Bioresource Technology 385 (2023) 129445

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Effect of composition of volatile fatty acids on yield of polyhydroxyalkanoates and mechanisms of bioconversion from activated sludge

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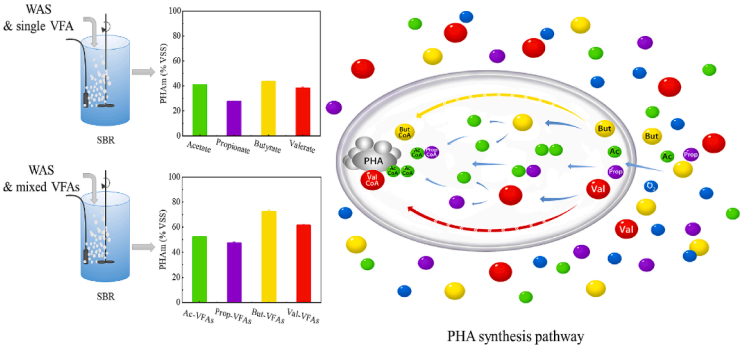
HIGHLIGHTS

- Waste active sludge could be acclimated for PHA production within 60 h.
- Butyrate-dominated substrate maximized PHA content at 72.08% of VSS.
- Preference order for PHA synthesis: butyrate > valerate > acetate > propionate.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Biomass
Volatile fatty acid
Polyhydroxyalkanoate
Butyrate

ABSTRACT

Polyhydroxyalkanoates (PHA) is green biodegradable natural polymer. Here PHA production from volatile fatty acids (VFAs) was investigated in sequential batch reactors inoculated with activated sludge. Single or mixed VFAs ranging from acetate to valerate were evaluated, and the dominant VFA concentration was 2 times of that of the others in the tests. Results showed that mixed substrates achieved about 1.6 times higher yield of PHA production than single substrate. The butyrate-dominated substrates maximized PHA content at 72.08% of VSS, and the valerate-dominated substrates were followed with PHA content at 61.57%. Metabolic flux analysis showed the presence of valerate in the substrates caused a more robust PHA production. There was at least 20% of 3-hydroxyvalerate in the polymer. *Hydrogenophaga* and *Comamonas* were the main PHA producers. As VFAs could be produced in anaerobic digestion of organic wastes, the methods and data here could be referred for efficient green bioconversion of PHA.

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https://doi.org/10.1016/j.biortech.2023.129445
Received 4 June 2023; Received in revised form 29 June 2023; Accepted 30 June 2023
Available online 1 July 2023

1. Introduction

Traditional chemical plastics are derived from petroleum. According to statistics, the plastics industry consumed 8% of the world's petroleum and caused 1.29 billion tons of carbon dioxide emissions in 2019. The production and use of plastics accounted for 3.8% of global carbon dioxide emissions. In addition, disposal methods such as landfill or incineration also produced 3.12 tons of CO_2 per ton of plastic (Tang et al., 2021). One strategy to reduce global plastic greenhouse gas emissions and plastic pollution is to adopt biodegradable polymers.

Polyhydroxyalkanes (PHAs) are such a type of natural polymer that could be biosynthesized and degraded. It possesses fundamental characteristics similar to polypropylene (Fang et al., 2019). Therefore, it has the potential to replace existing chemical plastics and tackle the problem of plastic pollution (such as microplastics) (Wu et al., 2020). As the first plastic derived from microbial technology, PHA is produced entirely from renewable natural resources. Sugars, fatty alcohols, fatty acids, and even carbon dioxide, biogas can be used as carbon sources for microbial synthesis of PHA (Bian et al., 2020; Lagoa-Costa et al., 2017). Since it is an osmotically inert polymeric material, numerous microbes can accumulate the substance without any physiological constraints (Munir & Jamil, 2018).

However, the primary obstacle to PHA commercialization is its high investment in production in comparison to conventional chemical plastics (Wen et al., 2020b). Pure culture or genetic engineering for PHA production requires complex operations as well as high costs (Qiao et al., 2021). Waste resourcing is a hot research topic in line with sustainable development (He et al., 2020; Zhao et al., 2022). PHA can be produced at a significantly lower cost by using activated sludge as mixed cultures (MCs) and organic-rich wastewater (such as fruit waste, brewing waste, rubber wood hydrolysate, and cheese whey) as the raw materials (Yukesh Kannah et al., 2022). However, the instability of the yield and polymer characteristics during MCs-PHA production are also significant challenges to overcome. Thus, the current research on PHA synthesis by activated sludge focuses on the improvement of PHA yields or obtaining better-performing biopolymers.

PHA yield can be effectively improved by altering operating parameters. For example, weak alkaline reaction conditions, lower temperature (20℃), and longer sludge residence time (SRT = 5 ~ 10 d) facilitate the microbial synthesis of PHA (Obruca et al., 2021). Besides, the substrate supply method also affects the production of PHA by MCs. Pulse feed strategy research obtained a PHA content at 85 wt% (on a dry cell weight basis), which showed that periodic periods of famine were not necessary (Marang et al., 2018). Only if the enrichment of PHA producers in the reactor is already high enough might the famine phase be abolished, allowing for a smoother operation. For the initial enrichment phase of PHA producers, a famine phase is necessary. A feeding strategy that separated the supply of carbon (C) and nitrogen (N) sources was applied, with C-source being dosed at the start of the feast while the N-source at the start of the famine, and the maximum PHA content achieved 0.53 g PHA/g VSS (Lorini et al., 2020).

Obtaining better-performing polymers is also a research emphasis of the MCs-PHA progress. The composition of the PHA monomer and the ratio of each component were the major factors controlling the mechanical and processing properties of PHA. In MCs-PHA production progress, hydroxybutyrate (HB) and hydroxyvalerate (HV) were the most frequent monomers (Sagong et al., 2018). Among them, HB showed high crystallinity and brittleness, as well as strong hardness and water resistance. However, the fragility and poor thermal stability were not conducive to the subsequent processing applications. It was found that a certain percentage of HV in the copolymer could improve elasticity and ductility, which could greatly improve the polymer's processing qualities (Wei & Fang, 2021).

