Dependence of poly-β-hydroxybutyrate accumulation in sludge on biomass concentration in SBRs

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ABSTRACT

The combination of wastewater treatment with polyhydroxyalkanoate production has attracted increasing interest in the context of the circular economy. Recent studies have thus attempted to optimize the conditions for polyhydroxyalkanoate accumulation in sludge when treating wastewater. The effects of biomass concentration and sludge morphologies in reactors on PHB storage, however, were neglected in the literature. Therefore, in this study settling time and organic loading rate were manipulated to adjust sludge morphology and biomass concentration in sequential batch reactors (SBRs) to investigate their influence on PHB storage in the feast phase. Our study shows that reducing settling times in SBRs from 10 to 0 minutes under organic loading rate of 3 g L^{-1} d^{-1} resulted in the decrease in biomass concentration at steady states from 4.2 to 1.0 g L^{-1} and the change of sludge morphology from well-settled granules to poorly settled pinpoint flocs, but PHB content in sludge at the end of feast phase increased from 7.7 to 26.7%. The well-fitted regression lines between PHB content, SRT, feast/famine and food/microorganisms ratios and biomass concentration under different settling times suggest that PHB was highly dependent on biomass concentration but independent on sludge morphology. Under settling time of 0 minutes, the increase in OLR from 3 to 7.5 g L^{-1} d^{-1} resulted in an increased biomass concentration from 1.0 to 2.1 g L^{-1} and an increase in PHB content from 26.7 to 33.8%. The batch and fed-batch experiments with different biomass concentrations also showed the influence of biomass concentration on PHB accumulation in sludge. The conclusion of the dependence of PHB content on biomass concentration under a fixed OLR and varied OLRs drawn from this study enables sludge PHB content as high as possible by adjusting biomass concentration in SBRs apart from the selective enriching strategies for PHB accumulating organisms when treating VFA-rich wastewater.

Keywords: poly-β-hydroxybutyrate (PHB), biomass concentration, settling time, SBR, biomass loading shock, OLR

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Introduction

Using bio-based and biodegradable plastics such as PHB to replace petroleum-based plastics is believed as one of the most important strategies to solve plastic pollution problems. However, the price of PHB produced from fermentation with pure culture and well-defined media at present is at least three times higher than that of petroleum-based plastics (Gholami et al., 2016) although decades of research has been done. With the popularity of the circular economy concept and its implementation, more studies on PHB have being directed from pure culture to open mixed culture with waste streams to reduce costs of both feedstock and operation by eliminating sterilisation demand (Sabapathy et al., 2020). Even more, it has been gradually realised that PHB production could be integrated with the treatment of conventional wastewater and waste activated sludge (WAS) and thus transform wastewater treatment plants into resource recovery plants (or biorefinery plants) (Morgan-Sagastume et al., 2015). In this case, PHB is produced as one of the recovered products from wastewater when achieving the main function of wastewater treatment plants, i.e. pollutant removal from wastewater. This concept was demonstrated in several pilot-scale wastewater treatment plants in the last few years (Sabapathy et al., 2020). However, the integration of PHB production with conventional wastewater treatment is still very new. The fundamental study is desirable to move PHB production from wastewater forwards for real application in the context of a circular economy.

Although PHB storage was originally found in biological phosphate removal sludge (Fuhs and Chen, 1975), PHB accumulation in non-phosphate removal sludge was later reported as well (Liu et al., 1996). Gradually, people realised that intracellular polymer PHB storage in activated sludge is a common phenomenon. Sludge from conventional wastewater treatment plants without any particular enrichment could accumulate PHB up to 7.9-24% in acetate-fed 24-h batch experiments under aerobic and nitrogen- and phosphorus-limited conditions (Sakai et al., 2015). PHB is more easily stored in sludge under transient conditions such as sequential batch reactors (SBR) operation (Fang and Liu, 2000). SBR operation which creates feast and famine phases (i.e. transient conditions with substrate gradient over time) is favourable for the selection and enrichment of PHB accumulating organisms because organisms with PHB storage outcompete those without intracellular polymer storage during the famine phase for stored PHB to be used as a carbon source for growth. Thus, SBR with aerobic dynamic feeding is widely adopted for the enrichment of PHB accumulating organisms. For wastewater treatment, SBR is also extensively used with either activated sludge or aerobic granular sludge. PHB storage with variable contents from 9% to 68% in well-settled aerobic granules in SBRs fed with palm oil mill effluent (Gobi and Vadivelu, 2014), domestic wastewater (Karakas et al., 2020), and synthetic wastewater (Wang et al., 2014) was reported while bulking sludge with poor settleability was found to have high PHB storage response (Beccari et al., 1998). From these reports, we can see that high PHB storage could be obtained from either well-settled granular sludge or poor-settled bulking sludge, implying that PHB storage might not be dependent on sludge settleability. The relationship between PHB storage in cells and filamentous or non-filamentous bulking of sludge under different conditions was studied to shed light on the mechanism of ecological selection for the control of sludge bulking in conventional wastewater treatment plants because sludge bulking can cause biomass loss from reactors and costly liquid-solid separation after wastewater treatment in downstream. However, when the enrichment of PHB accumulating organisms or PHB production is one of the main research purposes, sludge settleability or sludge characteristics are mostly neglected and not well studied and reported. It seems more focuses were put on the achievement of PHB content in cells as high as possible or the effects of operating conditions such as sludge retention time (SRT), pH, acetate concentration (Chua et al., 2003), temperature (Chinwetkitvanich et al., 2004), feast/famine (F/F) ratio (Hao et al., 2018), organic loading rate (Lorini et al., 2020) on PHB accumulation. PHB production efficiency from sludge depends on both PHB content in cells and harvested sludge amount during a certain period (Johnson et al., 2009; Valentino et al., 2015). Recently, this problem has attracted some attention from researchers and the effects of sludge settleability and biomass concentration on PHB accumulation were investigated.

