Pesante *et al.*
Biotechnology for Biofuels and Bioproducts (2026) 19:53
https://doi.org/10.1186/s13068-026-02773-3

Biotechnology for Biofuels and Bioproducts

RESEARCH

Open Access

Process optimisation for polyhydroxyalkanoates production with tailored hydroxyvalerate content from agri-food residues using mixed microbial cultures

Giovanna Pesante[1*], Claudia Magonara[1], Alessia Zucca[1], Elvis Montagnese[1], Davide Bertasini[1], Gaia Salvatori[2], Lionel Nguemna Tayou[2], Luca Di Leva[2], Marianna Villano[2] and David Bolzonella[1]

Abstract

Background Polyhydroxyalkanoates (PHAs) are promising bio-based and biodegradable polymers that can contribute to the transition towards a circular bioeconomy, particularly when produced from renewable feedstocks. Nevertheless, the use of mixed microbial cultures (MMC) and real agri-food residues poses challenges related to substrate variability, process stability and control of polymer composition. In this study, a three-stage process was investigated to produce poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) from agri-food residues. The process consisted of: (i) acidogenic fermentation of cattle slurry and corn residues to generate a carboxylic acid (CA)-rich stream; (ii) microbial selection under feast–famine conditions to enrich PHA-accumulating microorganisms; and (iii) PHA accumulation in a fed-batch reactor. Two operational setups, differing in the pretreatment type, were compared to evaluate possible effects on PHA production: setup 1 used centrifuged fermentate, whereas setup 2 included an additional 0.22-µm filtration step.

Results Acidogenic fermentation produced up to 18.6 gCOD/L of CAs, mainly acetic, butyric and propionic acids, with production strongly influenced by substrate ratio, hydraulic retention time and pH. The CA-rich fermented liquid was used to select MMC enriched with PHBV-accumulating microorganisms and to tune the PHBV composition during the subsequent accumulation phase. In setup 1, storage yield (mean 0.33 ± 0.23 $gCOD_{PHA}/gCOD_{CA}$), PHA titre (mean 0.81 ± 0.33 g/L) and intracellular PHA contents (4.0–21.0% w/w of cell dry weight) remained relatively low, likely due to the use of unfiltered fermentation effluent. In setup 2, the introduction of a filtration step and improved microbial selection led to higher and more stable conversion of CAs into PHAs, reaching mean storage yields of 0.78 ± 0.27 $gCOD_{PHA}/gCOD_{CA}$, mean PHA titre of 1.70 ± 0.44 g/L and intracellular PHA values up to 57.0% w/w. The hydroxyvalerate (HV) monomer content showed temporal variability, ranging from 14.0–32.0% w/w in setup 1 and from 13.0–35.0% w/w in setup 2, with values generally within or close to the target range required to improve polymer flexibility and processability. Microbial community analysis revealed a marked reduction in genus richness following feedstock

*Correspondence:
Giovanna Pesante
giovanna.pesante@univr.it
Full list of author information is available at the end of the article

filtration and the progressive dominance of specialised PHA-storing genera, indicating effective selection pressure under feast–famine operation and supporting the observed improvements in polymer accumulation.

Conclusions Overall, the results indicate that MMC-based PHBV production from agri-food residues can achieve polymer contents comparable to those reported for similar systems using complex substrates, while highlighting the importance of feedstock pretreatment and process control. The study provides insights into the integration of acidogenic fermentation with MMC-based PHA production, and identifies operational aspects that require further optimisation to enhance process robustness and product consistency.

Keywords Polyhydroxyalkanoates, Hydroxyvalerate, Carboxylic acids, Agricultural residues, Cow manure, Mixed microbial cultures, Waste valorisation

Introduction

The agri-food sector generates a great quantity of residues each year from both primary production (agricultural activities such as crop cultivation and livestock farming, generating residues like straw or manure) and food processing activities (industrial transformation of raw materials into food products, generating residues such as peels, pulp, or wastewater) [1]. These residues include by-products (secondary outputs with potential further use, e.g., bran from milling), co-products (multiple valuable outputs generated simultaneously, e.g., oil and meal from oilseed processing), and wastes (materials with no immediate economic value requiring disposal, e.g., spoiled food or processing sludges). Collectively referred to as agri-food residues, they are frequently mismanaged, leading to high disposal costs and posing risks to human health and the environment [2, 3]. Global estimates indicate that agricultural residues may represent up to 50% of the fresh biomass harvested [4], amounting to nearly 3700 million tonnes annually, while the European Union alone produces more than 950 million tonnes [5]. In addition, approximately one-third of the food produced globally is lost or wasted along the food chain, resulting in further co- and by-product generation [6, 7]. Within the framework of a circular bioeconomy, these residues should be regarded not as waste but as a valuable source of renewable carbon that can be transformed into high-added-value biomolecules and materials [1, 8]. Their efficient utilisation may contribute to mitigating greenhouse gas emissions from the agricultural sector—currently estimated to represent about 11% of the European Union's total carbon footprint [9]—and to reduce dependency on fossil-based raw materials and energy-intensive production processes.

Among the wide range of bioproducts that can be obtained from agri-food residues—such as proteins, polysaccharides, organic acids, antioxidants and biofertilisers [10, 11]—biopolyesters like polyhydroxyalkanoates (PHA) have attracted particular interest. PHA are intracellular storage polymers synthesised by numerous microorganisms as energy and carbon reserves under unbalanced nutrient conditions [12, 13]. These polymers, accumulated in the form of cytoplasmic granules known as carbonosomes [14], display a wide spectrum of physicochemical properties depending on their monomeric composition, making them suitable alternatives to conventional plastics [15]. Poly(3-hydroxybutyrate) (PHB), composed solely of the 3-hydroxybutyrate monomer, is typically brittle and thermally unstable [16]; therefore, its applications are mainly restricted to rigid biomedical devices, bioresorbable sutures, and controlled drug-delivery systems where dimensional stability and slow degradation are required [17, 18]. On the contrary, copolymers such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) possess greater flexibility and processability due to the presence of hydroxyvalerate (HV) units [19], making them more suitable for flexible packaging films, disposable consumer products, agricultural mulching films, and tissue-engineering scaffolds [18, 20]. Overall, PHB is preferred for applications demanding rigidity and mechanical stability, whereas PHBV is favoured when compliance, toughness, flexibility and industrial processability are critical [19, 21].

PHAs can be produced using pure or mixed microbial cultures, each approach offering specific advantages and limitations. Industrial production is currently dominated by pure-culture systems, which enable high polymer yields and consistent product quality [21]. However, mixed microbial cultures (MMC) present several important benefits, including the ability to operate without sterilisation, higher process resilience, and the potential to utilise waste-derived feedstocks [15, 22]. These features translate into environmental advantages, since the use of organic waste streams avoids the upstream impacts associated with the production of refined substrates, while non-sterile operation reduces energy demand and process inputs. Consequently, MMC-based systems can reduce key life-cycle impact categories, such as global warming potential and cumulative energy demand, compared with conventional pure-culture processes [23–25] This aspect is particularly relevant in the context of a sustainable circular economy, as it enables the valorisation

of complex, real-world waste streams, such as animal manures or crop residues, that are otherwise costly to manage [26]. At present, to the best of our knowledge, one of the only commercially operating processes specifically producing PHBV via MMC from organic residual streams is offered by Paques Biomaterials (https://www.paquesbiomaterials.nl/). The Dutch company has developed a technology that uses non-genetically modified MMC to accumulate PHA from wastewater and other organic side streams (e.g., food industry, paper industry, sewage sludge) as feedstock [27].

Despite their promise, using agri-food residues as carbon sources for PHA production remains technically challenging. These substrates typically exhibit compositional variability, nutrient imbalances and the presence of inhibitory compounds that may hinder microbial activity and polymer accumulation [21, 28]. Nonetheless, several studies have shown that fermentation effluents derived from manure or crop-processing residues can be rich in carboxylic acids (CA) [29]—key precursors for PHA biosynthesis—thereby offering a viable route for their bioconversion into value-added polymers [19, 30, 31]. Cow manure, for instance, has a high potential since it is very abundant: there are around 1.57 billion cattle worldwide [32], which have an estimated manure production potential of at least 3 billion tons per year [33]. Utilising these real feedstocks not only improves the environmental footprint of PHA production but also enhances its techno-economic feasibility, aligning with current European strategies for waste valorisation and carbon neutrality [34].

In this context, the present study investigates the valorisation of agri-food residues as substrates to produce PHBV using mixed microbial cultures. Attention is given to assessing how variations in feedstock composition affect the incorporation of the HV monomer, with the objective of producing PHAs suitable for agricultural applications. The experimental work presented here is a continuation of a previous study [35], in which PHBV production by MMC was demonstrated using model CA-based synthetic media, with the HV content of the polymer effectively controlled by adjusting the ratio of propionate and valerate in the feed. That study reported PHBV with HV content ranging from 7.1 to 53.1% w/w, and maximum polymer accumulation of approximately 50.0–55.0% w/w under optimal conditions. These results established baseline strategies for achieving targeted HV content and high PHA storage yields in MMC systems fed with defined substrates. In contrast, the present work extends these findings to real agri-food residues, which exhibit compositional variability, nutrient imbalances, and potential inhibitors. We examine how such substrate heterogeneity influences HV incorporation and assess process performance using real waste streams rather than model media. While several studies have investigated PHA production from waste-derived substrates using mixed microbial cultures, most have focused on maximising polymer yield, with limited attention to controlling monomer composition, particularly HV content, under variable and complex feedstock conditions [36, 37]. This shift from synthetic to real residues, including evaluation of operational challenges and polymer quality under more variable conditions, represents an important step towards practical waste valorisation and broader applicability of MMC-based PHBV production. Therefore, the novelty of this work lies in demonstrating the simultaneous production of PHBV with controlled HV content and high accumulation using real, compositionally variable agri-food residues, while addressing the operational constraints associated with non-defined substrates.

Materials and methods

Experimental setup

PHA production (illustrated in Fig. 1) was performed using three reactors: (1) a fed-batch anaerobic reactor (FBR) to produce a fermentation fluid rich in CAs from agri-food residues; (2) a sequencing batch reactor (SBR1) for the selection of the PHA-producing bacteria consortium starting from MMC; (3) a second SBR (SBR2) to maximise the content of PHA in the bacterial cells (accumulation phase). Details on these three bioreactors' characteristics and on their respective working parameters are given in the following paragraphs. A multiparameter controller (Lange SC1000, Hach Italia) operated the peristaltic pumps during each phase of the reactors' cycles according to a pre-set programme. Dissolved oxygen (DO) levels were monitored using DO probes (Hach LDO sc Probe, Model 2) in SBR1 and SBR2, and the temperature was maintained at a constant level with water heaters (Klima Evo IPX8, Amtra, Italy). The reactors were aerated and mixed through air pumps and diffusers, whereas the FBR was operated under anaerobic conditions and mixed by mechanical agitation. Samples from the three reactors were analysed regularly to assess performance through the following parameters of the bioreactors' influents and effluents: pH, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), chemical oxygen demand (COD), CA, and PHA content (methodologies explained below). Due to the nature of continuous reactor operation, no biological replicates were performed; instead, data are reported as mean values ± standard deviation based on three measurements ($n = 3$) collected over stable operational periods.