The proportions of volatile fatty acids (VFAs) among the feedstock affected the composition of polyhydroxyalkanoates. VFAs, which were abundant in the anaerobic fermentation broth of organic wastewater, were favored substrates for PHA synthesis compared to other carbon sources (De Donno Novelli et al., 2021). Depending on the number of carbon atoms in the backbone, VFAs could be classified into even-numbered carbon VFAs (E-FAs) and odd-numbered carbon VFAs (O-FAs) (Li et al., 2020). The ratio of E-FAs to O-FAs could be altered by directed acid production to suit the percentage of polyhydroxybutyrate (PHB) to polyhydroxyvalerate (PHV) in PHA formation (Fang et al., 2020). It had traditionally been assumed that PHA producers preferentially use E-FAs over O-FAs and that E-FAs generally result in higher PHA production and productivity (Lemos et al., 2006). O-FAs were often considered to facilitate the synthesis of HV (Huang et al., 2018). When the ratio of E-FAs to O-FAs in the VFAs was 60: 40, the percentage of HV in the obtained copolymer reached about 40% (Fra-Vázquez et al., 2019). Li et al. (2020) used acetate, propionate, and butyrate as substrates. The ratio of E-FAs to O-FAs was changed by adjusting the ratio of propionate. When the E-FAs to O-FAs ratio was 88: 12, the PHA content could reach 50.3 wt%, while only 6% of HV was in polymers. When the ratio was modified to 48: 52, the PHA content was 44.7 wt%, and the HV in polymers increased by 10%. So here comes the question: which substrate composition was better for obtaining polymers with a higher yield and better properties?

In this study, PHA production using either single VFA or mixed ones including from acetate to valerate was investigated in sequential batch reactors (SBRs). In order to acclimatize and enrich the PHA producers, the culture mode of aerobic dynamic feed (ADF) coupled nitrogen limiting was applied. The PHA production response of mixed cultures (MCs) enrichment in various substrates was investigated. The PHA yield was determined, as well as the proportion of HB and HV in the polymers. The reasons for the various PHA production responses of MCs were investigated from the perspectives of metabolic analysis and microbial community. Finally, the optimized feeding strategy for MCs-PHA production was briefly discussed.

2. Materials and methods

2.1. PHA accumulation assays

PHA producers were enriched using SBR reactors with 1L operating volumes. Waste activated sludge (WAS) was collected from the secondary sedimentation tank of a sewage treatment facility (Hunan, China) and filtered using a sieve with a pore size of 0.45 μm. The screened WAS was used as inoculum for SBRs. The total suspended solid (TSS) concentration in the reactor was about 3000 mg/L. This study included two batch experiments, one with a single carbon source and one with a mixed carbon source. Glucose, acetate, propionate, butyrate, and valerate (analytical reagent) were used as substrates. Mixed VFA substrates were set up in 5 types: uniform VFA mixture (U-VFAs, Ac/Prop/But/Val = 1: 1: 1: 1, based on g COD, same below), acetate dominated VFA mixture (Ac-VFAs, Ac/Prop/But/Val = 3: 1: 1: 1), propionate dominated VFA mixture (Prop-VFAs, Ac/Prop/But/Val = 1: 3: 1: 1), Butyrate dominated VFA mixture (But-VFAs, Ac/Prop/But/Val = 1: 1: 3: 1), Valerate dominated VFA mixture (Val-VFAs, Ac/Prop/But/Val = 1: 1: 1: 3). Two parallel experiments were set for each reactor to run under the same conditions. The butyrate included n-butyrate and *iso*-butyrate with a ratio of 3:1. Nutrient components were composed of NH_4Cl, K_2HPO_4, KH_2PO_4, $MgSO_4$, and NaCl. 1 mL of trace element stock solution was added, which consisted of (g/L): 2 g amino-triacetic acid, 1 g $MnSO_4{\cdot}H_2O$, 0.8 g $Fe(SO_4)_2(NH_4)_2{\cdot}6H_2O$, 0.2 g $CoCl_2{\cdot}6H_2O$, 0.2 mg $ZnSO_4{\cdot}7H_2O$ and 20 mg $CuCl_2{\cdot}2H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$, 20 mg $Na_2MoO_4{\cdot}2H_2O$, 20 mg Na_2SeO_4 and 20 mg Na_2WO_4, and 1 mL vitamin stock solution was added, which composed of (g/L): 2 mg biotin, 2 mg folic acid, 10 mg pyridoxine hydrochloride, 5 mg thiamine-HCl, 5 mg riboflavin, 5 mg nicotinic acid, 5 mg calcium D-(+)-pantothenate, 0.1 mg vitamin B12, 5 mg p-aminobenzoic acid, and 5 mg thioctic acid. The carbon source concentration was about 1.3 g COD/L, and the C, N and P ratio (on a molar basis) was held consistent at 100: 3: 1. The addition of

1 mL of 40 g/L thiourea (analytical reagent) was used to restrain nitrification in the SBRs (Deng et al., 2022).

The initial pH value was modified to 7 ± 0.5 by adding 3 mol/L HCl or NaOH solution. The reactor was mechanically agitated throughout the whole process at 200 rpm and 30 ± 1℃ without pH control. For all the SBRs, SRT was held constant at 10 d, while hydraulic retention time was fixed at 1 d. A conventional ADF culturing mode was employed: all SBRs operated on a 9-hour operational cycle that consisted of four phases: feeding (5 min), aeration (480 min), sedimentation (50 min), and withdrawal (5 min). The compressed-air pump provided air through a ceramic diffuser.

2.2. Analysis of general parameters

The sCOD, total suspended solids (TSS), and volatile suspended solids (VSS) were measured according to the standard methods. 10 mL aliquots of mixed sludge were extracted from each SBR, and subsequently centrifuged at 3700 g and 4 °C for 10 min. The supernatant was extracted to determine sCOD and VFAs. The sludge was resuspended in 2.5 mL acetone. After thorough mixing, the samples were rinsed with deionized water and freeze-dried for 48 h by a vacuum freeze-dryer. The extraction and detection of PHA were based on previous research (Fang et al., 2021). The measurements of VFAs were performed by gas chromatograph (AOC-20i, Shimadzu, Japan) with a flame ionization detector (FID) (Nie et al., 2023).

2.3. Parameters calculations

The PHA storage capability of microorganisms was assessed by PHA content, specific substrate uptake rate (q_s、g COD_{-S}/g X/h), PHA production rate (q_{PHA}、g COD_{-PHA}/g X/h), yield of PHA production on substrate ($Y_{PHA/S}$,g COD_{-PHA}/g COD), biomass growth yield on substrate ($Y_{X/S}$,g COD_{-X}/g COD) were calculated according to Eqs. (1)– (5) (Wen et al., 2020b). The average and standard deviation were calculated for the data obtained from duplicate reactors.

$$PHA\ (\%\ VSS) = \frac{PHA}{VSS} \times 100\% \tag{1}$$

$$q_{PHA} = (PHA_1 - PHA_0)/X/T \tag{2}$$

$$q_s = (S_1 - S_2)/X/T \tag{3}$$

$$Y_{PHA/S} = (PHA_1 - PHA_0)/(S_1 - S_0) \tag{4}$$

$$Y_{X/S} = (X_1 - X_0)/(S_1 - S_0) \tag{5}$$

The biomass (X) concentration (g/L) was translated to g COD/L according to the conversion factor of 1.42 g COD/g X. The PHA concentration (g/L) was converted to g COD/L based on the oxidation stoichiometry: 1.67 g COD/g polyhydroxybutyrate (PHB) and 1.92 g COD/g polyhydroxyvalerate (PHV), respectively (Frison et al., 2015). A one-way ANOVA test was executed by the IBM SPSS statistics program (v 29.0, IBM, United States).