It was reported that bulking sludge could accumulate PHB to a comparable content with well-settled activated sludge and thus saved energy required for aeration in the culture enrichment stage due to the lower dissolved oxygen demand of filamentous bacteria (Wen et al., 2012). However, poor sludge settleability can lead to less biomass concentration in reactors, higher biomass loading rate (i.e. high F/M ratio), longer feast phase in a single cycle, which in turn might affect PHB yield and productivity. In addition, harvesting bulking sludge for PHB extraction could be more expensive. On the contrary, aerobic granular sludge with good settleability could overcome the disadvantages of bulking sludge. Meanwhile, PHB content in granular sludge could reach comparable contents to bulking and flocs-formers under appropriate conditions (Wen et al., 2012; Gobi and Vadivelu, 2014). It seems that sludge morphology and settleability is not an important factor affecting PHB content in sludge, but the studies on bulking and granular sludge were conducted under different conditions, making the fair comparison hard. Furthermore, there is a requirement of hydraulic selective pressure to maintain granular sludge structure, which makes the operation of reactors for the enrichment of PHB accumulating organisms a bit more complicated because both maintaining granule structure and enriching PHB should be achieved under the same conditions. Thirdly, sludge settleability affects biomass retention in SBRs, and further influences biomass loading rate, feast/famine ratio, SRT, substrate gradient, which are believed to be important for PHB storage. To understand the relationship between sludge characteristics and PHB accumulation, (Li et al., 2019) correlated PHB storing capacity with sludge characteristics such as sludge flocs size, biomass concentration and extracellular polysaccharide concentration, but this correlation was only limited to PHB accumulation stage rather than enrichment stage. The correlation of biomass with PHB content in the enrichment stage is more critical when PHB enrichment is combined with wastewater treatment.

This study thus aimed to investigate the effects of sludge settleability and morphology on PHB storage under the same operating conditions by using different settling times in SBRs to form granular sludge or suspended sludge with different SVI. To further understand the interaction between sludge settleability, biomass concentration in reactors, and PHB storage, three levels of organic loading rates were applied. The ultimate purpose of this study was to deepen the understanding of the interaction between different factors caused by sludge settleability and biomass concentration in reactors, and corresponding PHB storage during the culture enrichment stage, guiding engineering operating conditions for the integration of PHB enrichment with wastewater treatment.

2. Materials and Methods

2.1 The enrichment of PHB accumulating organisms in Sequence batch reactors

Three bubble column reactors with a working volume of 2 L as triplicates were inoculated with the excess activated sludge from the Millbrook wastewater treatment plant in Southampton, United Kingdom, for the enrichment of PHB accumulating microorganisms. The reactors were operated in sequential batch mode as shown in Figure 1 with a cycle of 6 hours including 8-minute feeding, 337-327-minute aeration, 0-10-minute settling, and 5-minute discharging. With an exchange ratio of 0.32, the hydraulic retention time (HRT) was maintained at 0.8 days. Due to the variation of biomass concentrations in the reactors under different settling times, SRT ranged between 1 to 10 days. Air was continuously supplied with a flow rate of 3.0 L min^{-1} to provide sufficient oxygen. The temperature was kept at 28℃ with heating tapes and pH was not controlled with a variation from 7.5 to 8.4.

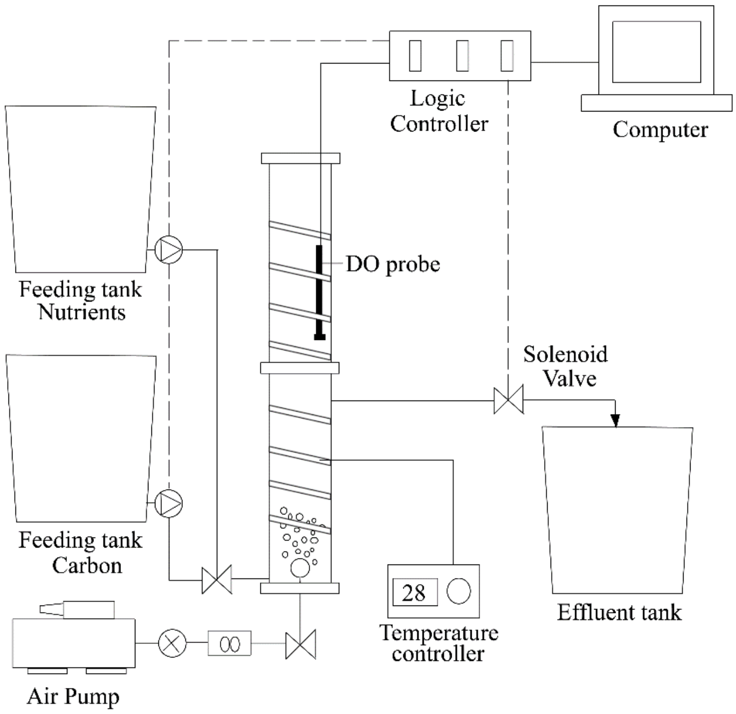

Figure 1. Schematic diagram of sequential batch reactor operation

The inoculated excess activated sludge was acclimatized for 38 days by increasing the concentration of COD in the synthetic wastewater until 2.4 g COD L^{-1} to allow the sludge to adapt to higher influent COD concentration and SBR operation. After the acclimatization, the reactors were fed with 0.65 L of synthetic wastewater, which composed of 5.0 g L^{-1} $CH_3COONa{\cdot}3H_2O$ as the carbon source (equivalent to 2.4 g COD L^{-1}), 560 mg L^{-1} $(NH_4)_2SO_4$, 53 mg L^{-1} KH_2PO_4, and 67 mg L^{-1} K_2HPO_4 with carbon, nitrogen, and phosphorus ratio (C: N: P ratio) at 100:5:1, and micronutrients such as 40 mg L^{-1} of $CaCl_2.2H_2O$, 12.5 mg L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.307 mg L^{-1} of $FeSO_4 \cdot 7H_2O$, 0.045 mg L^{-1} of H_3BO_3, 0.009 mg L^{-1} of $CuSO_4 \cdot 5H_2O$, 0.054 mg L^{-1} of KI, 0.036 mg L^{-1} of $MnC1_2 \cdot 4H_2O$, 0.018 mg L^{-1} of $Na_2MoO_4 \cdot 2H_2O$, 0.036 mg L^{-1} of $ZnSO_4 \cdot 7H_2O$, 0.045 mg L^{-1} of $CoCl_2 \cdot 6H_2O$, 3 mg L^{-1} of EDTA. 20 mg L^{-1} Thiourea was added to inhibit nitrification (Wang et al., 2017). The reactors were operated with an organic loading rate of 3.0 g COD L^{-1} d^{-1}.