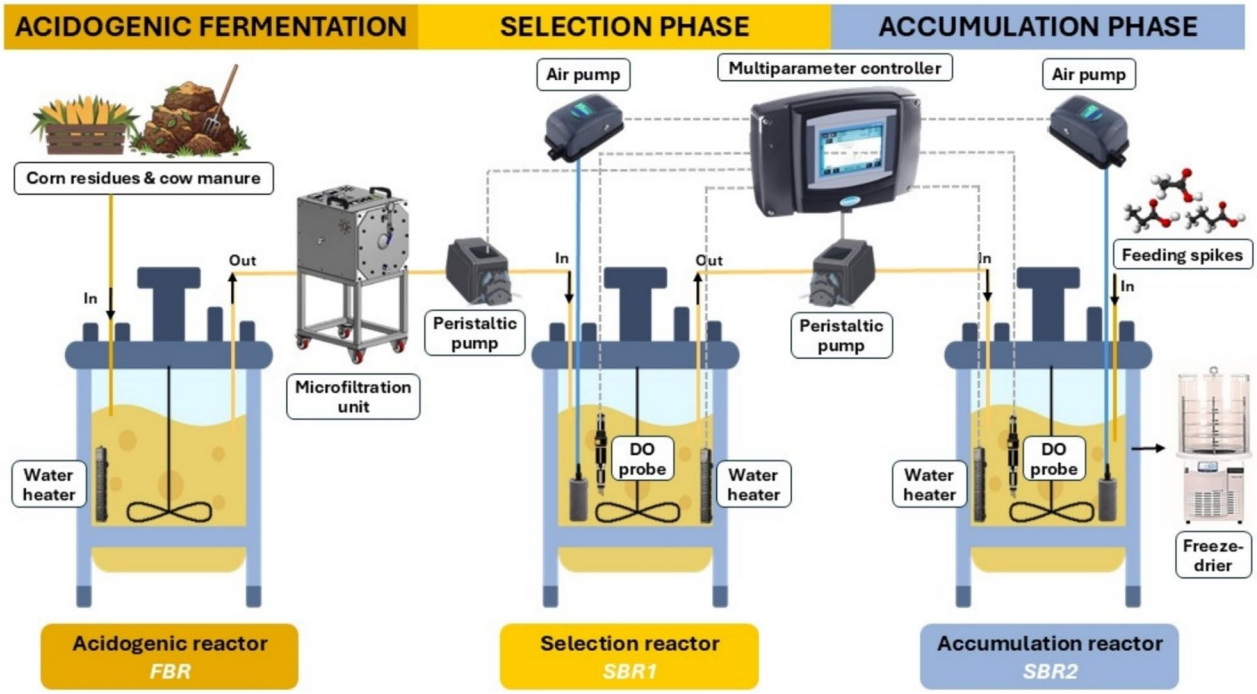

Fig. 1 Schematic representation of the experimental setup for PHA production. DO: dissolved oxygen; SBR: sequencing batch reactor; FBR: fed-batch reactor

Feedstock and acidogenic fermentation phase (FBR)

The experimental work was divided into two consecutive operational periods (referred to as setup 1 and 2), between which the selection reactor was reseeded to initiate the next operational period. During both periods, the feedstock used to run both SBR1 and SBR2 originated from the acidogenic conversion of agri-food residues. Agricultural digestate from a local anaerobic digestion plant was used as inoculum, and was added only at the beginning of operations and when the reactor was re-inoculated after the first period. The substrate (whose characterisation is shown in Table 1) consisted of cattle slurry and corn residues (maize silage obtained by anaerobic fermentation of chopped corn biomass, compacted and stored under oxygen-free conditions) at the ratio of 20:80, 50:50 or 40:60 (on a VS basis) depending on the experimental period. The mixture ensured a proper C/N balance and helped prevent ammonia-induced inhibition [38, 39]. The fermentate was produced in a semi-continuous plexiglass reactor (total volume 35 L, working volume 30 L) equipped with mechanical agitation, whose operational parameters are listed in Table 2. The reactor was maintained at the upper mesophilic range of 38 °C to enhance acidogenic activity and carboxylic acid production from complex substrates [40], and the pH was checked daily to confirm that the conditions fell within the optimal range for dark fermentation (5.5–6.5) (Tagne et al. 2024). The unit was operated with daily withdrawal and fresh feeding, with a hydraulic retention time (HRT) between 2.86 and 5.00 days, chosen to promote acidogenesis while suppressing methanogen activity due to their high abundance in cattle slurry [41]. The organic loading rate (OLR) varied from 9.2 to 12.4 g COD/Ld. Total solids were kept below 5% to prevent mixing limitations throughout the process. The produced fermentate was adjusted, when necessary, with synthetic CAs to

Table 1 Characterisation of the bacterial inoculum (digestate) and the agri-food residues utilised as feedstock of the fermentation reactor

	TS (% ± SD)	TVS (% ± SD)	TVS/TS (% ± SD)	COD (gCOD/KgTS ± SD)
Digestate (inoculum)	6.3 ± 0.1	3.7 ± 0.2	58.9 ± 0.2	26.5 ± 0.3
Corn residues	33.5 ± 0.3	31.9 ± 0.2	95.3 ± 0.3	772.1 ± 5.9
Cattle slurry	5.4 ± 0.1	4.1 ± 0.1	57.3 ± 0.1	883.4 ± 23.94

TS: total solids; TVS: total volatile solids; COD: chemical oxygen demand

Table 2 Operational parameters for the fermentation reactor

	14/10/24 to 17/12/24	13/01/25 to 10/02/25	11/02/25 to 20/03/25	21/03/25 to 13/06/25
Setup	**1**	**2**	**2**	**2**
Parameters	**Value**			
Volume (L)	30	28	28	20
HRT (d)	3.00	4.00	4.00	2.86
OLR (gCOD/Ld)	12.46	9.53	9.23	9.23
TS (%)	5	5	5	5
Feed volume (L/d)	10	7	7	7
Feed (VS) (gVS/d)	377.27	277.14	277.14	277.14
Cow slurry (%)	20 (days 1–17), then 50	50	40	40
Corn residues (%)	80 (days 1–17), then 50	50	60	60
Cow slurry VS (gVS)	188.64	138.57	110.86	110.86
Corn residues VS (gVS)	188.64	138.57	166.29	166.29
Water (g/d)	3655.25	4051.90	4467.97	4467.97

match a total CA concentration of 10.0 g COD/L and a CA distribution that emulated the composition typically obtained from the fermentation of agro-residual biomass (75% acetic, 13% propionic, and 12% butyric acid) [42]. This CA distribution was also preferred because it enabled obtaining a PHA with the targeted HV content, as previously demonstrated with the same experimental setup [35]. In practice, the adjustment primarily involved the addition of acetic acid, which was frequently below the target concentration in the fermentate compared to the desired level of 7.32 g COD/L, whereas propionic and butyric acids generally required limited or no supplementation. Prior to using it in the SBR1, the produced fermentate was centrifuged (3900 rpm, 8 min, Eppendorf centrifuge 5810R) and, for setup 1, the clarified supernatant was supplied to both SBRs without further filtration. During setup 2, filtration at a nominal size of 0.22 µm was performed using a rotating ceramic membrane system (model MMSL LAB from Ju.cla.s Srl, Italy); this step was necessary to eliminate suspended solids, bacteria and the non-biodegradable particulated carbon fraction found in the fermentation fluid.

Selection phase (SBR1)

The enrichment of PHA-accumulating mixed microbial cultures was carried out in a 28-L sequencing batch reactor, following the same two-setup operational procedure as for the FBR. Operational conditions are reported in Table 3. SBR1 was maintained at mesophilic conditions of 25 °C using a water-heating system (Klima Evo IPX8, Amtra, Italy) to promote stable microbial selection [40]. Its HRT was 3.28 days, and the solid retention time equalled the HRT, since no settling period was applied.

Table 3 Operational parameters for the selection SBR

Parameters	Value
Ideal feast/famine ratio (F/F)	0.2
Cycle length (h)	6
Feast phase length (h)	1
Famine phase length (h)	5
N° cycles/d	4
Hydraulic retention time (d)	3.00
Reactor volume (L)	28
Feed volume (L/cycle)	2.33
Feed volume (L/d)	9.32
Feedstock concentration (g COD/L)	9.7
COD_{IN} (g/cycle)	22.6
COD_{IN} (g/day)	90.4
Organic loading rate (kg COD/m^3d)	3.28
Temperature (°C)	25.0
pH	8.0–9.0

The pH was monitored but when left uncontrolled, it generally fluctuates between 8 and 9.

The inoculum consisted of activated sludge collected from a municipal wastewater treatment plant located in Verona (Italy), added to the reactor to obtain a starting concentration of 3 gTS/L. Enrichment of PHA-storing microorganisms was achieved by imposing cyclic feast–famine (F/F) conditions [43]. Four 6-h cycles were run each day, consisting of a 1-h feast phase [36], where the total substrate was supplied as a single, rapid feed (approximately 2 min), followed by a consumption phase during which the microbial community depleted the available substrate, and a subsequent 5-h famine period,

corresponding to an F/F ratio of 0.2. This value has been described as optimal for selecting microorganisms that preferentially channel carbon towards PHA storage, whereas F/F ratios above 0.55 are known to favour biomass growth [15].

The fermentation fluid supplied to SBR1 was supplemented, when required, with ammonium bicarbonate to adjust the COD:N:P ratio to 100:10:1.

Accumulation phase (SBR2)

A 5-L fed-batch system was used to maximise the PHA content within the biomass generated during the selection step. The reactor operated at room temperature (approximately 22 °C) and was equipped with both aeration and a dissolved oxygen (DO) probe (Hach LDO sc Probe, Model 2). The increase in DO was used as an indicator of CA depletion due to declining microbial oxygen uptake.

Each accumulation run was initiated with biomass taken from the selection reactor at the end of the famine phase. The carbon feed supplied during this stage had the same CA composition as the selection feed but was not adjusted with ammonium bicarbonate, thereby favouring storage over growth. A multi-pulse feeding regime was adopted, with spikes of fermentate consisting of 1.0 g COD_{CA}/L. Individual accumulation experiments lasted 5–6 h, and additional substrate pulses were introduced each time a rise in DO signalled carbon exhaustion. On average, 5 or 6 spikes were added for each accumulation test.