Metabolic fluxes analysis (MFA) was conducted to figure out the metabolic characteristics of reactors with VFAs mixture in the PHA accumulation assays. The corresponding reaction formulas for the metabolic fluxes were listed in the Supplementary materials. Related calculation methods were used for the MFA (C mol based). Based on the molecular weights of HB and HV, which were 21.5 and 20 g/C mol, respectively, the units of concentration of PHA were transformed from mg/L to C mol/L (Huang et al., 2020). According to stoichiometric calculations, the active biomass formulation was $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight of 25.1 g/C mol, containing 2% ash (Lemos et al., 2006). Matrix calculations in MFA were executed with MATLAB (R2018a, MathWorks, United States).

2.4. Microbial community analysis

Microbial samples were collected from the SBRs (inoculum, reactors with glucose, and different proportions of VFAs) for microbial community analysis. Total genomic DNA was extracted and quantified using an E.Z.N.A. soil DNA kit (Omega Bio-tek, Norcross, GA, U.S.) following the manufacturer's recommended protocol. Shanghai Majorbio Bio-pharm Technology Co., Ltd (Shanghai, China) was commissioned to perform high-throughput sequencing of 16S rRNA gene with Illumina MiSeq PE300 sequencing platform (Illumina, Inc., CA, USA). Primers 308F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) were used to amplify the V3-V4 region of the 16S rRNA gene. Tables for the operational taxonomic unit (OTU) were generated after data sets were rarefied to 30,000 reads per sample (see Supplementary materials). Raw sequence data is available at NCBI BioProject (PRJNA987419).

3. Results and discussion

3.1. Effect of substrate composition on PHA yield

In the present study, all mixed cultures (MCs) in the reactors showed different PHA production responses with short-term acclimation. After 20 cycles of operation, the PHA production gradually decreased or even disappeared in the reactor fed with glucose (see Supplementary materials). However, an increased response in PHA production occurred after the same acclimation time when fed with a single VFA (see Supplementary materials). Especially, after four cycles in the reactor with valerate as the substrate, PHA synthesis increased significantly. When acetate or butyrate was utilized, the microorganisms responded more quickly to synthesize HB using butyrate. Both HB and HV were synthesized when propionate or valerate was employed as a carbon source, while the yield of HV was not significantly higher than that of HB. The HV yield gradually increased as the HB yield declined when valerate was utilized as a single substrate.

Reaction parameters, such as pH and dissolved oxygen (DO), could noticeably affect the metabolic process of PHA synthesis and the enrichment of PHA producers. In the reactor fed with glucose, continuous monitoring revealed that the pH value had fallen to 3.87 after 30 min of aeration (see Supplementary materials). Due to the production of pyruvate or VFAs during the metabolism of glucose (Cui et al., 2016), the pH decreased rapidly in the reactor. The acidic environment not only discouraged bacterial PHA synthesis but also hindered the growth and enrichment of PHA producers. However, the pH value of the entire reaction process was 8 ~ 10 in the reactors that used VFAs as substrates. The alkaline environment was beneficial for PHA synthesis by MCs (Montiel-Jarillo et al., 2017). Besides, different substrates could be synthesized into PHA through different metabolic pathways. Wang et al. (2017a) discovered that longer-chain VFAs (such as butyrate and valerate) might be utilized by MCs to synthesize PHA more quickly, which might be that it uses less energy. This could also explain why the reactor fed with butyrate or valerate had a quicker response to enhanced PHA production.

Considering glucose was unsuitable for PHA production under the operational conditions of this work, it was excluded when the substrates were mixed. A similar PHA synthesis response was observed following the short-term acclimatization with mixed VFAs (see Supplementary materials). Besides, it was found that the amount of HB synthesized rose in the early cycles, but declined subsequently along with the HV content rose in each SBR.

The preliminary enrichment of PHA producers in the reactor was another important aspect that influences the efficiency of PHA production. It took months if not more, to enrich PHA producers initially in several investigations (De Donno Novelli et al., 2021), which raised the investment of money and time. Similar PHA accumulation responses were also observed after the short-term domestication of WAS in other

studies. Ike et al. (2019) discovered that WAS from a municipal sewage treatment facility dramatically improved the power to synthesize PHA and glycogen with short-term acclimation (14 cycles). Through selective staining and image analysis, Pei et al. (2022) revealed that about 55% of microorganisms in the municipal waste activated sludge could store PHA using organic matter. Direct inoculation of WAS for the synthesis of PHA can realize the resourcing of residual sludge and reduce the cost of WAS treatment. Despite some minor differences in the reactors fed with VFAs, it was clear that WAS could synthesize PHA after short-term acclimation, demonstrating the presence of PHA producers in WAS, which quickly assumed supremacy under the ADF coupled nitrogen limiting culture mode. The operational mode in this study was simpler compared to the aerobic dynamic discharge mode proposed by Inoue et al. (2021).

3.2. Effect of substrate composition on metabolic characteristics

3.2.1. PHA production under various substrates

The results of continuous sampling during the cycle were shown in Fig. 1. The PHA content synthesized by MCs reached the maximum with the degradation of the substrate except for the reactor with glucose. Propionate and valerate produced by the degradation of glucose might lead to a minor amount of HV (0.27% VSS) being synthesized (Fig. 1 (a)). The maximum PHA content (PHA_m) reached 40.95% VSS before