The reactor operation was divided into two stages. In the first stage from day 0 to day 151, settling time was set as 10 minutes, 1 minute and 0 minute, respectively, to provide different hydraulic selective pressure and thus control the sludge concentration and morphology in the reactors. In the second stage from day 151 to day 189, OLR was increased from 3.0 g COD L^{-1} d^{-1} to 6.0 g COD $L^{-1}d^{-1}$, and further to 7.5 g COD L^{-1} d^{-1} by increasing the concentration of $CH_3COONa{\cdot}3H_2O$ in the influent from 5.0, to 10, and 12.5 g L^{-1} $CH_3COONa{\cdot}3H_2O$, respectively, while COD: N : P ratio was still maintained at 100: 5: 1. Only after reactors reached steady states with stable values of MLSS, PHB content in sludge, SVI, effluent ammonium and phosphate concentrations and stable sludge morphology, reactor operational conditions were changed. In both stages, samples were collected from the beginning of the aerating phase when the bacteria started to consume the carbon and nutrients, the end of the feast phase when the provided external carbon source in the medium was depleted which could be identified by a sharp increase in dissolved oxygen (DO) by an online measurement with a DO meter (YSI 5100), and the end of the aeration phase. From the depletion of the external carbon source to the end of the aerating phase, it was defined as the famine phase.

Table 1. Summary of operational conditions in two stages with variable settling times and OLRs

	Operation stage 1			Operation stage 2		
Phase	I	II	III	I	II	III
Settling time (min)	10	1	0	0	0	0
OLR (g COD $L^{-1}d^{-1}$)	3.0	3.0	3.0	3.0	6.0	7.5
Influent COD concentration (g COD L^{-1})	2.4	2.4	2.4	2.4	4.8	6.0

2.2 PHB accumulation in the fed-batch reactors

Fed-batch experiments depending on a feed-on-demand method (Valentino et al., 2015; Zeng et al., 2018) were conducted to examine the maximum PHB storage ability of the cultures enriched in SBRs. Airflow rate and temperature were controlled at the same levels as those in SBRs. 200 mL of the enriched culture harvested at the end of the cycle of SBR was inoculated into a 1L fed-batch reactor for overnight aeration to reduce PHB content in the cells from the SBRs to facilitate the maximum PHB accumulation in the subsequent fed-batch experiment. The first fed-batch experiment used the culture from the steady-state of reactors enriched with OLR 3.0 g L^{-1} d^{-1} and settling time of 1 minute, the second with the same OLR but 0 settling time, and the third experiment with OLR 6.0 g L^{-1} d^{-1} and 0 settling time. 5 g L^{-1} $NaAc·3H_2O$ (equivalent to 2.4 g CODL-1) and 10.0 g L^{-1} $NaAc·3H_2O$ (equivalent to 4.8 g COD L^{-1}) were prepared, respectively, for the first fed-batch experiment and the second and third fed-batch experiments accordingly. The substrates had the same concentration of COD as the feeding stock of their respective enrichment processes but without nutrients. There was no residual acetate at the beginning of the fed-batch experiments. An online DO probe was used to monitor the change in DO concentration in the fed-batch reactors. 10 mL of the substrate was initially added into the fed-batch reactors. Once the reduced DO concentration due to the addition of carbon source started to rise sharply, an additional 10 mL of the substrate was added (Dionisi et al., 2006). According to (Dionisi et al., 2001) (Valentino et al., 2017) and our own results, PHB content in cells could not increase any more after DO could not drop further with the addition or presence of an external carbon source. Thus, the fed-batch operation was stopped based on stable DO values with the maximum PHB storage that cells could get.. It should be pointed out that there was no nutrient limitation in fed-batch experiments due to high residual ammonium and phosphate concentrations in effluent from SBRs. The main purpose of the fed-batch experiments in this study is to investigate if high substrate concentration in SBRs could inhibit PHB accumulation in cells.

2.3 Analytical methods

Mixed liquor suspended sludge (MLSS), sludge volume index (SVI_{30}), COD, ammonium, and phosphate were measured in accordance with standard methods (Eaton et al., 2005).

Sludge samples taken from the SBR reactors were placed in centrifuge tubes with each 5 drops of formaldehyde to stop all biological activity before centrifugation, which was conducted in a centrifuge (Thermo, Sorvall Legend XTR) at 8500 rpm (equivalent to 7916 RCF) for 15 minutes. The supernatant was then removed, and the sludge pellets were vortexed with deionized water and centrifuged at the same condition to remove sodium acetate residuals from the sludge. The washed sludge pellets were frozen at -80 ℃ and freeze-dried (VirTis® BenchTop™, K) at a condenser temperature of -80 ℃ for 10 to 14 hours. 5 to 8 mg dried sludge sample was placed into a 15 mL vial and then mixed with 1.5 mL of dichloromethane solution containing 300 mg L^{-1} benzoic acid as an internal standard, and 1.5 mL acidified 1-propanol (4 volumes of hydrochloric acid and 1 volume of 1-propanol). The vial was then kept at 65 °C for 12 hours for the esterification of the extracted PHB and benzoic acid with 1-propanol. After that, the vial was cooled down at ambient temperature and vortexed with 2 mL deionized water to separate the dichloromethane layer, in which esterified PHB and benzoic acid were dissolved. 1 mL of dichloromethane layer was placed into a GC vial with 50 to 80 mg of Na_2CO_3 to dry the residual water in the organic layer. 1 μL of the dried dichloromethane solution was injected into a gas chromatograph (Varian CP – 3800) equipped with a flame ionization detector (FID) and an Agilent DB-FFAP 0.53mm capillary column with a thickness of 0.25 μm and a length of 30 m. Helium was used as the carrier gas with a 50:1 split volume ratio between helium and sample. The temperatures of the sample injector and the detector were set at 230°C and 250°C, respectively. The temperature of the oven with the column was increased from 80°C by a rate of 10°C min^{-1} until 120 °C, and then the heating rate was increased to 45°C min^{-1} until the temperature reached 240°C, at which it was held for three minutes.

Poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid) solutions with 5 different concentrations (sigma-Aldrich) were prepared as external standards against the peaks of internal standard (i.e. benzoic acid) for the quantification of PHB and PHV in sludge samples.

2.4 Calculation of stoichiometric parameters

The feast/famine (F/F) ratio was calculated by dividing the length of the feast Phase by the length of the famine phase in the same cycle. PHB content (wt%) in sludge was calculated by dividing PHB mass with the dry mass of sludge as shown in equation 1.

$$\text{PHB content (wt\%)} = \frac{\text{PHB amount (g)}}{\text{dry mass of a sample (g)}} \times 100 \qquad \text{(eq1)}$$

PHB yield, PHB productivity, biomass yield and the specific uptake rate of acetate were calculated as shown in equations 2-5, respectively. In the calculation, the mass of PHB was converted into Cmol based on that PHB monomer with a chemical formula of $C_4H_6O_2$ and a molecular weight of

21.5 g C mol^{-1} (Marang et al., 2014). The mass of biomass was converted into Cmol based on the assumption that biomass had a general chemical formula of $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight of 24.6 g C mol^{-1} (Beun et al., 2002).

$$\text{PHB yield (PHB Cmol/Acetate Cmol)} = \frac{\Delta \mathrm{PHB_{feast}}\ (\mathrm{wt\%}) * \mathrm{MLSS_r}\ (\mathrm{g\ L^{-1}}) * 68\ (\mathrm{g\ Cmol^{-1}})}{\text{Influent acetate } (\mathrm{g\ L^{-1}}) * \text{Exchange ratio} * 21.5 (\mathrm{g\ Cmol^{-1}}) * 100} \quad \text{(eq2)}$$

$$\text{PHB productivity}_{\text{(g PHB/L·h)}} = \frac{\Delta \mathrm{PHB_{feast}}\ (\mathrm{wt\%}) * \mathrm{MLSS_r}\ (\mathrm{g\ L^{-1}})}{6\mathrm{h}} \quad \text{(eq3)}$$

$$\text{Biomass yield}_{\text{(xCmol/Acetate Cmol)}} = \frac{\Delta \mathrm{NH4{-}N_{feast}} (\mathrm{g\ L^{-1}}) * 68\ \mathrm{g\ C\ mol^{-1}}}{\text{Influent Acetate } (\mathrm{g\ L^{-1}}) * \text{Exchange ratio} * 0.114 * 24.6\ \mathrm{g\ C\ mol^{-1}}} \quad \text{(eq4)}$$

$$\text{Sodium acetate specific uptake rate} = \frac{\Delta \text{Acetate concentration } (\mathrm{g}\ L^{-1}) * 24.6\ \mathrm{g\ C\ mol^{-1}}}{MLSS_r (\mathrm{g}\ L^{-1}) * 68\ \mathrm{g\ C\ mol^{-1}} * \text{Length of feast Phase (h)}} \quad \text{(eq5)}$$

Where ΔPHB_{feast} (wt%) the different of PHB contents in cells between the initial and ends of feast phase, $MLSS_r$ biomass concentration in the reactors at the end of a cycle, V_r effective reactor volume, reactor exchange ratio in this study is 0.32, 0.114 is nitrogen percentage of biomass, 68 g $\mathrm{Cmol^{-1}}$ is the molecular weight of $CH_3COONa \cdot 3H_2O$, $\Delta \text{Acetate concentration } (\mathrm{g}\ L^{-1})$ is the difference between acetate concentrations in a cycle. The specific uptake rate of sodium acetate per unit of biomass was calculated with assumptions of a zero-order sodium acetate consumption rate and a constant active biomass concentration in the cycle (Beun et al., 2002). It needs to point out that only MLSS at the end of a cycle as a single reference point was used for PHB productivity, so the productivity values were only used for the purpose of comparison under different conditions.

3. Results and discussion

3.1 Sludge morphology and settleability, biomass retention in SBRs and PHB storage in sludge under different settling times

After reactors reached a steady state, i.e., MLSS, PHB content, ammonium and phosphate concentrations in reactors were stable for at least three SRTs, sludge samples were taken for morphology observation as shown in Figure 1. Activated sludge in SRBs with a settling time of 10 min was gradually converted into aerobic granular sludge as shown in Figure 2a. The formed granular sludge had a compact structure and large size, and the SVI of granules was less than 90 mL g^{-1}, indicating good sludge settleability. Short settling time is usually believed to be critical for the formation or maintenance of aerobic granular sludge (Liu and Tay, 2015). After a 100-day operation, the settling time was reduced from 10 min to 1 min to create stronger hydraulic selective pressure. Aerobic granules, however, disintegrated due to the extreme condition (i.e. settling time of 1min) applied. At the steady-state with a settling time of 1 minute, SVI was 194 mL g^{-1}, a significantly increased SVI, indicating a dramatically reduced sludge settleability compared with that with a 10-minute settling time. It can be seen from Figure 2b that at this condition, large suspended flocs were dominant without aerobic granules observed. This might be due to the extreme hydraulic selective pressure exerted on reactors, causing aerobic granules to be unstable. Although a short settling time is usually required as a selective pressure for the cultivation of aerobic granular sludge, the settling time used for the operation of SBRs is usually longer than 5 minutes to maintain the long-term stability of granular sludge (Chen et al., 2015; Franca et al., 2018). After a 29-day operation with a settling time of 1 min, the settling time was further reduced to 0 min (i.e. no settling time applied). It was found that the settleability of sludge deteriorated further, resulting in an SVI of 273 mL g^{-1}. Pinpoint flocs were dominant in SBRs at the steady state as shown in Figure 2c. Since only settling time was changed, it can be concluded that settling time is a critical factor to control the settleability of sludge in SBRs when other operating conditions are the same. A longer settling time is favourable to form more compact sludge with lower SVI.