At the end of each accumulation run, the broth was acidified to pH 1–2 with concentrated sulfuric acid (98%, w/v) to halt microbial activity and prevent intracellular PHA degradation. Biomass was harvested by centrifugation at 3900 rpm for 20 min and washed four times with distilled water. The resulting pellet was freeze-dried (Lio 5P, CinquePascal Srl, Italy), manually ground, and stored at − 80 °C for subsequent analyses.

Analytical methods

PHA quantification and polymer characterisation

PHA content and monomer composition (3HB and 3HV) were determined following the method of Braunegg et al. [44]. Dried biomass (2–5 mg) was subjected to acid-catalysed methanolysis to convert polymeric 3-hydroxyacyl units into their corresponding methyl esters, which were then analysed by gas chromatography. One microliter of the organic phase was injected into an Agilent 8860 GC equipped with an HP-5 column (30 m × 320 µm × 0.25 µm). Nitrogen was used as the carrier gas at a flow rate of 10 mL/min. The oven temperature program consisted of an initial hold at 100 °C for 1 min, followed by heating to 130 °C at 12 °C/min and then to 250 °C at 25 °C/min. The flame ionisation detector operated at 320 °C. Quantification of 3HB and 3HV was performed using a PHBV standard containing 12% (w/w) 3HV (Sigma-Aldrich), with benzoic acid as the internal standard.

The molecular weight distribution (weight-average molecular weight, Mw, and number-average molecular weight, Mn and the polydispersity index, PDI = Mw/Mn) was determined for the polymer extracted from the dried biomass using chloroform and methanol, following the method described by Braunegg et al. [44]. After extraction at 70 °C for 4 h, the solution was filtered, and PHAs were precipitated by adding methanol (5 mL per mL of chloroform). The precipitate was isolated by filtration, transferred onto glass Petri dishes, and left to evaporate overnight. The purified polymer was analysed by gel permeation chromatography (GPC) equipped with a JASCO PU-4180 pump, a guard column, two TSKgel® columns (G6000-HHR and GMHHR-H), a JASCO CO-4060 column oven, and a JASCO RI-4030 detector. Chloroform was used as the eluent at a flow rate of 1 mL/min. Column and detector temperatures were set to 40 °C and 35 °C, respectively. Calibration was performed using polystyrene standards (0.013–3.05 × 10^6 g/mol).

Solids, chemical oxygen demand and CA measurements

MLSS, MLVSS and COD were measured according to standard methods [45]. Prior to soluble-phase analyses, samples were filtered through 0.45-µm membranes. CAs were analysed by ion chromatography (Dionex™ ICS-1100, Thermo Fisher Scientific) equipped with an IonPac ICE-AS1 column, following procedures described by Raposo et al. [46]. Individual CAs (acetic, propionic, butyric, isobutyric, pentanoic, isopentanoic and hexanoic acids) were quantified, and total CA values were obtained as their sum.

Standard errors for all stoichiometric and kinetic parameters were calculated via conventional error propagation formulas.

Microbial community analysis

Changes in the bacterial community within the selection reactor were monitored throughout the experimental period. A total of 11 samples were collected. The first sample consisted of centrifuged fermentation fluid obtained from the FBR, whereas the remaining samples were collected from SBR2 at approximately 2- to 3-week intervals. Samples 2 to 4 correspond to operations with fermentation fluid subjected only to simple centrifugation. Samples 5–11 represent the experimental phase in which an additional 0.22-µm filtration step was introduced prior to feeding, to prevent the introduction of external bacteria into SBR2 that could disrupt the

microbial balance established through the speciation process.

Two millilitres of each sample were centrifuged at maximum speed for 2 min, and the pellet was sent to BMR Genomics (Padua, Italy) for 16S rRNA gene amplicon sequencing (www.bmr-genomics.it). DNA extraction was performed using the DNeasy® 96 PowerSoil® Pro QIAcube® HT Kit (Qiagen), followed by purification with the QIAcube HT system. The V4 region of the 16S rRNA gene was amplified using universal primers suitable for bacteria and archaea [47]. Amplicons were purified using Thermolabile Exonuclease I (NEB). Library preparation was performed using the Nextera XT DNA Library Preparation Kit (Illumina). Afterwards, the samples were normalised, pooled, and sequenced on an Illumina MiSeq platform. Sequence quality was assessed using PASTQC, and taxonomic assignment was performed through the QIIME2 pipeline against the Greengenes 13_8, Silva 132, and RDP reference databases. Community analyses focused on genus-level dynamics; unknown or uncultured taxa were excluded, and for each sample only the ten most abundant genera were considered.

Calculations

The feast-to-famine ratio (F/F) was calculated as:

1.

$$\frac{F}{F}\left(\frac{\text{min}}{\text{min}}\right) = \frac{\text{Feast phase length}}{\text{Faminephaselength}}.$$

The food-to-microorganism ratio (F/M) was computed as the daily CA load (LCA, expressed as g COD) divided by the average mass of MLVSS (MMLVSS):

2.

$$\frac{F}{M}\left(\frac{g}{g} * d\right) = \frac{L_{\text{CA}}}{M_{\text{MLVSS}}}.$$

COD removal per cycle was determined using influent and effluent CA-COD concentrations:

3.

$$\text{COD}_{\text{removal}}(\%) = \frac{\text{COD}_{\text{IN,CA}} - \text{COD}_{\text{OUT, CA}}}{\text{COD}_{\text{IN,CA}}} * 100.$$

The daily biomass growth yield ($Y_{\text{MLVSS/S}}$) was obtained as:

4.

$$Y_{\text{MLVSS/S}}(\text{gMLVSS/gCOD}) = \frac{\text{MLVSS}}{\text{COD}_{\text{IN, CA}} - \text{COD}_{\text{OUT, CA}}}.$$

PHBV purity was calculated from the sum of 3HB and 3HV masses relative to the input dried biomass (m_{dw}):

5.

$$\text{PHBV}\left(\%\frac{w}{w}\right) = \frac{(\text{m3HB+m3HV})}{\text{m}_{\text{dw}}}.$$

3HV content within the polymer was derived as:

6.

$$\text{3HV}\left(\%\frac{w}{w}\right) = \frac{\text{m3HV}}{(\text{m3HB} + \text{m3HV})}.$$

The storage yield ($Y_{\text{PHA/CA}}$) was obtained as:

7.

$$Y_{\text{PHA/CA}}(\text{gCODPHA/gCODCA}) = \frac{(\Delta\text{COD}_{\text{PHA}})}{(\Delta\text{COD}_{\text{CA}})}.$$

Stoichiometric conversion factors were: 1.67 g COD/g for 3HB and 1.92 g COD/g for 3HV.

Results and discussion

Carboxylic acid production by acidogenic fermentation

The FBR was operated for 109 days during setup 1 and 151 days in setup 2, with reinoculation between the two operational periods. The obtained results are therefore robust, as system performance was maintained over several consecutive solids retention times (SRTs), a commonly used indicator of process stability in MMC systems [48]. Figure 2 shows the pH values and CA production for the first (top figure) and second (bottom figure) setups. During the first 13 days of setup 1, the fermenter was fed exclusively with water and corn residues. Throughout this initial acclimation phase, only the pH was monitored, which decreased from 7.5 to 6.5. On day 14, cattle slurry was introduced into the substrate at a 20:80 ratio (on a VS basis) relative to corn residues. After day 17, the proportion of cattle slurry was increased to 50:50 and maintained until the end of setup 1, with the aim of valorising cattle slurry within a circular-economy approach. During setup 2, after a 6-day acclimation phase, the substrate ratio was maintained at 50:50. However, on day 29, the ratio was adjusted to 40:60 in response to a decrease in acetic acid production. The decline persisted, with acetic acid concentrations dropping to 0.45 g COD/L, likely due to fermentative pathways preferentially degrading the corn residues before they were added to the fermenter. To enhance acetic acid production, the hydraulic retention time (HRT) was therefore reduced on day 67 from 4.00 to 2.90 days by decreasing the working volume of the fermenter from 28 to 20 L. This operational adjustment successfully restored acetic acid production, which rapidly increased to the highest values of both setups.

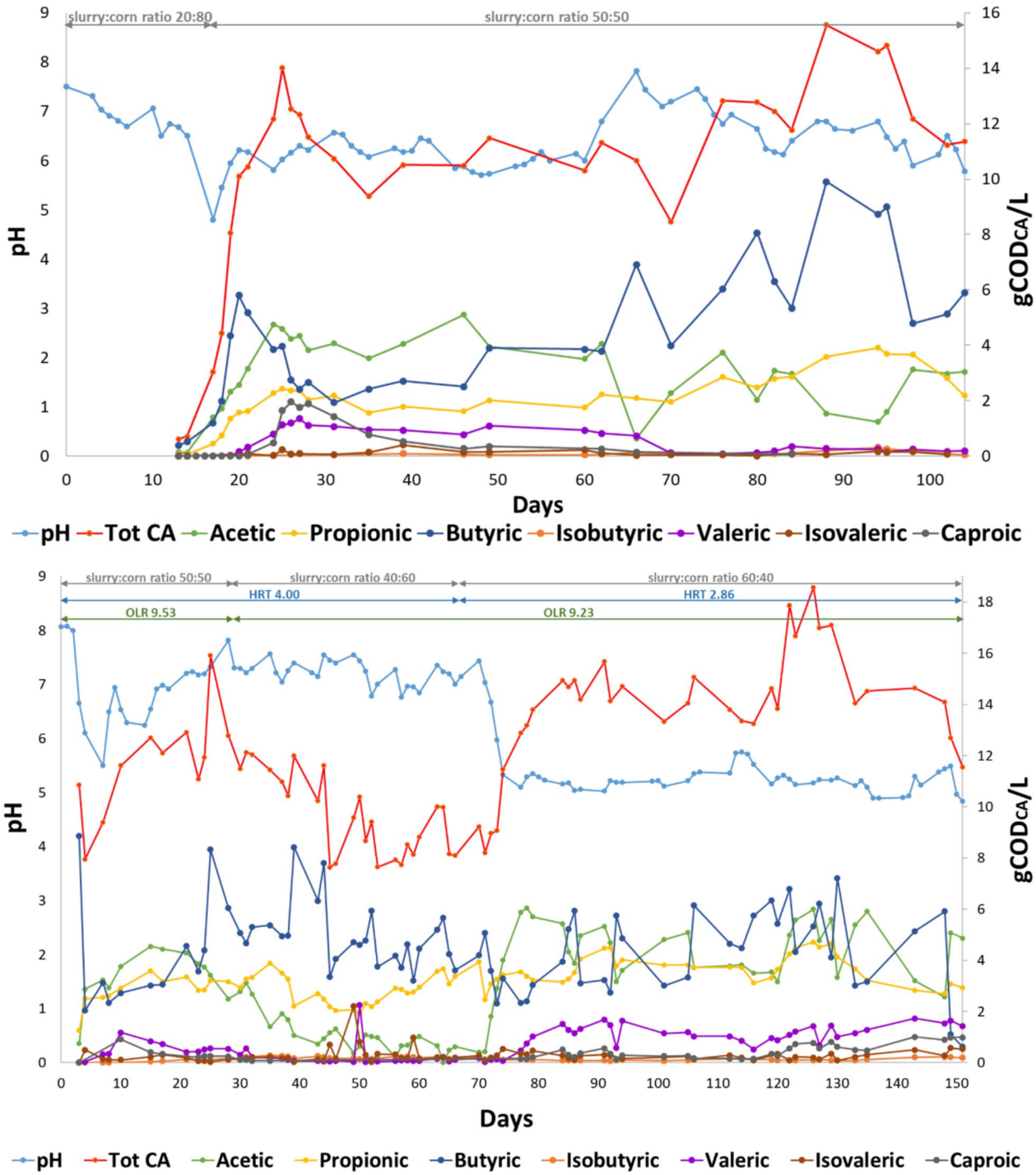

Fig. 2 pH values and CA (carboxylic acid) production for the acidogenic reactor (FBR). The top figure shows the results for setup 1, and the bottom figure shows those for setup 2