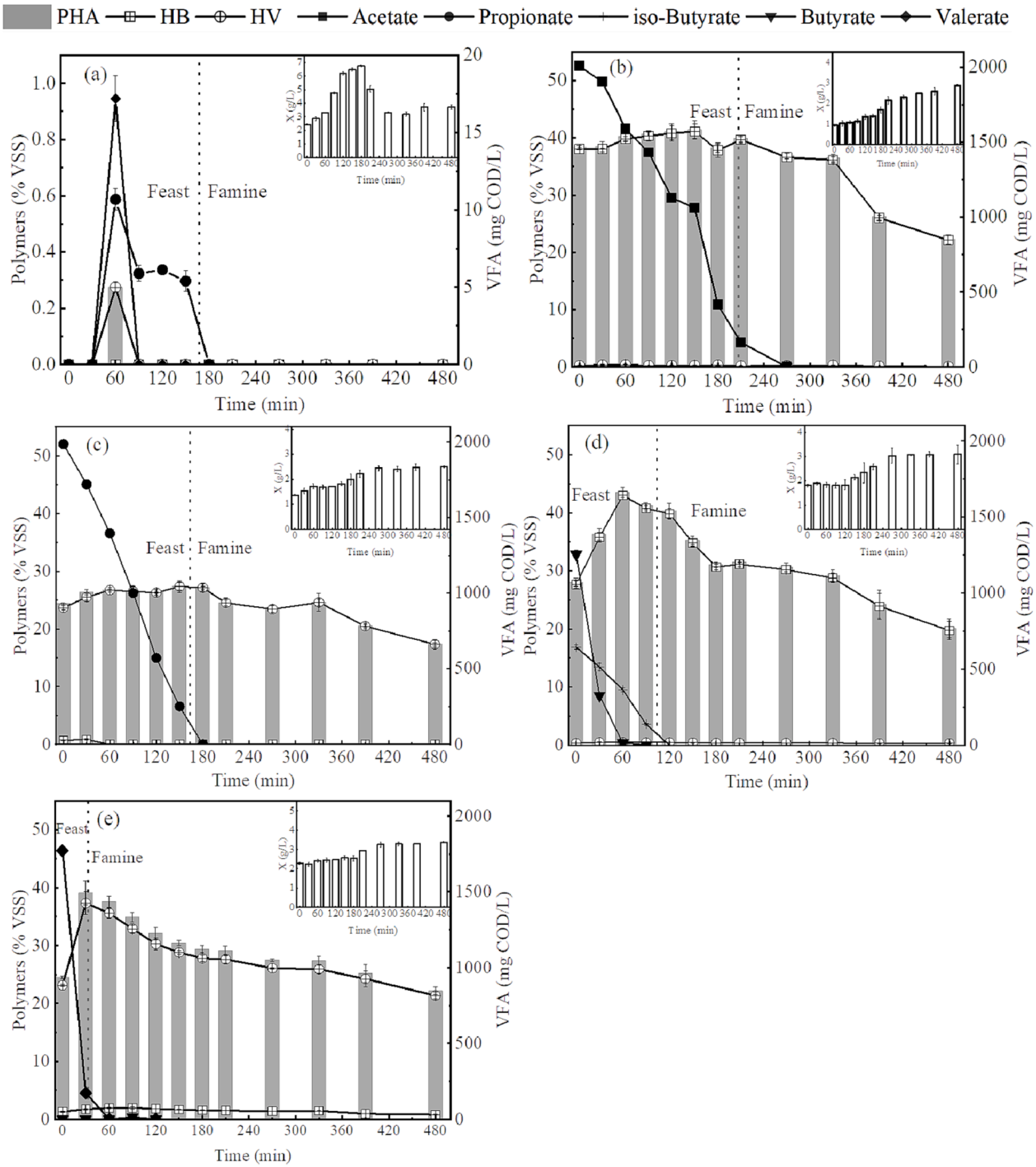

Fig. 1. Substrate uptake, PHA synthesis and microbial growth over the cycle: (a) Glucose; (b) Acetate; (c) Propionate; (d) Butyrate; (e) Valerate.

the acetate depletion (see Fig. 1 (b)). The absorption rate of n-butyrate was 0.9994 g COD/g X/h in the first 30 min. n-Butyrate was consumed after 60 min of aeration, while *iso*-butyrate was still present and was exhausted after 100 min. The PHA_m reached 43.63% VSS with n-butyrate depleted rather than at the end of the feast stage (see Fig. 1 (d)). The presence of *iso*-butyrate might prolong the length of the feast stage. The PHA content decreased slightly (-3.35% VSS) before *iso*-butyrate depletion. It might be that MCs did not need to consume excessive internal carbon sources to maintain cell activities in the presence of *iso*-butyrate. PHA content sharply decreased (-9.25% VSS) and microbial biomass increased obviously at the start of the famine stage. It was probable that there was high microbial activity at the end of the feast stage and internal carbon sources were consumed for life-sustaining activities. Similar phenomena were observed in other reactors.

Acetate and butyrate were common in anaerobic fermentation and were ideal carbon sources for PHB synthesis. Acetate was absorbed by MCs and activated into acetyl-CoA. Acetyl-CoA was synthesized into PHB through condensation, reduction, and polymerization (Meng et al., 2014). Butyrate could be activated into butyryl-CoA directly after being absorbed by MCs and then oxidized into hydroxybutyryl-CoA, which could be directly used in the synthesis of PHB (Wang et al., 2018). However, the acetate was absorbed at a slower rate (0.3314 g COD/g X/h) than the others (see Table 1). It might be that the initial PHA content (38.31% VSS) in MCs closed to its maximum value (or saturation value), which limited the conversion rate of acetate to HB. Furthermore, 1 mol acetate required 1 mol ATP to produce acetyl-CoA (De Donno Novelli et al., 2021), which was more energy-intensive than other VFAs. Energy limited the substrate uptake rate under the same aeration rate conditions. In particular, the dissolved oxygen (DO) fell to zero during the feast phase in this work (data not shown).

With propionate as substrate, the specific substrate uptake rate (q_s) in the feast stage was 0.4417 g COD/g X/h, while its maximum PHA content was only 27.63 % of dry cell weight (Fig. 1 (c)). The valerate was absorbed at the fastest rate and was almost completely consumed within the first 30 min of the reaction (Fig. 1 (e)), and the specific substrate uptake rate (q_s) reached 1.4222 g COD/g X/h. While the maximum PHA content (38.27% VSS) did not show similar superiority.

Propionate and valerate were also commonly found in anaerobic fermentation effluent. These O-FAs were absorbed by MCs and utilized for the production of HV. The propionate absorbed by MCs was activated to propionyl-CoA, which would decarboxylate to acetyl-CoA, resulting in the loss of carbon (Scolamiero et al., 2014). Acetyl-CoA would preferentially undergo selective condensation with propionyl-CoA to form HV, and the remaining acetyl-CoA would form HB (Lemos et al., 2006). Therefore, the yield of PHA was low but HV still dominated. Valerate could be converted to valeryl-CoA directly for the synthesis of HV, a pathway that required lower energy (Sagong et al., 2018). Hence, it could be absorbed in the shortest time. The greatest selection pressure was created by the low F/F ratio at 0.06. During the long-lasting famine phase of MCs, the famished microorganisms had a stronger appetite for the substrate, allowing valerate to be absorbed in a high rate. It had long been found that the ratio of the duration of the feast phase to the entire cycle should not exceed 0.25 (Wen et al., 2020a). However, there was an F/F ratio threshold in the enrichment system, and the maximal PHA accumulation capacity of MCs did not increase considerably when the F/F ratio was reduced further after reaching the critical point (Huang et al., 2020).

VFAs with longer carbon chains (such as butyrate and valerate) required less energy to produce PHA than acetate and propionate (Wang et al., 2017a). What's more, acetate and propionate were more inclined to be used for microbial growth. The biomass production yield on substrate ($Y_{X/S}$) were 0.6664 and 0.2481 g COD/g COD respectively (see Table 1). Therefore, different mixing ratios of VFAs were set to explore the possibility of obtaining PHAs with higher yields and better performance.