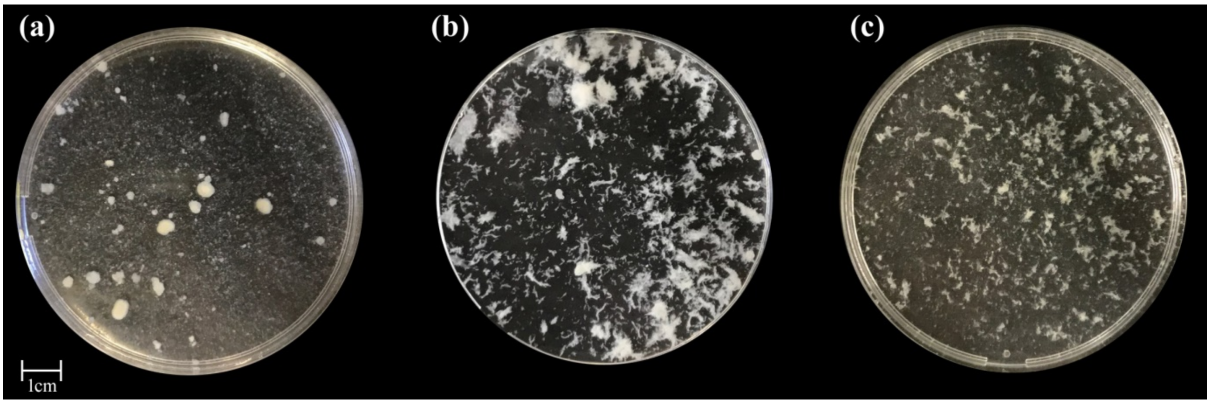

Figure 2. Morphologies of sludge at steady states in SBRs with a settling time of (a) 10 minutes, (b) 1 minute, and (c) 0 minute

Due to the change in sludge settleability with different settling times, biomass retention was changed accordingly with more biomass washed out at a shorter settling time, resulting in lower biomass concentrations in the reactors. Table 2 shows biomass concentration in reactors, sludge PHB storage and other parameters in the PHB enrichment stage from three different settling times. It can be seen that the change in sludge settleability caused by settling time led to the changes in biomass concentration in the reactors and the effluent, thus the changes in biomass loadings (F/M ratio), feast/famine (F/F) ratio, and SRT, which further affected PHB storage in sludge, biomass yield and PHB yield and productivity. It is widely believed that higher biomass concentration results in lower biomass yield (Liu and Liu, 2004), which was demonstrated as well by the biomass yields shown in Table 2. This might benefit the direction of carbon flux from new biomass growth to PHB accumulation. However, higher biomass concentration might be unfavorable to the enrichment of PHB accumulating organisms in sludge because of the retention of biomass in reactors. It has been widely reported that aerobic granules can retain slow-growing bacteria and anaerobic bacteria into the reactors due to their compact structure, large size and excellent retention (Tay et al., 2002; Liu et al., 2004b; Liu et al., 2007). Thus, it is reasonable to speculate that non-PHB accumulating microorganisms could be retained in aerobic granules while aerobic granules were retained in the reactors, leading to a very low PHB content even at low F/F. In addition, more carbon is demanded for aerobic respiration at higher biomass concentrations.

Table 2. Comparison of sludge settling ability, PHB storage and other reactor parameters at steady states with different settling times

Settling time (min)	SVI (mL g^{-1})	MLSS in reactors (g L^{-1})	MLSS in effluent (g L^{-1})	SRT (day)	F/M (g COD g^{-1} MLSS·day^{-1})	F/F (feast/ famine)	Biomass yield (xCmol $Cmol^{-1}$ Acetate)	PHB content (wt%)	PHB yield (PHB Cmol $Cmol^{-1}$ Acetate)	PHB productivity (g PHB $L^{-1} \cdot h^{-1}$)
10	89	4.23 ±0.20	0.74 ±0.11	4.47	0.71	0.07	0.03	7.7 ±0.5	0.28	0.41
1	194	1.55 ±0.11	0.80 ±0.08	1.51	1.94	0.28	0.15	22.0 ±1.4	0.33	0.14
0	273	0.98 ±0.11	1.06 ±0.15	0.72	3.07	0.30	0.18	26.7 ±1.0	0.27	0.11

* biomass yield, PHB content, PHB yield and PHB productivity are at the end of feast phase

3.2 Correlation of PHB content, F/F and F/M ratios and SRT with biomass concentration in reactors with different settling times during the long-term operation period

Variable PHB contents in well-settled aerobic granule sludge from 9% to 68% were reported (Fang et al., 2009; Gobi and Vadivelu, 2014; Wang et al., 2014; Karakas et al., 2020). In addition, comparable PHB content such as 53% was found in poorly-settled bulking sludge with well-settled sludge (Wen et al., 2012). Thus, it could be speculated that the low PHB content in aerobic granular sludge in this study was partially caused by high biomass concentration, which reduced the ratios of F/F and F/M, and increased SRT accordingly. To validate this, PHB content in cells at the end of feast phase, SRT, F/F and F/M during transient and steady states from three triplicate reactors were correlated with biomass concentration at three different settling times from 10 min to 0 min as shown in Figure 2. It can be seen that although sludge morphologies (Figure 2) and settleability at the settling times of 10, 1 and 0 minutes (Table 2) were different, experimental data between PHB content, F/F and F/M and biomass concentration were fitted very well with regression models with R^2 above 0.93, suggesting that sludge morphology (e.g. granular sludge or suspended sludge) and sludge settleability (high or low SVI) are not decisive factors to affect PHB storage in cells. Although higher contents of extracellular polymeric substances (EPSs) were reported in aerobic granular sludge (Liu et al., 2004a), there is no evidence to show that the synthesis of more EPS would reduce the storage of PHB in cells although it was reported that EPS removal in granules facilitates PHB recovery in downstream (Gobi and Vadivelu, 2015). Thus, sludge morphologies and settleability are not critical for PHB accumulation. The well-fitted regression lines against biomass concentration with different sludge morphologies and settleability indicate that biomass concentration plays an important role while effects of sludge morphologies and settleability were negligible in this study.