In setup 1, total CA production remained relatively stable throughout the operational period, reaching a maximum of 15.56 gCOD/L, with an average concentration of 11.63 ± 1.79 gCOD/L. These values are within the range reported for acidogenic fermentation of manure- and agro-residues-based systems, where CA concentrations typically range from 8 to 18 gCOD/L under mesophilic conditions, depending on HRT and pH control [49].

Although the acetic-to-butyric acid ratio varied over time, an increase in pH was generally associated with a shift towards higher butyric acid proportions relative to acetic acid, in agreement with observations reported by Kim et al. [50, 51]. In setup 2, total CA production could be divided into two distinct phases, likely associated with the HRT reduction implemented on day 67. During the first phase (HRT = 4.00 days), total CA concentrations reached up to 15.91 gCOD/L, with an average of 10.28 ± 1.97 gCOD/L. In the second phase (HRT = 2.86 days), CA production increased further, reaching a maximum of 18.57 gCOD/L and an average concentration of 13.83 ± 1.66 gCOD/L. Comparable increases in CA yields following HRT optimisation have been reported in semi-continuous fermenters treating manure-based substrates, where shorter HRTs enhanced hydrolysis and acidogenic activity [49]. In the first phase of setup 2, the acetic-to-butyric acid ratio was generally in favour of butyric acid, likely due to pH values above 5.5, which promote butyrate-type fermentation pathways [50]. In contrast, during the second phase, the acetic-to-butyric acid ratio was more variable, likely reflecting the concurrent fluctuations in pH observed after the HRT adjustment.

Overall, across both setups, acetic, propionic and butyric acids were the predominant CAs produced (although their relative proportions varied over time), whereas caproic, valeric, isovaleric and isobutyric acids contributed only minor fractions to the total CA composition. This distribution is consistent with the findings of Cavinato et al. [52], who reported a similar CA profile when treating a comparable feedstock under analogous operational conditions, particularly temperature and HRT.

From a process-design perspective, stable CA production in MMC-linked systems requires: (i) controlled substrate ratios; (ii) careful optimisation of HRT to balance hydrolysis and acidogenesis; (iii) appropriate feed conditioning steps such as removal of suspended solids from the fermentation effluent prior to its use in the downstream SBR reactors (e.g., filtration or centrifugation) to avoid biomass and inhibitor carryover; and (iv) gradual rather than abrupt operational changes, since sudden shifts can destabilise microbial pathways and CA profiles [40].

Selection phase

The SBR1 operated for a total of 216 days, comprising 70 days under setup 1 and 146 days under setup 2 (Fig. 3). The prolonged experimental period allowed for obtaining robust results.

During setup 1, the HRT was maintained at 3.65 days, whereas in setup 2 it was generally kept at 3.03 days, with minor deviations between days 86 and 104 due to the reactor's pump clean-up activities (Fig. 3, top panel). The OLR remained relatively stable during setup 1, ranging from 3.10 to 3.70 kgCOD m^{-3} d^{-1}. In contrast, larger OLR fluctuations were observed during the second setup (3.32–6.08 kgCOD m^{-3} d^{-1}), likely attributable to variations in the CA composition of the fermentation effluent used as feed for SBR1, despite corrective measures such as dilution or supplementation with synthetic CA being taken when necessary. OLR ranges of this magnitude are consistent with MMC selection systems reported in the literature (2–8 kgCOD m^{-3} d^{-1}), where stable PHA enrichment has been achieved under similar feast–famine regimes [53, 54]. The F/F ratio remained stable within the range 0.1–0.3, indicating favourable conditions for the selection of PHA-producing microorganisms [43, 55]. Occasional increases in the F/F ratio, caused by aeration issues that extended the feast phase, were addressed by cleaning or replacing the air diffuser. MLVSS, an indicator of active bacterial biomass in the bioreactor, increased rapidly following the addition of centrifuged fermentate, reaching a maximum concentration of 10.3 g/L. These values are comparable to those reported during the previously published experimental phase, in which SBR1 was fed with real substrate [35]. The observed biomass increase is likely attributable to the introduction of additional microbial biomass and suspended solids with the fermentation fluid, as previously reported in similar systems [56]. Similar biomass increases in selection reactors fed with waste-derived substrates have been reported by Valentino et al. [37], who observed rapid biomass build-up due to both microbial growth and solids carryover from complex feeds. However, after approximately 30 days of operation, MLVSS concentrations declined progressively despite no other relevant operational parameter changes during this period, reaching approximately 4.3 g/L. These findings may be associated with the progressive acclimation and selection of the microbial community for the complex and heterogeneous substrates present in the solid fraction of the fermentate. In particular, the presence of particulate and slowly biodegradable compounds may have limited substrate availability for rapid growth, while favouring maintenance metabolism over biomass accumulation [57]. In addition, the continuous input of unfiltered fermentate, containing suspended solids and exogenous microorganisms from the acidogenic phase, may have contributed to a partial destabilisation of the MMC in SBR1, by interfering with the selection pressure imposed by the feast–famine regime. Such disturbances have been reported to affect microbial community structure and reduce the enrichment efficiency of PHA-storing populations [36, 58]. In setup 2, the filtration step markedly reduced the

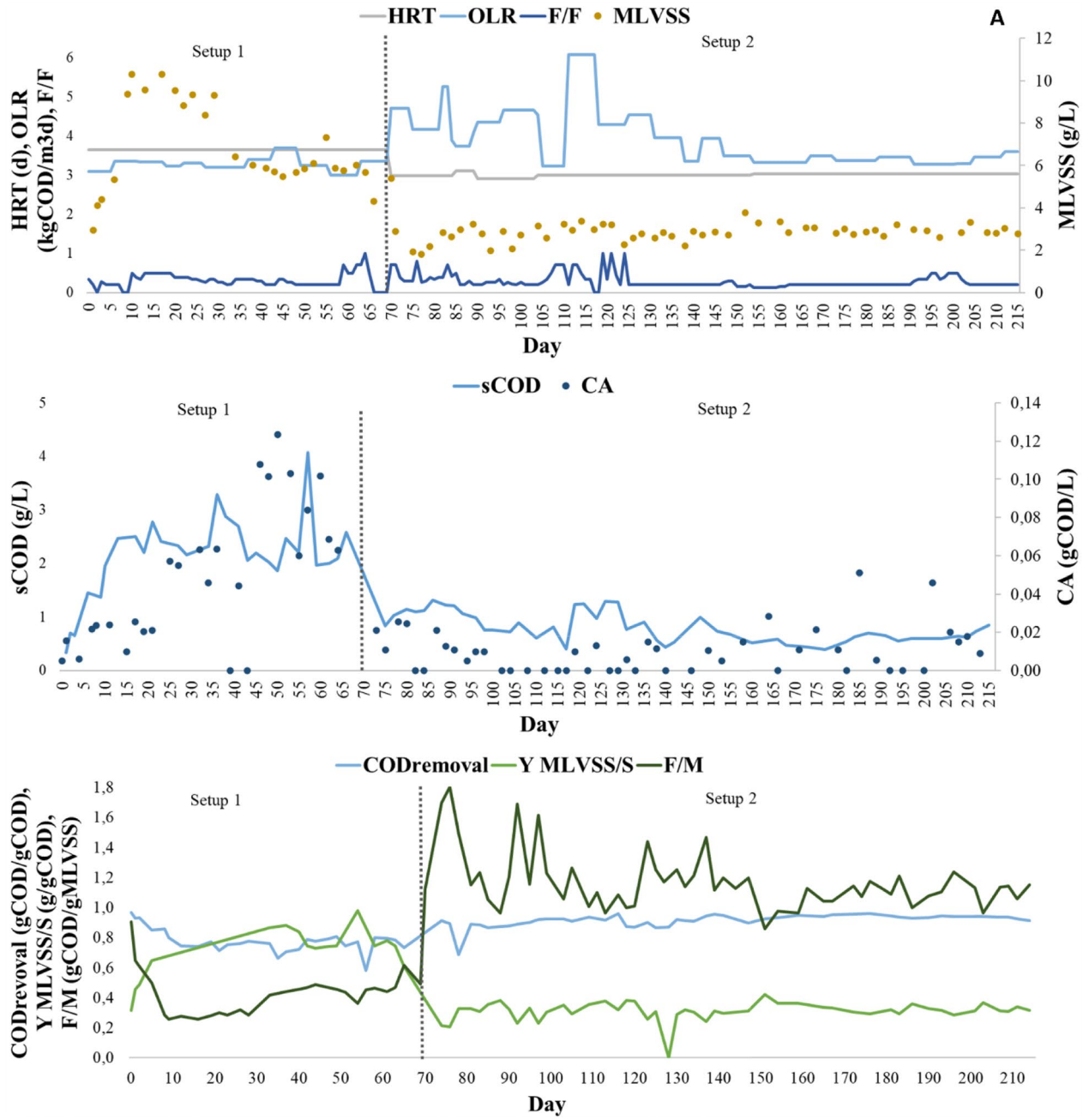

Fig. 3 Performance of the selection reactor across setups 1–2. Top panel: hydraulic retention time (HRT), organic loading rate (OLR), feast/famine ratio (F/F) and mixed liquor volatile suspended solids (MLVSS). Middle panel: carboxylic acids (CA) and soluble chemical oxygen demand (sCOD) concentrations. Bottom panel: COD removal, daily biomass growth yield ($Y_{MLVSS/S}$) and food-to-microorganism ratio (F/M)

introduction of exogenous biomass and particulate matter into the reactor. As a result, MLVSS concentrations remained relatively stable throughout the experimental period, averaging 2.8 g/L, suggesting improved control over microbial selection and process stability.