Fig. 2 showed the PHA synthesis and substrate consumption respectively in the reactors fed with mixed VFAs. Similar to the single substrate, the specific substrate uptake rate and PHA_m were greater in But-VFAs and Val-VFAs than others (0.6628 g $COD_{.S}$/g X/h, 0.6251 g $COD_{.S}$/g X/h, and 72.80 % VSS, 61.57 % VSS, respectively). The PHA_m remained at the minimum (47.43 % VSS) in the propionate-rich reactor (see Table 2). Nevertheless, the yield of PHA with mixed VFAs as substrate was obviously increased compared to that of a single substrate, with about 1.6 times higher yield. Microorganisms competed fiercely for the same single carbon source, which reduced the flux to PHA production (Huang et al., 2022). For example, when butyrate or valerate was introduced as the sole carbon source, since acetyl-CoA was an important precursor for cellular metabolism, they had to undergo β-oxidation to produce acetyl-CoA (and propionyl-CoA) for cell maintenance via the TCA cycle, while the remainder was utilized for PHA storage. However, with other substrates, such as acetate and propionate, available for cell growth, microorganisms could use butyrate and valerate more efficiently for PHA synthesis without going through the β-oxidation pathway (Albuquerque et al., 2013).

The yield of HV in the feast stage was higher than that of HB in all other reactors except for the reactors rich in E-FAs. A similar situation occurred with the consumption of HB and HV during the famine phase (see Table 2). However, the HB to HV ratio at the end of the feast phase was close to 1: 1 except for the butyrate-rich reactor, regardless of the E-FAs to O-FAs ratio. Even in the butyrate-dominated reactor, the proportion of HV reached 20% (see Table 2). Unlike the study by Li et al. (2020) with HB to HV ratios of (84 to 94): (6 to 16) at different E-FAs/O-FAs ratios, the HV yields were evidently lower. It might be mainly owing to the fact that the VFAs in their substrates were acetic acid, propionic acid and butyric acid, and propionic acid was the only odd VFA. Thus, the condensation of acetyl-CoA with propionyl-CoA was the primary process of HV synthesis. Similarly, even with the mixed substrates of propionate and acetate (1: 1), the HB fraction in PHA was far more than HV in all tests (Miao et al., 2016). In the presence of other carbon sources, valerate was mainly metabolized to PHA precursors for co-polymerization and not for growth. Therefore, it was presumed that the presence of valerate was more favorable for the synthesis of HV than propionate. For further validation, the synthetic pathways of HB and HV in different reactors were analyzed by metabolic flux calculations.

3.2.2. *Synthesis pathway of PHA monomer*

The metabolic flux analysis (MFA) revealed the main synthesis pathways of HB and HV, which was beneficial for judging a better

Table 1
Yields and rates obtained of the cultures enriched from the single carbon sources.

Substrate	$-q_{s\text{-feast}}$ g $COD_{.S}$/g X/h	$q_{HB\text{-feast}}$ g $COD_{.PHA}$/g X/h	$q_{HV\text{-feast}}$ % VSS	PHA_m g $COD_{.PHA}$/g COD	$Y_{PHA/S\text{-feast}}$ g $COD_{.X}$/g COD	$Y_{X/S\text{-feast}}$
Glucose*	0.3133^{c}	0.0000^{d}	0.0061^{c}	0.27^{e}	0.0190^{e}	0.9253^{a}
Acetate	0.3341^{c}	0.2810^{b}	0.0011^{c}	40.95^{b}	0.8143^{d}	0.6664^{b}
Propionate	0.4417^{b}	$(0.0073)^{d}$	0.4619^{b}	27.63^{d}	0.9958^{b}	0.2481^{c}
Butyrate	0.5093^{b}	0.7632^{a}	0.0092^{c}	43.63^{a}	1.5347^{a}	0.0109^{d}
Valerate	1.4222^{a}	0.0371^{c}	1.2168^{a}	38.27^{c}	0.8390^{c}	0.0143^{d}
Pooled standard error	0.4175	0.2944	0.4733	15.9073	0.4862	0.3651

* Calculated only from the data when PHA synthesis was available Note: Vertical data marked with different alphas indicate significant differences ($p < 0.05$).