If there was no extra control of biomass concentration in reactors by sludge discharge, biomass concentration would be determined by sludge settleability, which was influenced by settling time in this study. Other parameters such as SRT and F/F ratio have been intensively investigated for PHB storage capacity. It was found that generally shorter SRT was beneficial to PHB production capability (Chua et al., 2003; Johnson et al., 2010), but too short SRT such as 0.5 days led to reduced PHB storage and unstable reactor operation (Johnson et al., 2010). Some researchers reported that a lower F/F ratio less than 0.28 in SBRs allowed the good selection of culture with high polymer storage capacities (Dionisi et al., 2006; Johnson et al., 2009; Albuquerque et al., 2010; Jiang et al., 2011; Moita et al., 2014) while F/F ratios higher than 0.55 increased the biomass growth and the storage mechanisms started to be negligible. F/F ratios in this study under OLR of 3.0 g L^{-1} d^{-1} ranging between 0.02-0.2 as shown in Figure 3 fell well within the range of less than 0.28. Compared with SRT and F/F ratio, the studies on the effects of F/M on sludge PHB storage were much less even though a wide range of F/M ratios from 0.03 to 6.0 were used in the literature (Serafim et al., 2004; Qu and Liu, 2009; Chakravarty et al., 2010; Khumwanich et al., 2014). As shown in Figure 3, F/F and F/M ratios decreased exponentially with the increase in biomass concentration while SRT increased linearly with biomass concentration. Therefore, it could be interpreted that in this study biomass concentration affected PHB storage capacity by changing SRT, F/F and F/M ratios. It has to be pointed out that biomass concentration, SRT, F/F and F/M ratios interact with each other while they are also affected by OLR and initial substrate concentration in cycles. Thus, it is very challenging to derive the decisive factor for PHB storage capacity. Even though, the experiment designed in this study is helpful to understand the importance of biomass concentration, which is widely ignored and much less reported in PHB studies with open mixed culture compared with other parameters. Although the effects of biomass concentration on enrichment or PHB accumulation were occasionally touched in literature (Chakravarty et al., 2010; Chen et al., 2017), all of them just focused on the comparison of PHB storage under two or three different biomass concentrations, resulting in difficulty to look into the precise relationship between biomass concentration and PHB storage or other parameters. In addition, those very limited studies on biomass concentration did not consider the determining effects of biomass concentration on SRT, F/F and F/M ratios to elucidate the interaction of different parameters. Our experimental design in this study with triplicate reactors at three different settling times allowed us to obtain dozens of data under multiple biomass concentrations to establish reliable correlations as shown in Figure 3. As far as we know, this is the first report about the correlations between PHB storage and biomass concentration, and between other parameters such as SRT, F/F and F/M and biomass concentration during the long-term operation period, which shed light on the physiological response of sludge for PHB storage on the top of the enrichment theory on PHB accumulating organisms.

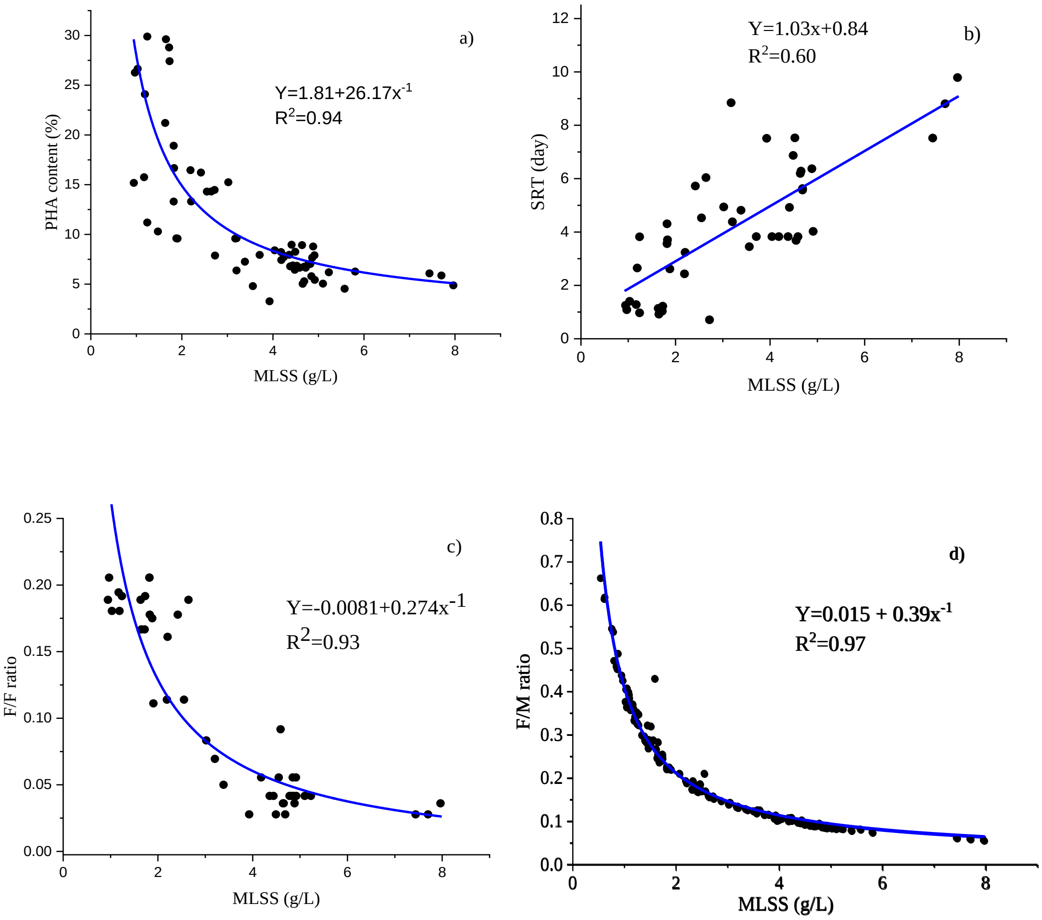

Figure 3. Correlations between (a) PHB content, (b) SRT, and (c) F/F ratio d) F/M ratio and biomass concentration at OLR of 3 g $L^{-1}d^{-1}$ in three reactors with different settling times at transient and steady states: ●, experimental data; — fitted regression lines

3.3 The immediate response of reactor sludge regarding PHB storage to biomass loading shock caused by the change in the settling time