Carboxylic acid concentrations in the SBR1 effluent remained close to zero for most of the operational period (Fig. 3, middle panel), indicating efficient uptake and utilisation by the microbial community. In contrast, during the latter phase of setup 1, CA concentrations increased slightly, concomitantly with the observed decrease in biomass concentration. The soluble chemical oxygen demand (sCOD) concentration was also low in setup 2, further confirming the effective assimilation

of the supplied organic substrates by the microbial biomass. Conversely, sCOD was consistently higher in setup 1, likely due to the presence of a non-biodegradable or slowly biodegradable fraction (soluble or colloidal organic compounds) introduced with the fermentation fluid, such as recalcitrant intermediates or soluble microbial products, which may not be readily assimilated and can accumulate in the liquid phase [59, 60].

As expected, COD removal values were generally inversely related to sCOD concentrations (Fig. 3, bottom panel), as higher COD removal corresponds to lower sCOD levels, due to its consumption for bacterial metabolism. Biomass growth yield increased in setup 1 following the use of the real substrate after centrifugation, concomitantly with a decrease in the F/M ratio (Fig. 3, bottom panel). Despite some variability observed over time, this trend can likely be attributed to the increase in MLVSS, resulting from the additional microbial biomass contributed by the fermentation fluid [56]. Similar trends were observed by Magonara et al. when switching from a synthetic to a real, non-filtered substrate [35]. Conversely, the filtration step implemented in setup 2 led to a reduction in biomass growth yield and a concomitant increase in the F/M ratio.

Accumulation batches, PHA composition and its characteristics

A total of 38 accumulation batches were performed during the study period, yielding 255.5 g of lyophilised PHA-rich biomass. As illustrated in Fig. 4, the storage yield, representing the efficiency of conversion of CAs into PHAs, was lower in the first period compared to the second one, with a mean value of 0.33 ± 0.23 $gCOD_{PHA}/gCOD_{CA}$ in setup 1 and of 0.78 ± 0.27 $gCOD_{PHA}/gCOD_{CA}$ in setup 2. This improvement is attributable to more favourable conditions for PHA production in the accumulation reactor during this period, including efficient speciation towards PHA producers, as discussed in paragraph 3.4. Overall, the storage yields in setup 1 were in line with those obtained with the same experimental arrangement in the previously published manuscript [35] and also with similar work performed using MMC and other low-cost substrates such as molasses [61] and fermented paper mill wastewaters [62], both characterised by the presence of complex carbon substrates. The values of setup 2 are similar to the yields reported for more complex feedstocks, such as fermented food waste [63], sludge hydrolysate [55] and candy factory effluent or municipal sludge [64]. In addition to storage yield, PHA titre was evaluated to provide a quantitative measure of polymer production. PHA concentrations followed a similar trend to intracellular accumulation, with higher values achieved in setup 2 (1.70 ± 0.44 g/L) compared to setup 1 (0.81 ± 0.33 g/L, Fig. 4), reflecting improved substrate conversion and biomass storage capacity under filtered feedstock conditions. The obtained PHA titers are consistent with values reported for MMC systems fed with waste-derived substrates. For example, batch studies using mixed cultures and CA mixtures report relatively low PHA concentrations, typically in the range of 0.1–0.3 g/L [65]. In more complex systems using real waste streams, such as organic fraction of municipal solid waste or pulp industry effluents, PHA titers generally range between 0.5 and 2.0 g/L, depending on substrate concentration and process configuration [22, 66]. Similarly, studies using fermented manure or wastewater-derived substrates report PHA concentrations typically below 1.5 g/L, despite achieving relatively high intracellular contents [67]. These values remain significantly lower than those obtained in pure-culture systems, where titers often exceed 10–50 g/L under optimised conditions [23]. In this context, the PHA titers obtained in the present

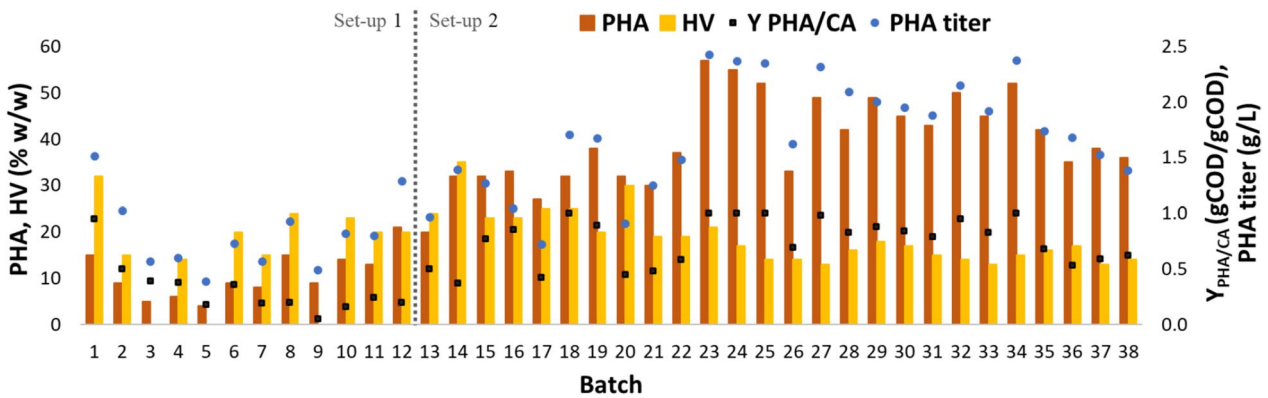

Fig. 4 PHA storage yield, PHA titre, intracellular PHA and HV content of the biomass obtained from the accumulation bioreactor. Accumulation batch numbers 3, 5 and 9 have no value for HV content due to problems during data acquisition

study fall within the expected range for MMC-based processes treating dilute and variable waste streams, reflecting the well-known trade-off between the use of non-refined substrates and volumetric productivity.

As a consequence of the variation in storage yield, intracellular PHA content was low during setup 1 (mean 10.7 ± 5.0% w/w), likely due to the use of unfiltered fermentate [58]. In contrast, PHA accumulation increased markedly during setup 2, reaching values close to 60.0% (mean 39.8 ± 9.4% w/w). These values are at the high end or exceed typical values reported for MMC-fed complex or waste-derived substrates. Numerous studies using MMCs with real feedstocks such as agricultural residues, CAs from waste fermentation, or wastewater, generally report PHA contents of ~ 30.0–50.0% (w/w) under standard enrichment and accumulation conditions [68, 69]. Higher values, similar to or exceeding those in setup 2, have been reported only under highly optimised or non-standard conditions such as high salinity or specifically enriched cultures [70, 71]. These comparisons indicate that the accumulation levels in setup 2 compare favourably with the literature and are notable for processes using real complex substrates.

The hydroxyvalerate (HV) monomer fraction also exhibited temporal variability throughout the study, with more pronounced fluctuations observed during setup 1 (mean 20.3 ± 5.6% w/w). During setup 2, higher HV contents were recorded in the initial phase (mean 24.0 ± 4.9% w/w), followed by lower but more stable levels in the second phase (mean 15.1 ± 1.7% w/w). Overall, the HV content largely fell within the target range established by the AgriLoop project (25.0–35.0% w/w), which confers thermochemical properties suitable for downstream applications, such as the production of agricultural mulching films. Indeed, the incorporation of five-carbon or longer-chain monomers into the poly(3-hydroxybutyrate) backbone is known to significantly reduce polymer melting temperature and crystallinity [72], thereby improving mechanical properties, including flexibility and toughness, of the resulting bioplastic. Achieving elevated HV fractions was primarily accomplished in this study through the control of the carbon source composition: the use of carboxylic acids with odd carbon numbers, such as propionic and valeric acids, in preference to even-carbon CAs (e.g., acetic and butyric acids), promotes the synthesis of PHAs with higher HV content [30, 62, 73]. More broadly, the HV content is directly linked to the composition of CAs supplied to the culture, and several studies have demonstrated that adjusting the ratio of odd- to even-carbon CAs enables targeted manipulation of PHBV composition [36, 37]. In the present work, the use of real agri-food residues resulted in variable CA profiles that did not always match the desired composition, and therefore supplementation with selected CAs was applied to achieve the targeted HV range. This variability is consistent with previous studies showing that CA production during acidogenic fermentation is strongly influenced by substrate type and operating conditions such as pH, hydraulic retention time, and organic loading rate [37, 40]. Nevertheless, this approach highlights the potential for process integration, as optimisation of the upstream acidogenic fermentation could allow tailoring of CA composition directly from waste streams, thereby enabling controlled HV incorporation without the need for external adjustment. Further research is required to better understand and control these interactions under realistic operating conditions.

The molecular weight distribution of the extracted PHBV (determined for each setup by mixing the polymers produced from single batches) showed high weight-average molecular weights in both experimental setups (Mw = 772.9 kDa and 720.5 kDa for setups 1 and 2, respectively), which fall within the range (340.0–800.0 kDa) typically reported for PHBV produced by MMC fed with waste-derived substrates [19, 30, 64, 74]. Such high Mw values are generally associated with adequate mechanical strength and suitability for thermoplastic processing, including film formation and melt processing [18, 21, 75]. In contrast, the number-average molecular weight decreased from 368.7 kDa in setup 1 to 234.4 kDa in setup 2, resulting in an increase in polydispersity index from 2.0 to 3.1. PDI values around 2 are commonly reported for relatively homogeneous MMC-derived PHAs, whereas higher PDI values indicate broader chain-length distributions, often linked to increased heterogeneity in microbial populations, substrate composition, or polymer extraction conditions [19, 58, 64, 76]. The broader molecular weight distribution observed in setup 2 may therefore reflect the greater variability in polymer chain lengths under the optimised accumulation conditions, while still remaining within the range reported for PHBV obtained from complex and real feedstocks [30, 62]. Broader molecular weight distributions can influence thermal transitions and mechanical behaviour in polymer films, affecting crystallisation, melt viscosity, and drawability during processing [77].

Bacterial community analysis

Analysis of the bacterial communities in the fermentation fluid and SBR1 identified the ten most abundant bacterial genera across 11 sampling events spanning the study period (Fig. 5). These data highlight changes in bacterial composition during the selection process and in response to variations in feeding characteristics.