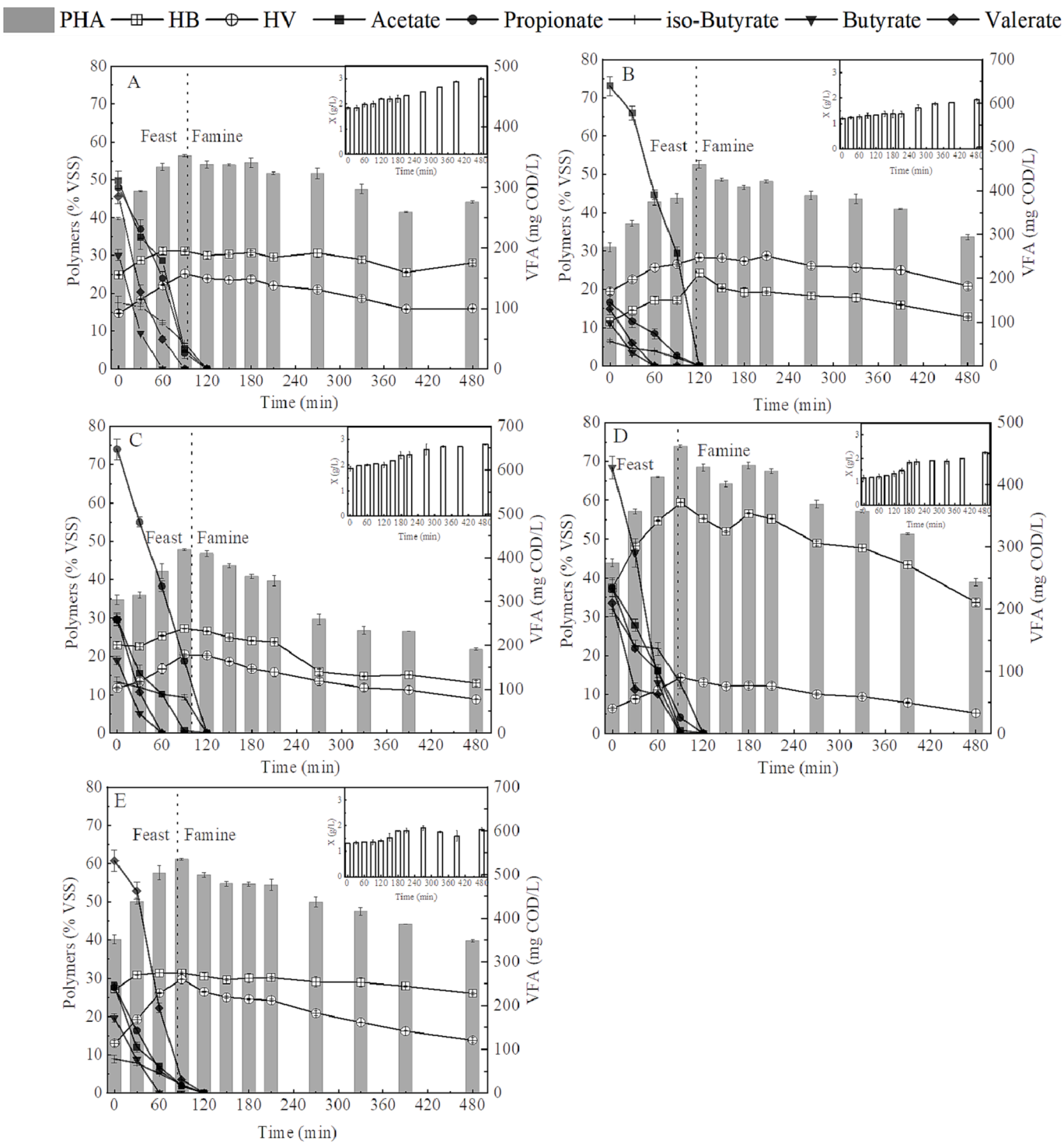

Fig. 2. Substrate uptake, PHA synthesis and microbial growth over the cycle: Ac/Prop/But/Val (based on g COD) =(A): 1: 1: 1: 1; (B): 3: 1: 1: 1; (C): 1: 3: 1: 1; (D): 1: 1: 3: 1; (E): 1: 1: 1: 3.

feeding strategy. Considering the minor concentration of *iso*-butyric acid in the real wastewater, the above study also showed that the synthesis of PHA during the feast stage was almost negligible when *iso*-butyrate was at a low level. Therefore, the metabolic pathway of *iso*-butyrate for PHA synthesis was ignored in the metabolic flux calculation.

Fig. 3 displayed the outcomes of the metabolic flux calculation. Condensation of acetyl-CoA and propionyl-CoA was one of the main pathways of HV synthesis, and the carbon flux of this process was represented by r_{11}. The propionyl-CoA decarboxylation process was shown by the flux r_5. The minimum flux r_5 (0.0019C mol/C mol/h) combined with flux r_{11} (0.0596C mol/C mol/h) in Ac-VFAs indicated that most propionyl-CoA was utilized for HV synthesis. It might be that a substantial amount of acetyl-CoA was produced with plentiful acetate, which allowed propionyl-CoA to be condensed with acetyl-CoA instead of decarboxylation (Jiang et al., 2011). Similarly, the flux r_{11} was the most significant in Prop-VFAs (0.1064C mol/C mol/h). Combined with flux r_5 (0.0314C mol/C mol/h) and flux of propionyl-CoA activation (r_{10}, 0.0639C mol/C mol/h), indicated that the condensation of acetyl-

Table 2
Yields and ratios obtained of the cultures enriched from the mixed VFAs.

Substrate	$-q_{s\text{-}feast}$	$q_{HB\text{-}feast}$ g COD/g X/h	$q_{HV\text{-}feast}$	PHA_m % VSS	$Y_{X/S\text{-}feast}$ g COD_X/g COD	E-FAs: O-FAs	%HB: %HV-$_{feast}$	$-q_{HB\text{-}famine}$ g COD/g X/h	$-q_{HV\text{-}famine}$
U-VFAs	0.3945^{c}	0.1515^{d}	0.2763^{b}	56.07^{c}	0.1826^{c}	58: 42	55: 45	0.0270^{c}	0.0739^{b}
Ac-VFAs	0.3620^{d}	0.2240^{c}	0.2061^{c}	52.29^{d}	0.2088^{b}	77: 23	46: 54	0.0903^{b}	0.0764^{b}
Prop-VFAs	0.3928^{c}	0.1140^{e}	0.2132^{c}	47.43^{e}	0.2449^{a}	39: 61	57: 43	0.0751^{d}	0.0739^{b}
But-VFAs	0.6628^{a}	0.7337^{a}	0.2739^{b}	72.80^{a}	0.0978^{d}	69: 31	80: 20	0.1388^{a}	0.0669^{b}
Val-VFAs	0.6251^{b}	0.3155^{b}	0.6802^{a}	61.57^{b}	0.0509^{e}	42: 58	51: 49	$(0.0219)^{d}$	0.1310^{a}
Pooled standard error	0.1292	0.2242	0.1777	8.7399	0.0722			0.0576	0.0243

Note: Vertical data marked with different alphas indicate significant differences ($p < 0.05$).

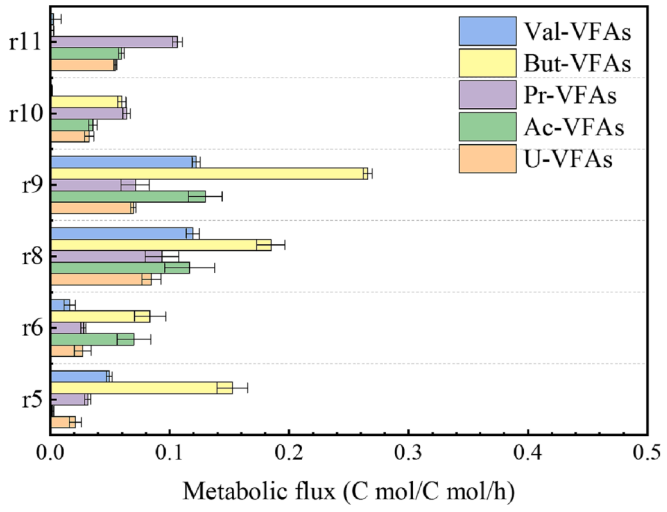

Fig. 3. Metabolic flux analysis of mixed VFAs for the synthesis of PHA (r_5: decarboxylation; r_6: catabolism; r_8: oxidative phosphorylation; r_9: HB monomer; r_{10}: activation of propionyl-CoA; r_{11}: condensation of acetyl-CoA with propionyl-CoA).

CoA with propionyl-CoA was the main contributor of HV. It also verified that the decarboxylation of propionyl-CoA led to carbon loss, which in turn led to a decrease in PHA content. Since the build-up of propionyl-CoA in microorganisms might inhibit the acetyl-CoA-dependent enzymes essential for growth, it was considered to suppress the operation of the TCA cycle, resulting in limiting the production of $NADH_2$ and ATP and further hindering the aggregation of HB and HV monomers (Huang et al., 2020). High fluxes in the condensation of propionyl-CoA and acetyl-CoA (r_{11}) and decarboxylation of propionyl-CoA (r_5) pathways avoided the accumulation of propionyl-CoA.