Figure 4 shows the changes in biomass concentration and PHB content in sludge with a reduced settling time from 10 minutes, to 1 and 0 minutes during the operating period from day 39 to 150. Two peaks regarding PHB content were observed just after the reduction in the settling time from 10 minutes to 1 minute and from 1 minute to 0 minute, i.e. around days 100 and 129. Once the settling time was reduced from 10 minutes to 1 minute on day 100, biomass was immediately washed out due to a very short settling time, which resulted in a drastic decrease in biomass concentration and thus a significant increase in biomass loading shock (i.e. a surge of F/M ratio) immediately. Meanwhile, sludge PHB content at the end of the feast phase surged from 7% to 28.5% within one day and further increased to 32.4% within 3 days. Sludge PHB content after three days, however, reduced and stabilised at around 15%. On day 129, the setting time was reduced from 1 minute to 0 minutes, which again resulted in a quick biomass wash-out and a biomass loading shock. Within 3 days, sludge PHB content surged from 15% to 32.5% again, but decreased later and stabilized at around 25%. The surge of sludge PHB content and the subsequent decrease to respond to the change in the settling time is hard to be explained by the enrichment theory of PHB accumulating organisms. To a large extent, this surge of sludge PHB content should come from the quick physiological response of cells to a sudden increase in biomass loading shock. Once biomass adapted to the new biomass loading rate, PHB storage was reduced.

As far as we know, this is the first study of settling time effects on PHB content and the first report about the surge of PHB content responding to biomass loading shock. According to (Mannina et al., 2020), under appropriate conditions, once PHB accumulating organisms were enriched, stable PHB content could be achieved while it was also reported that PHB accumulation was unstable at some conditions such as high OLRs (Valentino et al., 2014). However, there was no report in the literature about the surge of PHB content during a very short period, i.e. within 1-3 days by changing settling times and biomass loading rates. It could be speculated that some microorganisms in sludge that do not accumulate PHB at a steady state could synthesize PHB or those PHB accumulating organisms could synthesize more by a rapid stress response mechanism when suddenly being exposed to excessive loading pressure. When excessive biomass loading pressure becomes routine, these microorganisms could adapt to the new biomass loading pressure without synthesizing PHB at all or only synthesizing reduced PHB amount. This speculation needs to be validated by further study. But the phenomenon of PHB content surge under biomass loading shock could be tapped into by future studies for the increased sludge PHB content before the purge of sludge particularly for the simultaneous enrichment and accumulation of PHB in a single reactor.

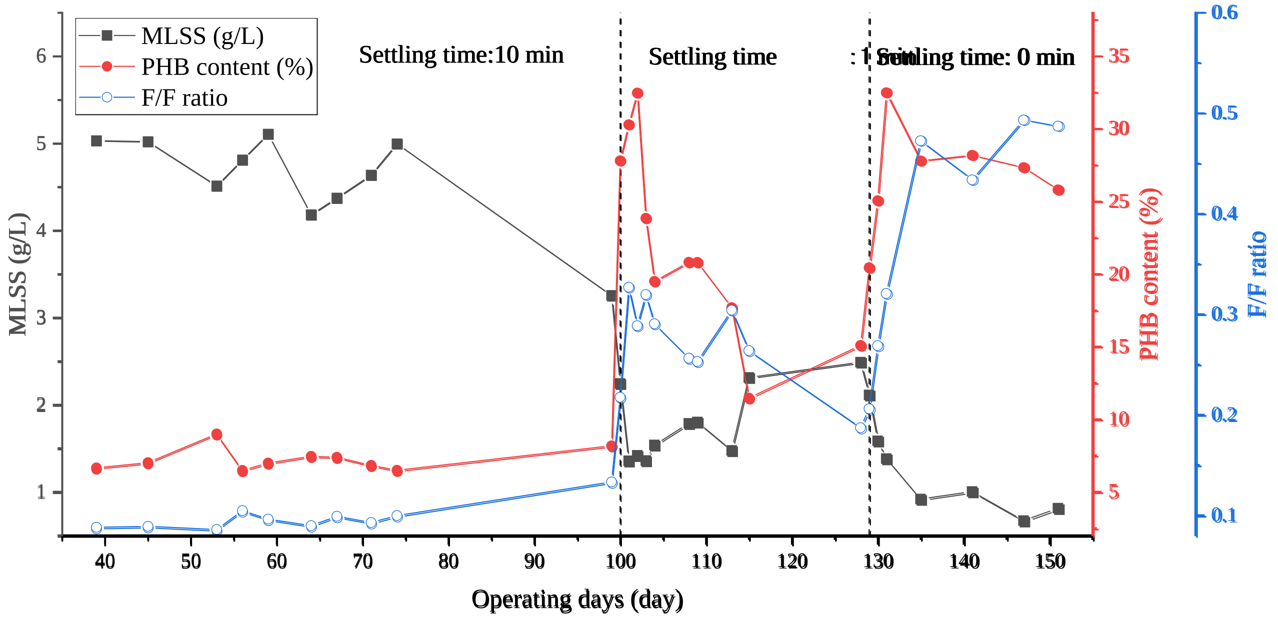

Figure 4. The profiles of biomass concentration, sludge PHB content and biomass loading rate with a reduced settling time from 10 minutes to 1 and 0 minutes during the operating period from days 39 to 150 under OLR of 3 g $L^{-1}d^{-1}$

3.4 Effects of organic loading rate on sludge settleability, biomass retention and PHB storage

To further study the effects of biomass concentration on sludge PHB content, OLR was increased from 3.0 g $L^{-1}d^{-1}$ to 6.0 g $L^{-1}d^{-1}$ and then to 7.5 g $L^{-1}d^{-1}$ from operating days 151 to 189 with a settling time of 0 minutes. The profiles of biomass concentration and sludge PHB content under different OLRs are shown in Figure 5. It can be seen that biomass concentration in the reactor increased with the rise in OLR almost linearly. For instance, the average biomass concentration increased by 95% from 0.93 to 1.81 g L^{-1} and by 24% from 1.81 to 2.45 g L^{-1}, respectively, corresponding to the increase of OLR by 100% from 3.0 to 6.0 g $L^{-1}d^{-1}$ and by 25% from 6.0 to 7.5 g $L^{-1}d^{-1}$, respectively. A similar result was found by (Dionisi et al., 2006), who reported that an increase in an OLR from 8 to 12.8 g $L^{-1}d^{-1}$ (by 50%) and 12.8 to 20.0 g $L^{-1}d^{-1}$ (by 56%) resulted in an increase in biomass concentration by 49% and 52%, respectively. (Kanimozhi and Vasudevan, 2014) also found the corresponding increase in biomass concentration when