Sample 1 represents the centrifuged fermentation fluid produced from the FBR. Most genera detected

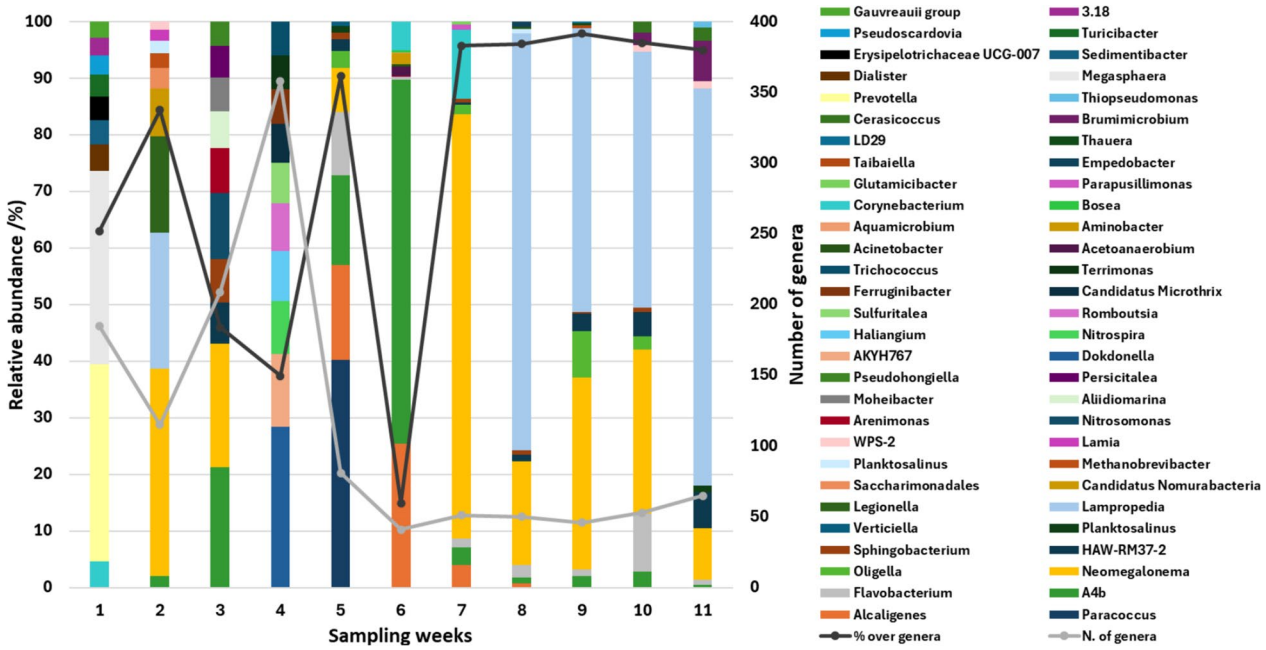

Fig. 5 Bacterial community analysis at the genus level in the selection bioreactor across the study period. The ten most abundant genera per sample were considered, with their cumulative abundance shown by the dark grey marker line. The light grey line represents the total number of genera per sample

in this sample were eubacteria (99.3%), differing from those found in the other samples, with the exception of the genus *Corynebacterium* (4.7% of the top 10 genera), which is known to include PHA-producing species [78]. This microbial community distribution is typical for wastewater treatment plant sludge, which was used as inoculum for the FBR [79, 80].

Sample 2 was collected from SBR1, after it had been fed for 42 days with centrifuged fermentation fluid (days 1–17 correspond to the experimental period reported in the previous publication by Magonara et al. (2025); days 18 onward are included in the present work). This sample suggests that speciation towards PHA-producing genera has already taken place, with *Neomegalonema* and *Lampropedia* being the most abundant among the top ten genera (36.7% and 24.1%, respectively); both genera are known to comprise PHA producer species [53].

Across samples 2 to 4, during which SBR1 was still fed with centrifuged fermentation fluid (setup 1), no clear stability in the bacterial community was observed. Both the identity and the relative abundance of the genera varied considerably across sampling events. Among the most abundant genera were both those known to include PHA-producers (e.g., *Dokdonella, Haliangium, Neomegalonema, Lampropedia*) and genera not known to contain species able to synthesise PHAs (e.g., *Nitrospira, Romboutsia, Nomurabacteria*). Genus richness remained high (115–358 genera), likely due to the continuous influx of bacterial species from the fermentation fluid.

A marked shift in the microbial population is observed in sample 5 (setup 2), taken from SBR2 3 weeks after the introduction of a 0.22 µm filtration step for the fermentation fluid, to prevent the influx of external bacteria into the selection reactor. Genus richness dropped to 81 in sample 5 and remained low (46–56) up to sample 11. Meanwhile, the proportion represented by the ten most abundant genera increased substantially (90.4–97.9%). This shift suggests that the feast-and-famine regime exerted strong selective pressure on the bacterial community, promoting the dominance of a few specialised species capable of surviving due to their ability to produce PHAs [58, 81]. This is reflected in the higher amount of intracellular PHA incorporated by the bacteria after the introduction of the filtration step and corresponds to these samples, as already discussed in paragraph 3.3.

Similarly to what was reported by Magonara et al. [35], the genera *Neomegalonema* and *Lampropedia* appeared consistently at high abundances after the establishment of speciation towards PHA production, representing, respectively, 9.1–75.0% and 45.2–73.7% of the 10 most abundant genera in samples 7–11. In those same samples, many PHA-producers reported in other publications could be detected at the species level, such as *Paracoccus alcaliphilus* [82], *Neomegalonema perideroedes* [53],

Alcaligenes faecalis [83], *Corynebacterium glutamicum* [78] and *Lampropedia hyalina* [35].

Conclusions and future research directions

This study demonstrates the feasibility of producing PHA-rich bacterial biomass with high intracellular accumulation and tailored HV content using mixed microbial cultures fed with carboxylic acids derived from the acidogenic fermentation of agri-food residues. Setup 2, in particular, enabled a significant improvement in PHA storage, achieving up to 57.0% w/w of cell dry weight, values that compare favourably with those reported for MMC systems using real and complex substrates. At the same time, HV fractions were largely aligned with the target range required to obtain PHBV with suitable thermochemical and mechanical properties for downstream applications, such as biodegradable agricultural films.

The results highlight the critical role of substrate quality and composition, particularly the presence of odd-carbon CAs, in steering both polymer accumulation and monomer distribution. These findings further indicate that HV content is intrinsically linked to the CA profile generated during acidogenic fermentation, suggesting that improved control of upstream fermentation processes could enable targeted PHBV production directly from waste-derived substrates, thereby reducing or eliminating the need for external carbon supplementation. From a broader perspective, this work strengthens the case for MMC-based PHA production as a robust and scalable strategy for valorising agricultural residues within a circular bioeconomy, consistent with the aims of sustainable bioproduct development.

Future research should focus on further stabilising HV content over long-term operation, optimising feeding strategies under variable waste-derived CA compositions, and validating process performance at pilot and industrial scale. In addition, downstream processing, polymer characterisation, and techno-economic and life-cycle assessments will be essential to fully assess the industrial viability and environmental benefits of the proposed bioprocess.

Abbreviations

3HB	3-Hydroxybutyrate
3HV	3-Hydroxyvalerate
COD	Chemical oxygen demand
DO	Dissolved oxygen
F/F	Feast-to-famine ratio
F/M	Food-to-microorganism ratio
FBR	Fed-batch reactor (acidogenic fermentation reactor)
GPC	Gel permeation chromatography
HRT	Hydraulic retention time
HV	Hydroxyvalerate
MLSS	Mixed liquor suspended solids
MLVSS	Mixed liquor volatile suspended solids
MMC	Mixed microbial cultures
Mw	Weight-average molecular weight
Mn	Number-average molecular weight
NA	Not available
OLR	Organic loading rate
PDI	Polydispersity index
PHA	Polyhydroxyalkanoates
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
SBR	Sequencing batch reactor
SD	Standard deviation
sCOD	Soluble chemical oxygen demand
TS	Total solids
TVS	Total volatile solids
CA	Carboxylic acids
VS	Volatile solids
WWTP	Wastewater treatment plant

Acknowledgements

This work was carried out within the framework of the AgriLoop project (https://www.agriloop-project.eu/), which aims to develop safe and sustainable bioconversion pathways to transform diverse agri-food residues into biopolymers and other high-value bio-based products. We are grateful to Lorenzo Panariello, who helped with the data collection at the University of Verona, and to Flavia Marzulli, who contributed to PHA analysis at the Sapienza University of Roma.

Author contributions

Material preparation, data collection, curation and analysis were performed by GP, CM, AZ, EM, DB, GS, LNT and LDL. DB and MV took care of funding acquisition, project administration, supervision and validation. The first draft of the manuscript was written by GP, and all authors reviewed and commented on subsequent versions. All authors read and approved the final manuscript.

Funding

This project has received funding from the European Union's Horizon Europe research and innovation programme under the grant agreement No. 101081776, the UK Research and Innovation (UKRI) fund under the UK government's Horizon Europe funding guarantee, the Swiss State Secretariat for Education, Research and Innovation (SERI) and from the National Key Research and Development Program supported by the Ministry of Science and Technology of the People's Republic of China (No. 2023YFE0104900). Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of neither of the aforementioned Funding authorities. None of the cited funding authorities can be held responsible for them.

Availability of data and materials

The data that support the findings of this study are available on request to the corresponding author.

Declarations

Ethics approval and consent to participate

Not applicable. This study does not involve direct or indirect interactions with human participants, does not use personal data of identifiable individuals, and does not involve the use of animals. Therefore, no ethical approval or consent to participate is required.

Consent for publication

Not applicable. This study does not involve human subjects, therefore no consent to publish was needed.

Competing interests

The authors declare no competing interests.

Author details

[1]Department of Biotechnology, University of Verona, 37134 Verona, Italy.
[2]Department of Chemistry, Sapienza University of Rome, 00185 Rome, Italy.