Direct activation of valerate to valeryl-CoA was another pathway for the synthesis of HV. But-VFAs had the highest value of flux r_5 (0.1523C mol/C mol/h). Combined with flux r_{11}, it indicated that the HV generated in this reactor was almost from the direct activation of valeryl-CoA generated from valerate. The HV formed by the condensation of acetyl-CoA and propionyl-CoA was almost absent, which in turn led to a lower HV content than other reactors. Butyric acid was prioritized and required a greater proportion of propionic acid before the bacterium will tend to synthesize carbon into HV (Huang et al., 2020). Thus, low levels of propionyl-CoA tended to decarboxylate to acetyl-CoA for HB synthesis ($r_9 = 0.2657$C mol/C mol/h) in But-VFAs. A similar phenomenon was observed in Val-VFAs, where only a trace of propionyl-CoA was utilized for HV ($r_{11} = 0.0026$C mol/C mol/h). It was possible that the low levels of acetate were unable to generate sufficient acetyl-CoA and that the decarboxylation of propionyl-CoA was necessary to make up the difference. Despite the high rate of decarboxylation (flux r_5) in But-VFAs, there was still 20% of HV in the polymers, which primarily came from valerate metabolism. The pathway of HV synthesis from propionate was more susceptible to the influence of substrate components, such as acetate. The previous idea, that the presence of valerate in the substrate could result in a more stable HV production, was also validated.

Cellular respiration and proliferation were essential approaches to carbon consumption as well. In Ac-VFAs, the flux r_6 (0.0700C mol/C mol/h) was observed to be second only to But-VFAs, suggesting that the presence of large amounts of acetyl-CoA also leads to a partial flow of carbon to cellular respiration and growth. Butyrate also produced large amounts of acetyl-CoA for HB synthesis and respiration ($r_6 = 0.0834$C mol/C mol/h) by the process of β-oxidation. Through β-oxidation, valerate could also be transformed into acetyl-CoA and propionyl-CoA, which could enter the TCA cycle for microbial respiration. Acetyl-CoA was required for the maintenance of basic microbial life activities. As mentioned previously, when substrates could not provide sufficient acetyl-CoA, longer-chain VFAs (such as propionate, butyrate and valerate) tended to produce acetyl-CoA via β-oxidation. The remainder of the ingredients were converted into PHA.

3.3. Effect of substrate composition on microbial community

The outcomes of microbial community analysis using 16S rRNA gene amplicon sequencing were shown in Fig. 4 as a proportion of the relative abundance in the phylum. After 20 cycles of enrichment with diverse carbon sources, the F/F mechanism exerted selective pressure on MCs, and the microbial population altered considerably. A considerable decrease in microbial diversity was seen in the SBR using glucose as a single carbon source, according to changes in the Shannon index (see Supplementary materials). The MCs were mainly composed of *Actinobacteria* (46.22 %) and *Cyanobacteriia* (only including *norank_f_norank_o_Chloroplast*, a genus without a specific name and named by the Majorpio cloud platform, 46.38 %). PHA synthesis was severely hindered by the low amount of PHA producers present in the reactor as well as the acidic environment present during the process. Acidophilic microorganisms such as *Propionibacteriaceae*, *Acidocella* and *Acidipropionibacterium* (Jones et al., 2013) were also enriched, which was related to the low pH in the reactor.

In the SBRs using VFAs as the substrate, a slight reduction in microbial diversity was seen, however, principal component analysis (PCA) revealed a significant difference in the microbial communities (see Supplementary materials). These results suggested that the ADF coupled nitrogen limiting mode would produce a particular selection pressure that led to the succession of microbial communities and changes in biodiversity. *Gammaproteobacteria*, *Alphaproteobacteria,* and *Bacteroidia* were the main microorganisms at the class level in all reactors with mixed VFAs.

The composition ratio of VFAs had no noticeable impact on the type of microorganism but did have significant effects on their relative abundance (Li et al., 2020). *Cyanobacteria* (17.04 %) also appeared to be enriched in Ac-VFAs. It was shown that *Cyanobacteria* can synthesize PHB in the presence of acetic acid (Singh et al., 2016). Although the abundance of *Cyanobacteria* was greater when glucose was used as a single substrate, the alkaline environment in the Ac-VFAs system was more suitable for the synthesis of PHA. Moreover, *Pseudofulvimonas* (17.24 %) and *Brevundimonas* (8.15 %) were the main PHA producers in

