Stroot et al., 2001; Abraham et al., 2021).

Scaling the methodology for real-world applications could involve optimizing operational conditions, such as temperature, sludge

acclimation, and retention times, to maximize hydrolysis rates. The absence of negative impacts on methane production indicates that PBAT-contaminated waste could be co-digested with organic substrates without compromising biogas yields, making it feasible for implementation in industrial AD systems.

3.4. Microbial community structure

Macroscopic inspections revealed that powdered PBAT and its monomers did not significantly impact the performance of the methanogenic sludge. To evaluate the evolution of the microbiome community structure during incubations with powdered PBAT, 16S rRNA gene metabarcoding was conducted, amplifying V1-V8 bacterial and archaeal (*mcrA*) markers (Fig. 5).

For bacteria, the most abundant genera were *Coprothermobacter, Lentimicrobium, Fervidobacterium,* midas_g_88 (within *Steroidobacteraceae*), and *Acetomicrobium*, collectively constituting nearly 50 % of the total relative abundance of bacteria at all time-points.

The relative abundance of *Coprothermobacter* increased from 19.7 % in the inoculum to 34.1–34.7 % in the controls, and 31.4–33.8 % in the 2 g PBAT·L^{-1}, indicating that the enrichment of this genus was driven by the operational conditions and not linked to the presence of PBAT or its derived monomers.

Lentimicrobium, on the other hand, experienced a decline from a relative abundance of 15.7 % in the inoculum to 5.9–6.1 % in the controls and 3.2–5.4 % in 2 g PBAT·L^{-1}. This shift suggests a response to the experimental conditions rather than a direct influence from the presence of PBAT or its monomers.

For methanogenic archaea, the most abundant species identified was *Methanothermobacter tenebrarum*. Throughout the incubation period, the relative abundance of this species increased from 96.1 % to 98.8–99.3 % in the control group and 98.8–99.1 % in the 2 g PBAT·L^{-1} treatment, suggesting that the enrichment of *Methanothermobacter tenebrarum* was due to operational conditions rather than the presence of powdered PBAT.

The second most abundant species, *Methanothermobacter thermautotrophicus,* experienced a decline from 2.3 % to 0.4–0.6 % in the control group and 0.5–1 % in the experiment with 2 g PBAT·L^{-1}, which can also be attributed to operational conditions.

The analysis of the microbiome community structure indicates a stable bacterial and archaeal community throughout all the operational periods. Therefore, the hydrolytic and metabolic capacities involved in the PBAT biotransformation were either conducted by small groups of microbes or by organisms intrinsic to the AD sludge. Neither the raw PBAT nor its derived monomers had a negative effect on the population, consistent with what the macroscopic analyses had already demonstrated.

This study extends prior findings by not only confirming PBAT hydrolysis but also demonstrating the integration of its monomers into key metabolic pathways, with 1,4-BDO being rapidly metabolized. Unlike earlier studies that reported inconsistent biodegradation rates, this work establishes a clear pathway for PBAT breakdown and highlights the production of intermediate compounds such as succinic acid.

Future work should explore the role of specific enzymes in PBAT hydrolysis, leveraging environmental proteomics and metagenomic analyses to identify key microbial players and enzymatic pathways. Enhanced statistical analyses, such as incorporating error bars in quantification figures, would provide greater precision in assessing trends. Additionally, extending the findings to continuous flow systems or pilot-scale AD setups would provide critical insights into scalability and operational robustness.

4. Conclusions

This study demonstrates that the biodegradable plastic PBAT undergoes partial hydrolysis by thermophilic methanogenic sludge, releasing its three monomers. Among these, 1,4-BDO was rapidly depleted, with evidence of its assimilation into the biomass and conversion into CH_4 and CO_2. Succinic acid was identified as an intermediate product in this process, while AA and TPA accumulated. Approximately 10 % of the added PBAT was hydrolyzed within a 15-day operational period, a rate that is more promising compared to previous