Received: 4 March 2026 Accepted: 1 May 2026
Published online: 14 May 2026

References

1. Gontard N, Sonesson U, Birkved M, Majone M, Bolzonella D, Celli A, et al. A research challenge vision regarding management of agricultural waste in a circular bio-based economy. Crit Rev Environ Sci Technol. 2018;48(6):614–54. https://doi.org/10.1080/10643389.2018.1471957.
2. Abdel-Shafy HI, Mansour MS. Solid waste issue: sources, composition, disposal, recycling, and valorization. Egypt J Pet. 2018;27(4):1275–90. https://doi.org/10.1080/10643389.2018.1471957.
3. Babu S, Rathore SS, Singh R, Kumar S, Singh VK, Yadav S, et al. Exploring agricultural waste biomass for energy, food and feed production and pollution mitigation: a review. Bioresour Technol. 2022;360:127566. https://doi.org/10.1016/j.biortech.2022.127566.
4. Bancal V, Ray RC. Overview of food loss and waste in fruits and vegetables: from issue to resources. In: Fruits and vegetable wastes: valorization to bioproducts and platform chemicals. Springer; 2022. p. 3–29.
5. Camia A, Robert N, Jonsson K, Pilli R, Sara GC, Lopez Lozano R, et al. Biomass production, supply, uses and flows in the European Union: First results from an integrated assessment. Online; 2018. Report No.: 9279772368. https://doi.org/10.2760/539520
6. FAO. Global food losses and food waste – Extent, causes and prevention. Rome; 2011.
7. Fritsch C, Staebler A, Happel A, Cubero Márquez MA, Aguiló-Aguayo I, Abadias M, et al. Processing, valorization and application of bio-waste derived compounds from potato, tomato, olive and cereals: a review. Sustainability. 2017;9(8):1492. https://doi.org/10.3390/su9081492.
8. Geissdoerfer M, Savaget P, Bocken NM, Hultink EJ. The Circular Economy–a new sustainability paradigm? J Clean Prod. 2017;143:757–68. https://doi.org/10.1016/j.jclepro.2016.12.048.
9. Jensen L, Scalamandrè C. Climate impact of the EU agrifood system. 2023.
10. Priya A, Alagumalai A, Balaji D, Song H. Bio-based agricultural products: a sustainable alternative to agrochemicals for promoting a circular economy. RSC Sustain. 2023;1(4):746–62. https://doi.org/10.1039/D3SU00075C.
11. Vigneswari S, Kee SH, Hazwan MH, Ganeson K, Tamilselvan K, Bhubalan K, et al. Turning agricultural waste streams into biodegradable plastic: a step forward into adopting sustainable carbon neutrality. J Environ Chem Eng. 2024. https://doi.org/10.1016/j.jece.2024.112135.
12. Sabapathy PC, Devaraj S, Meixner K, Anburajan P, Kathirvel P, Ravikumar Y, et al. Recent developments in Polyhydroxyalkanoates (PHAs) production–a review. Bioresour Technol. 2020;306:123132. https://doi.org/10.1016/j.biortech.2020.123132.
13. Cabrera F, Torres-Aravena Á, Pinto-Ibieta F, Campos JL, Jeison D. On-line control of feast/famine cycles to improve PHB accumulation during cultivation of mixed microbial cultures in sequential batch reactors. Int J Environ Res Public Health. 2021;18(23):12611. https://doi.org/10.3390/ijerph182312611.
14. Bresan S, Sznajder A, Hauf W, Forchhammer K, Pfeiffer D, Jendrossek D. Polyhydroxyalkanoate (PHA) granules have no phospholipids. Sci Rep. 2016;6(1):1–13.
15. Freches A, Lemos PC. Microbial selection strategies for polyhydroxyalkanoates production from crude glycerol: effect of OLR and cycle length. New Biotechnol. 2017;39:22–8. https://doi.org/10.1016/j.nbt.2017.05.011.
16. Rivera-Briso AL, Serrano-Aroca Á. Poly (3-Hydroxybutyrate-co-3-Hydroxyvalerate): enhancement strategies for advanced applications. Polymers. 2018;10(7):732. https://doi.org/10.3390/polym10070732.
17. Chen G-Q. A microbial polyhydroxyalkanoates (PHA) based bio-and materials industry. Chem Soc Rev. 2009;38(8):2434–46. https://doi.org/10.1039/b812677c.
18. Bugnicourt E, Cinelli P, Lazzeri A, Alvarez VA. Polyhydroxyalkanoate (PHA): review of synthesis, characteristics, processing and potential applications in packaging. Express Polym Lett. 2014;8(11):791–808. https://doi.org/10.3144/expresspolymlett.2014.82.
19. Arcos-Hernández MV, Laycock B, Donose BC, Pratt S, Halley P, Al-Luaibi S, et al. Physicochemical and mechanical properties of mixed culture polyhydroxyalkanoate (PHBV). Eur Polymer J. 2013;49(4):904–13. https://doi.org/10.1016/j.eurpolymj.2012.10.025.
20. Laycock B, Arcos-Hernandez MV, Langford A, Buchanan J, Halley PJ, Werker A, et al. Thermal properties and crystallization behavior of fractionated blocky and random polyhydroxyalkanoate copolymers from mixed microbial cultures. J Appl Polym Sci. 2014. https://doi.org/10.1002/app.40836.
21. Koller M, Mukherjee A. A new wave of industrialization of PHA biopolyesters. Bioengineering. 2022;9(2):74. https://doi.org/10.3390/bioengineering9020074.
22. Colombo B, Favini F, Scaglia B, Sciarria TP, D'Imporzano G, Pognani M, et al. Enhanced polyhydroxyalkanoate (PHA) production from the organic fraction of municipal solid waste by using mixed microbial culture. Biotechnol Biofuels. 2017;10:1–15. https://doi.org/10.1186/s13068-017-0888-8.
23. Kourmentza C, Plácido J, Venetsaneas N, Burniol-Figols A, Varrone C, Gavala HN, et al. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering. 2017;4(2):55.
24. Gurieff N, Lant P. Comparative life cycle assessment and financial analysis of mixed culture polyhydroxyalkanoate production. Bioresour Technol. 2007;98(17):3393–403. https://doi.org/10.1016/j.biortech.2006.10.046.
25. Heimersson S, Morgan-Sagastume F, Peters GM, Werker A, Svanström M. Methodological issues in life cycle assessment of mixed-culture polyhydroxyalkanoate production utilising waste as feedstock. New Biotechnol. 2014;31(4):383–93. https://doi.org/10.1016/j.nbt.2013.09.003.
26. Chouhan A, Tiwari A. Production of polyhydroxyalkanoate (PHA) biopolymer from crop residue using bacteria as an alternative to plastics: a review. RSC Adv. 2025;15(15):11845–62. https://doi.org/10.1039/d4ra08505a.
27. Pei R, de Vries E, Estévez A, Sousa J, Dijkman H, Tamis J, et al. Demonstrating performance in scaled-up production and quality control of polyhydroxyalkanoates using municipal waste activated sludge. Water Res. 2025;275:123160. https://doi.org/10.1016/j.watres.2025.123160.
28. Wang J, Liu S, Huang J, Qu Z. A review on polyhydroxyalkanoate production from agricultural waste Biomass: development, advances, circular approach, and challenges. Bioresour Technol. 2021;342:126008. https://doi.org/10.1016/j.biortech.2021.126008.
29. Castro-Ramos JJ, Solís-Oba A, Solís-Oba M, Calderón-Vázquez CL, Higuera-Rubio JM, Castro-Rivera R. Effect of the initial pH on the anaerobic digestion process of dairy cattle manure. AMB Express. 2022;12(1):162. https://doi.org/10.1186/s13568-022-01486-8.
30. Wei L, Guho NM, Coats ER, McDonald AG. Characterization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) biosynthesized by mixed microbial consortia fed fermented dairy manure. J Appl Polym Sci. 2014. https://doi.org/10.1002/app.40333.
31. Tomás-Pejó E, González-Fernández C, Greses S, Kennes C, Otero-Logilde N, Veiga MC, et al. Production of short-chain fatty acids (SCFAs) as chemicals or substrates for microbes to obtain biochemicals. Biotechnol Biofuels Bioprod. 2023;16(1):96. https://doi.org/10.1186/s13068-023-02349-5.
32. FAO. Number of cattle worldwide from 2012 to 2023 (in million head)* [Graph]: Statista; 2025 [updated 13 November 2025 %| Released 28 January 2025. https://www.statista.com/statistics/263979/global-cattle-population-since-1990/?utm_source=chatgpt.com.
33. Weldesemayat Sileshi G, Barrios E, Lehmann J, Tubiello FN. An organic matter database (OMD): consolidating global residue data from agriculture, fisheries, forestry and related industries. Earth Syst Sci Data. 2025;17(2):369–91. https://doi.org/10.5194/essd-17-369-2025.
34. EEA. Progress and prospects for decarbonisation in the agriculture sector and beyond. 2022.
35. Magonara C, Montagnese E, Bertasini D, Vona C, Salvatori G, Tayou LN, et al. Mixed-culture polyhydroxyalkanoate production with variable hydroxyvalerate content from agri-food residues. Environ Sci Pollut Res. 2025. https://doi.org/10.1007/s11356-025-36316-4.
36. Serafim LS, Lemos PC, Albuquerque MG, Reis MA. Strategies for PHA production by mixed cultures and renewable waste materials. Appl Microbiol Biotechnol. 2008;81(4):615–28. https://doi.org/10.1007/s00253-008-1757-y.
37. Valentino F, Beccari M, Fraraccio S, Zanaroli G, Majone M. Feed frequency in a Sequencing Batch Reactor strongly affects the production of polyhydroxyalkanoates (PHAs) from volatile fatty acids. New Biotechnol. 2014;31(4):264–75.
38. Chen Y, Yin Y, Wang J. Recent advance in inhibition of dark fermentative hydrogen production. Int J Hydrogen Energy. 2021;46(7):5053–73. https://doi.org/10.1016/j.ijhydene.2020.11.096.
39. Yenigün O, Demirel B. Ammonia inhibition in anaerobic digestion: a review. Process Biochem. 2013;48(5–6):901–11. https://doi.org/10.1016/j.procbio.2013.04.012.