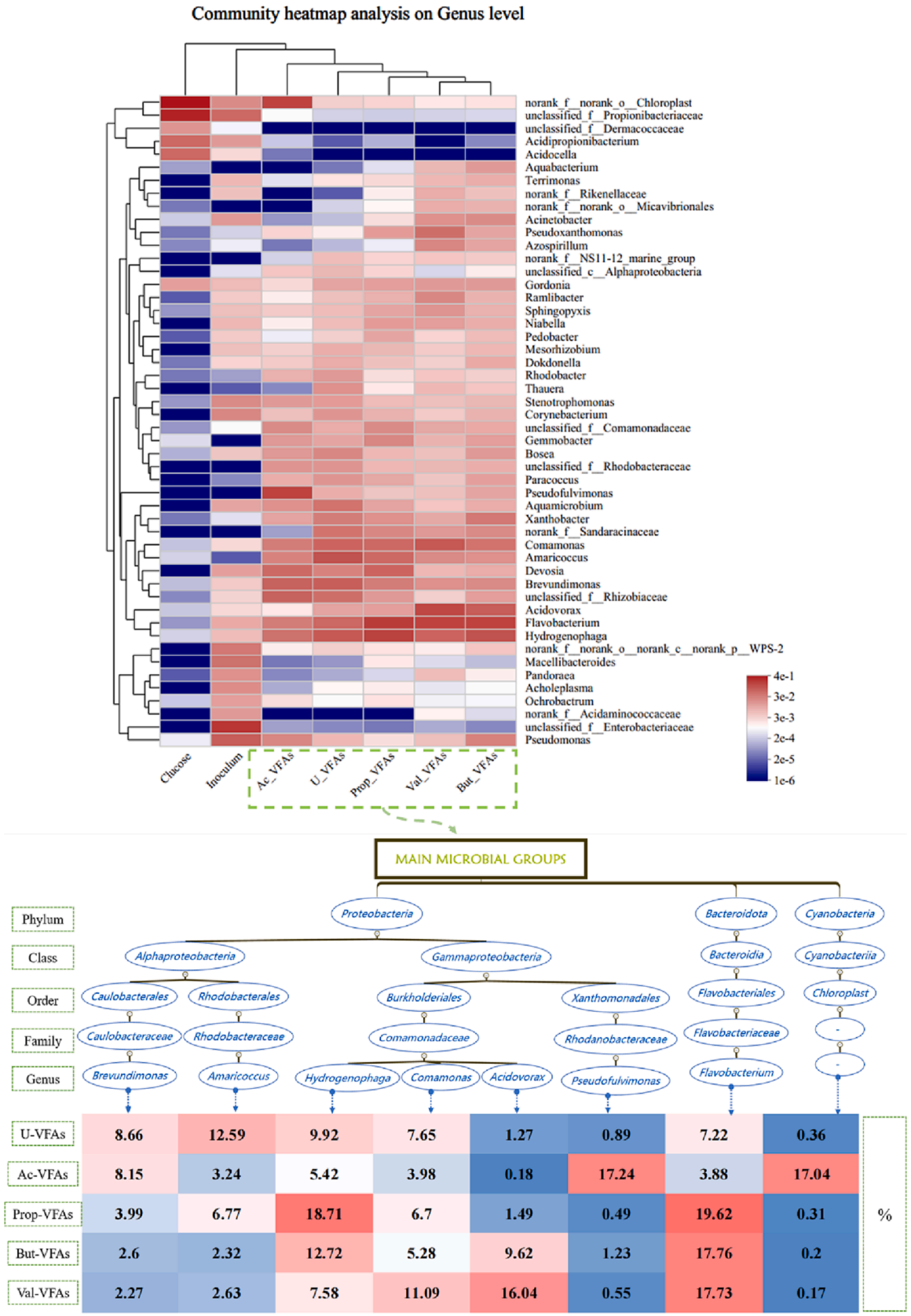

Fig. 4. Heatmap of the most abundant taxonomic groups identified in the MCs fed with various substrates.

Ac-VFAs. Similar PHA producers such as *Flavobacterium, Hydrogenophaga, Acidovorax, Comamonas,* and *Amaricoccus* were observed in the MCs of Prop-VFAs, But-VFAs and Val-VFAs with varied relative abundance (see Fig. 4).

Different microbes had a preference for different substrates. Acetate was the most favored substrate for *Pseudofulvimonas* (Cui et al., 2016), which could explain the high relative abundance. *Brevundimonas* could synthesize PHB with acetic acid as the only carbon source and the F/F mode did not affect its growth and reproduction (Liang et al., 2023). Therefore, it was more powerful in acetate-enriched conditions. Wang et al. (2017b) employed acetate as a carbon source to accumulate MCs for PHA synthesis in propylene oxide saponification wastewater and analyzed the changes in microbial communities. They found significant successional changes in microbial populations, with a significant increase in *Brevundimonas* especially. *Flavobacterium* was widely found in activated sludge from wastewater treatment plants and was the main microorganism that existed in the culture enriched for PHA production (Wang et al., 2017a; Wen et al., 2022). *Hydrogenophaga* could synthesize PHA with different carbon sources, including sugars, and VFAs (Wen et al., 2022). Unlike others, it was more advantageous at higher temperatures (30 °C) (Crognale et al., 2019), which operated in this work. Studies had shown that numerous *phaC* sequences related to PHA synthesis were related to those of *Hydrogenophaga* spp., *Acidovorax* spp., and *Pseudomonas* spp. (Crognale et al., 2019). *Comamonas* was a familiar group of PHA-accumulators as well, and organic acids were suitable carbon sources for its PHA production. PHA content reached 59 wt% with valerate as substrate and 30 wt% with acetate and propionate (Zakaria et al., 2010), which indicated that *Comamonas* had a preference for valerate. As a result, it had a greater relative abundance among Val-VFAs. *Amaricoccus* was also a frequently reported PHA producer and had a preference for propionate (Lemos et al., 2008). Thus, its relative abundance (6.77%) in Prop-VFAs was higher than in other reactors.

Unlike most studies, the relative abundance of two common PHA producers, *Paracoccus* and *Thauera*, was not dominant in either reactor of this study. *Thauera* did not take up acetate and only mildly ingested butyrate and valerate (Albuquerque et al., 2013), thus showing little enrichment in Ac-VFAs. *Paracoccus* could take up a wider range of substrates, with an intense preference for the uptake of propionate, butyrate, and valerate in particular (Wang et al., 2017a). The lack of superiority might be that the relative abundance of the two species in the inoculated sludge was relatively low and the short accumulation was insufficient for them to compete successfully. Also importantly, it was possible that the operating conditions (T = 30 °C, uncontrolled pH, low DO level in the feast stage) of this study were not optimal for their growth. With the exception of the substrate, the operating conditions during MCs enrichment, such as temperature, DO concentration, pH, and SRT, would affect the composition of microorganisms (Nguyen-huynh et al., 2021). However, the capacity of the system to produce PHA was mainly related to the degree of enrichment of PHA producers and not much to the type of PHA producers.

By studying the production of PHA under different substrate conditions, the maximum PHA content (% of VSS) was ranked as But-VFAs (72.08%) > Val-VFAs (61.57%) > U-VFAs (56.07%) > Ac-VFAs (52.29%) > Prop-VFAs (47.43%). PCA analyses revealed greater similarity between the enriched microbial communities with butyrate and valerate dominance (see Supplementary materials). This could also explain the high PHA yield in Val-VFAs. The addition of butyrate to the substrate could effectively improve the PHA yield, while the addition of valerate could effectively improve HV synthesis and obtain better-performing PHA polymers. Meanwhile, compared to the butyrate-rich, the valerate-rich substrates not only facilitated the synthesis of HV but also allowed a good yield of PHA. The anaerobic acid production could be adjusted to facilitate butyric or valeric acid-type fermentation by changing the operating conditions.

In addition, further studies are needed to clarify the feasibility of valerate in the PHA production process. For example, the synthesis of PHA in conditions of various valerate concentrations, and whether the inhibition effect occurs at high concentrations of valerate. The importance of valerate for improving HV production cannot be neglected.

4. Conclusions

In this study, the effects of substrate composition on the characteristics of PHA production were explored using a variety of single and mixed VFAs as carbon sources. Waste activated sludge was successfully acclimated to produce PHA within about 60 h via the mode of aerobic dynamic feed at nitrogen-limiting condition. In Val-VFAs, not only the maximum PHA content of 61.57% VSS was obtained second to But-VFAs, but also the polymers containing 49% of HV. MFA results showed that the presence of valerate in the substrates was beneficial to a more robust PHA production. *Hydrogenophaga* and *Comamonas* were main PHA producers.

CRediT authorship contribution statement

Ziying Zhang: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Yan Lin:** Methodology, Resources, Validation, Writing - original draft. **Shaohua Wu:** Methodology, Writing – original draft. **Xiang Li:** Validation, Writing – original draft. **Jay J. Cheng:** Methodology, Writing – original draft. **Chunping Yang:** Conceptualization, Methodology, Supervision, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant Nos.: 52270064, 51978178, and 51521006), Maoming Municipal Department of Science and Technology of Guangdong Province of China (Contract No.: 2018S0013), the Program for Innovative Research Teams of Guangdong Higher Education Institutes of China (Grant No.: 2021KCXTD043), Key Laboratory of Petrochemical Pollution Control of Guangdong Higher Education Institutes (KLGHEI 2017KSYS004), the Science and Technology Innovation Program of Hunan Province of China (Contract No.: 2021RC2058), and the Startup Fund of Guangdong University of Petrochemical Technology (Contract No.: 2018rc63).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.129445.

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