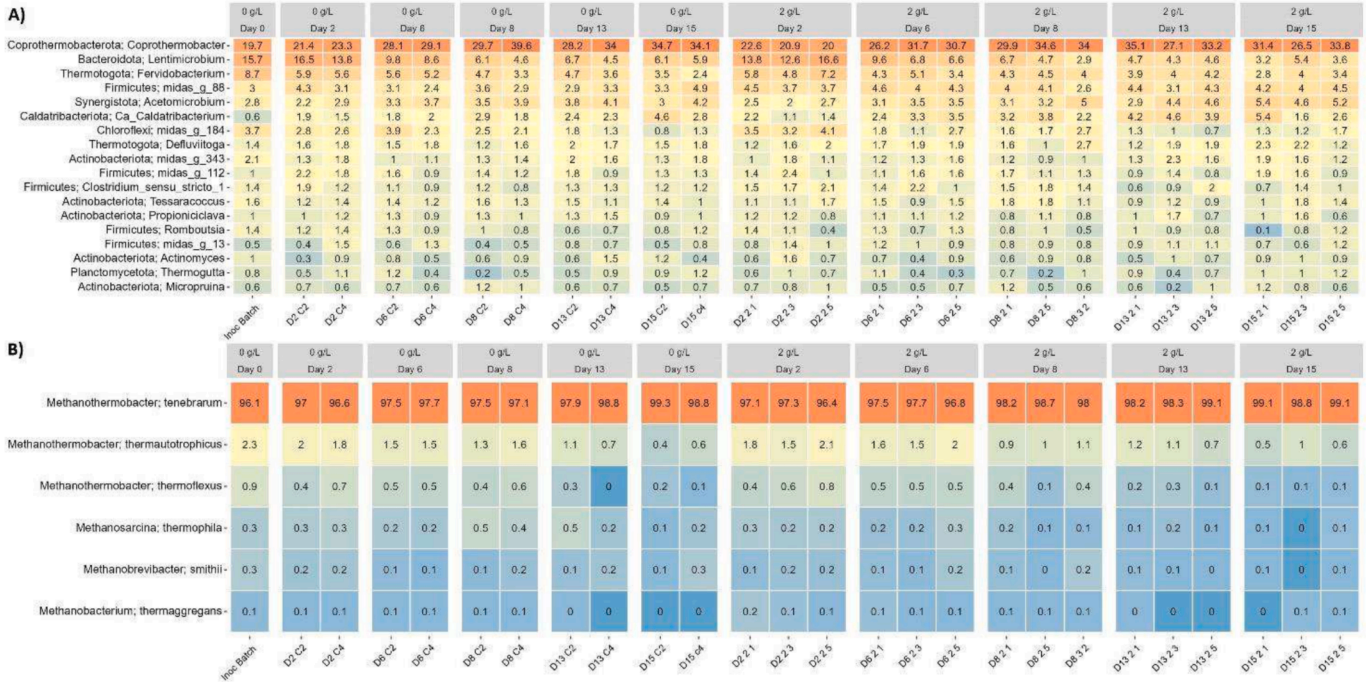

Fig. 5. Heatmaps illustrating core OTUs of bacteria and methanogenic archaea found over an incubation period of 15 days with either 0 or 2 g PBAT·L^{-1}. A) Top 18 most abundant genera of bacteria. B) Top 6 most abundant species of methanogenic archaea. Sample names (DxCy) indicate the day (x) and the concentration of PBAT in g·L^{-1} (y).

reports in the literature.

The findings highlight the potential for prolonged incubations with acclimated sludge to enhance PBAT hydrolysis further. Notably, the presence of PBAT and its derived monomers did not adversely affect the methane production capacity or significantly alter the microbial community structure, suggesting compatibility with industrial AD processes as bioplastic concentrations in waste streams increase.

CRediT authorship contribution statement

Alba Trueba-Santiso: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Reinhard Wimmer:** Writing – review & editing, Visualization, Resources, Investigation, Formal analysis. **Mathias Eskildsen:** Writing – original draft, Visualization, Investigation, Data curation. **Juan Cubero-Cardoso:** Visualization, Investigation. **Juan M. Lema:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Jeppe Lund Nielsen:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization.

Data availability

Amplicon sequencing data is available at the European Nucleotide Archive (ENA) under the project accession number PRJEB71743.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.132242.

Data availability

Data will be made available on request.

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