40. Bengtsson S, Hallquist J, Werker A, Welander T. Acidogenic fermentation of industrial wastewaters: effects of chemostat retention time and pH on volatile fatty acids production. Biochem Eng J. 2008;40(3):492–9. https://doi.org/10.1016/j.bej.2008.02.004.
41. Bi S, Hong X, Yang H, Yu X, Fang S, Bai Y, et al. Effect of hydraulic retention time on anaerobic co-digestion of cattle manure and food waste. Renew Energy. 2020;150:213–20. https://doi.org/10.1016/j.renene.2019.12.091.
42. Righetti E, Nortilli S, Fatone F, Frison N, Bolzonella D. A Multiproduct biorefinery approach for the production of hydrogen, methane and volatile fatty acids from agricultural waste. Waste Biomass Valoriz. 2020. https://doi.org/10.1007/s12649-020-01023-3.
43. Reis M, Albuquerque M, Villano M, Majone M. Mixed culture processes for polyhydroxyalkanoate production from agro-industrial surplus wastes as feedstocks. Comprehensive Biotechnology. Academic Press, Burlington, Canada 2011; 669; 2011.
44. Braunegg G, Sonnleitner B, Lafferty R. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur J Appl Microbiol Biotechnol. 1978;6(1):29–37. https://doi.org/10.1007/BF00500854.
45. APHA. Standard Methods for the Examinations of Water and Wastewater. Washington: American Public Health Association; 1998.
46. Raposo F, Borja R, Cacho JA, Mumme J, Mohedano ÁF, Battimelli A, et al. Harmonization of the quantitative determination of volatile fatty acids profile in aqueous matrix samples by direct injection using gas chromatography and high-performance liquid chromatography techniques: Multi-laboratory validation study. J Chromatogr A. 2015;1413:94–106. https://doi.org/10.1016/j.chroma.2015.08.008.
47. Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. PLoS ONE. 2014;9(8):e105592. https://doi.org/10.1371/journal.pone.0105592.
48. Serafim LS, Lemos PC, Oliveira R, Reis MA. Optimization of polyhydroxybutyrate production by mixed cultures submitted to aerobic dynamic feeding conditions. Biotechnol Bioeng. 2004;87(2):145–60. https://doi.org/10.1002/bit.20085.
49. Atasoy M, Owusu-Agyeman I, Plaza E, Cetecioglu Z. Bio-based volatile fatty acid production and recovery from waste streams: Current status and future challenges. Bioresour Technol. 2018;268:773–86. https://doi.org/10.1016/j.biortech.2018.07.042.
50. Kim H, Kim J, Shin SG, Hwang S, Lee C. Continuous fermentation of food waste leachate for the production of volatile fatty acids and potential as a denitrification carbon source. Bioresour Technol. 2016;207:440–5. https://doi.org/10.1016/j.biortech.2016.02.063.
51. Lee WS, Chua ASM, Yeoh HK, Ngoh GC. A review of the production and applications of waste-derived volatile fatty acids. Chem Eng J. 2014;235:83–99. https://doi.org/10.1016/j.cej.2013.09.002.
52. Cavinato C, Da Ros C, Pavan P, Bolzonella D. Influence of temperature and hydraulic retention on the production of volatile fatty acids during anaerobic fermentation of cow manure and maize silage. Bioresour Technol. 2017;223:59–64. https://doi.org/10.1016/j.biortech.2016.10.041.
53. Dionisi D, Majone M, Vallini G, Di Gregorio S, Beccari M. Effect of the applied organic load rate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor. Biotechnol Bioeng. 2006;93(1):76–88. https://doi.org/10.1002/bit.20683.
54. Johnson K, Kleerebezem R, van Loosdrecht MC. Influence of the C/N ratio on the performance of polyhydroxybutyrate (PHB) producing sequencing batch reactors at short SRTs. Water Res. 2010;44(7):2141–52.
55. Hao J, Wang H, Wang X. Selecting optimal feast-to-famine ratio for a new polyhydroxyalkanoate (PHA) production system fed by valerate-dominant sludge hydrolysate. Appl Microbiol Biotechnol. 2018;102:3133–43. https://doi.org/10.1007/s00253-018-8799-6.
56. Oliveira CS, Silva CE, Carvalho G, Reis MA. Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: feast and famine regime and uncoupled carbon and nitrogen availabilities. New Biotechnol. 2017;37:69–79. https://doi.org/10.1016/j.nbt.2016.10.008.
57. Henze M, van Loosdrecht MC, Ekama GA, Brdjanovic D. Biological wastewater treatment: IWA publishing; 2008. https://doi.org/10.2166/9781780401867.
58. Morgan-Sagastume F, Karlsson A, Johansson P, Pratt S, Boon N, Lant P, et al. Production of polyhydroxyalkanoates in open, mixed cultures from a waste sludge stream containing high levels of soluble organics, nitrogen and phosphorus. Water Res. 2010;44(18):5196–211. https://doi.org/10.1016/j.watres.2010.06.043.
59. Morgan-Sagastume F, Valentino F, Hjort M, Cirne D, Karabegovic L, Gerardin F, et al. Polyhydroxyalkanoate (PHA) production from sludge and municipal wastewater treatment. Water Sci Technol. 2014;69(1):177–84. https://doi.org/10.2166/wst.2013.643.
60. Bolzonella D, Battista F, Mattioli A, Nicolato C, Frison N, Lampis S. Biological thermophilic post hydrolysis of digestate enhances the biogas production in the anaerobic digestion of agro-waste. Renew Sustain Energy Rev. 2020;134:110174. https://doi.org/10.1016/j.rser.2020.110174.
61. Albuquerque M, Eiroa M, Torres C, Nunes B, Reis M. Strategies for the development of a side stream process for polyhydroxyalkanoate (PHA) production from sugar cane molasses. J Biotechnol. 2007;130(4):411–21. https://doi.org/10.1016/j.jbiotec.2007.05.011.
62. Bengtsson S, Werker A, Christensson M, Welander T. Production of polyhydroxyalkanoates by activated sludge treating a paper mill wastewater. Bioresour Technol. 2008;99(3):509–16. https://doi.org/10.1016/j.biortech.2007.01.020.
63. Li R-h, Huang J, Liu C-x, Yu K, Guo F, Li Y, et al. Genome-centric metagenomics provides new insights into metabolic pathways of polyhydroxyalkanoates biosynthesis and functional microorganisms subsisting on municipal organic wastes. Water Res. 2023;244:120512. https://doi.org/10.1016/j.watres.2023.120512.
64. Werker A, Bengtsson S, Korving L, Hjort M, Anterrieu S, Alexandersson T, et al. Consistent production of high quality PHA using activated sludge harvested from full scale municipal wastewater treatment–PHARIO. Water Sci Technol. 2018;78(11):2256–69. https://doi.org/10.2166/wst.2018.502.
65. Bravo-Porras G, Fernández-Güelfo LA, Alvarez-Gallego CJ, Carbú M, Sales D, Romero-García LI. Influence of the total concentration and the profile of volatile fatty acids on polyhydroxyalkanoates (PHA) production by mixed microbial cultures. Biomass Convers Biorefinery. 2024;14(1):239–53. https://doi.org/10.1007/s13399-021-02208-z.
66. Queirós D, Rossetti S, Serafim LS. PHA production by mixed cultures: a way to valorize wastes from pulp industry. Bioresour Technol. 2014;157:197–205.
67. Coats ER, Watson BS, Brinkman CK. Polyhydroxyalkanoate synthesis by mixed microbial consortia cultured on fermented dairy manure: effect of aeration on process rates/yields and the associated microbial ecology. Water Res. 2016;106:26–40. https://doi.org/10.1016/j.watres.2016.09.039.
68. Marchetti A, Lorini L, Salvatori G, Gianico A, Majone M, Villano M. Polyhydroxyalkanoates production by mixed microbial cultures in sequencing batch reactors operated under different feeding conditions. Chem Eng Trans. 2022;93:163–8. https://doi.org/10.3303/CET2293028.
69. Zhou T, Wang S, Zhang W, Yin F, Cao Q, Lian T, et al. Comparison of different aerobic sludge on enriching polyhydroxyalkanoate mixed microbial culture using lactic acid fermentation broth of agricultural wastes. Chem Eng J. 2023;475:146000. https://doi.org/10.1016/j.cej.2023.146000.
70. Chen Z, Huang L, Wen Q, Guo Z. Efficient polyhydroxyalkanoate (PHA) accumulation by a new continuous feeding mode in three-stage mixed microbial culture (MMC) PHA production process. J Biotechnol. 2015;209:68–75. https://doi.org/10.1016/j.jbiotec.2015.06.382.
71. Albuquerque M, Torres C, Reis M. Polyhydroxyalkanoate (PHA) production by a mixed microbial culture using sugar molasses: effect of the influent substrate concentration on culture selection. Water Res. 2010;44(11):3419–33. https://doi.org/10.1016/j.watres.2010.03.021.
72. Chang H-F, Chang W-C, Tsai C-Y. Synthesis of poly (3-hydroxybutyrate/3-hydroxyvalerate) from propionate-fed activated sludge under various carbon sources. Bioresour Technol. 2012;113:51–7. https://doi.org/10.1016/j.biortech.2011.12.138.
73. Duque AF, Oliveira CS, Carmo IT, Gouveia AR, Pardelha F, Ramos AM, et al. Response of a three-stage process for PHA production by mixed microbial cultures to feedstock shift: impact on polymer composition. New Biotechnol. 2014;31(4):276–88. https://doi.org/10.1016/j.nbt.2013.10.010.
74. Lorini L, Martinelli A, Capuani G, Frison N, Reis M, Sommer Ferreira B, et al. Characterization of polyhydroxyalkanoates produced at pilot scale from different organic wastes. Front Bioeng Biotechnol. 2021;9:628719. https://doi.org/10.3389/fbioe.2021.628719.
75. Blunt W, Shah P, Vasquez V, Ye M, Doyle C, Liu Y, et al. Production and characterization of poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

copolymers from a pre-fermented hardwood hydrolysate. Bioprocess Biosyst Eng. 2025;48(10):1679–92. https://doi.org/10.1007/s00449-025-03203-8.
76. Vermeer CM, Nielsen M, Eckhardt V, Hortensius M, Tamis J, Picken SJ, et al. Systematic solvent screening and selection for polyhydroxyalkanoates (PHBV) recovery from biomass. J Environ Chem Eng. 2022;10(6):108573. https://doi.org/10.1016/j.jece.2022.108573.
77. Luo S, Grubb D, Netravali A. The effect of molecular weight on the lamellar structure, thermal and mechanical properties of poly (hydroxybutyrate-co-hydroxyvalerates). Polymer. 2002;43(15):4159–66. https://doi.org/10.1016/S0032-3861(02)00242-2.
78. Tan G-YA, Chen C-L, Li L, Ge L, Wang L, Razaad IMN, et al. Start a research on biopolymer polyhydroxyalkanoate (PHA): a review. Polymers. 2014;6(3):706–54. https://doi.org/10.3390/polym6030706.
79. Hu M, Wang X, Wen X, Xia Y. Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresour Technol. 2012;117:72–9. https://doi.org/10.1016/j.biortech.2012.04.061.
80. Muszyński A, Tabernacka A, Miłobędzka A. Long-term dynamics of the microbial community in a full-scale wastewater treatment plant. Int Biodeterior Biodegrad. 2015;100:44–51. https://doi.org/10.1016/j.ibiod.2015.02.008.
81. Johnson K, Jiang Y, Kleerebezem R, Muyzer G, Van Loosdrecht MC. Enrichment of a mixed bacterial culture with a high polyhydroxyalkanoate storage capacity. Biomacromol. 2009;10(4):670–6. https://doi.org/10.1021/bm8013796.
82. Puri A, Bajaj A, Singh Y, Lal R. Harnessing taxonomically diverse and metabolically versatile genus *Paracoccus* for bioplastic synthesis and xenobiotic biodegradation. J Appl Microbiol. 2022;132(6):4208–24. https://doi.org/10.1111/jam.15530.
83. Sharma N, Saharan BS. Chemistry of bioplastic production by *Alcaligenes faecalis* NSBN10 using cane molasses: optimization and characterization. Discov Chem. 2025;2(1):12. https://doi.org/10.1007/s44371-025-00090-6.

Publisher's Note