OLRs were increased although the increase was not linear. Meanwhile, it was observed that sludge PHB content increased accordingly with the increase in OLRs, but sludge PHB content increased only by 16.8% and 8.3%, respectively. This is consistent with those found by (Reddy and Mohan, 2012) that an increase in OLR from 1.51 to 3.03 (100%) and from 3.03 to 4.54 kg $L^{-1}day^{-1}$ (50%) resulted in an increase in PHB content from 32 to 37.4 wt% (16.87%) and 40.3 wt% (7.75%), respectively, although only one batch instead of steady state was investigated in their study. This suggests that an increase in OLR could not lead to the same level of increase in sludge PHB content. In addition, it was found that the increase in sludge PHB content did not necessarily lead to an increase in PHB yield. As shown in Table 3, both biomass and PHB yields were more or less the same under three different OLRs, indicating that the carbon flux was neither directed to biomass nor sludge PHB with the increased OLRs. This result might have practical significance as reactors could be operated with different influent COD concentrations and OLRs within certain ranges to get similar biomass and sludge PHB yields with regard to carbon source utilization. But too-high influent COD concentrations and OLRs above 20 g L^{-1} d^{-1} would inhibit the activity of microorganisms and thus reduce PHB accumulation or stable PHB accumulation (Dionisi et al., 2006). Comparable results regarding biomass and sludge PHB yields when biomass loading rate (F/M) reduced from 3.07 to 1.43 g COD g^{-1} MLSS d^{-1} and F/F ratio increased from 0.32 to 0.62 are contradictory with that was reported by (Albuquerque et al., 2010), in which that PHB yield decreased linearly with the increase of F/F ratio in the range of 0.2-0.6 by increasing OLRs. Also, (Qu and Liu, 2009) found that an increased F/M ratio from 1.0 to 4.5 enhanced PHB storage in sludge in batch tests while PHB accumulation decreased when F/M was increased further to 6.0, but it has been pointed out the results from Qu and Liu (2009) were only from batch experiments instead of from steady-states of long-term SBR operation. Instead, in this study, PHB contents in sludge increased when F/M decreased from 3.07to 1.43 during the steady states of long-term operation with OLRs increased from 3.0 to 7.5 g L^{-1} d^{-1} (Table 3) while increased when F/M increased from 0.71 to 3.07 with OLR of 3.0 g L^{-1} d^{-1} under three different settling times (Table 2). Thus, the correlations in Figure 3 are only applicable to the constant OLR. Once OLR increased as shown in Table 3, the relationships between biomass concentration and PHB content was inverse. This partially suggests that the relationships between different parameters obtained in literature are only limited to the conditions where the results were obtained. Furthermore, these types of discrepancies indicate the intricated interaction between different operating conditions and the difficulty to identify critical factors or universal rules affecting PHB storage. Meanwhile, the discrepancies also suggest that F/F and F/M ratios could not be decisive factors to determine PHB contents or yields.

In addition, it was noticed that both SVI and sludge PHB productivity increased by 40-45%, which was much less than the OLR increasing percentage, i.e. 150%, in this study. An increase in SVI with OLR in SBRs was observed by some researchers as well (Kanimozhi and Vasudevan, 2014) although intermittently fed operations such as SBRs could be used to control filamentous growth and sludge bulking (Chiesa and Irvine, 1985). The increase in SVI would lead to an increased cost for liquid-solid separation for the sludge PHB extraction downstream while the increase in productivity would result in decreased capital and operating costs. Meanwhile, a higher sludge PHB content at higher OLRs is beneficial to PHB extraction. Thus, the optimal OLR should be determined based on the cost evaluation from the whole process with other specific operating conditions.

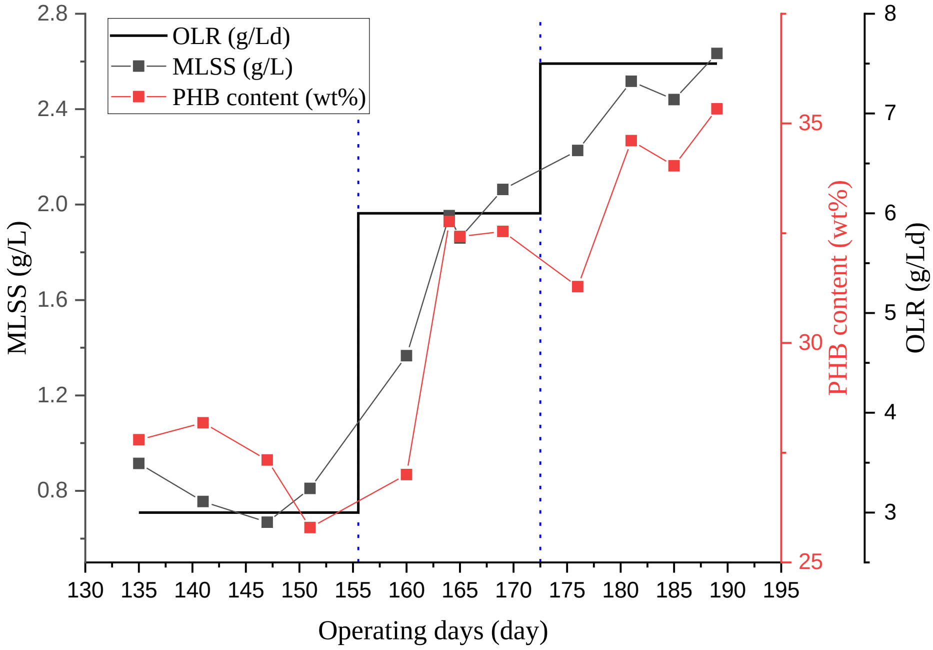

Figure 5. The profiles of biomass concentration and sludge PHB content under different OLRs from operating days from 135 to 189

Table 3. Comparison of sludge settleability, PHB storage and other reactor parameters at steady states with different OLRs and a settling time of 0 minutes

OLR (g L^{-1} day^{-1})	SVI (mL g^{-1})	MLSS in reactors (g L^{-1})	MLSS in effluent ((g L^{-1})	SRT (day)	F/M (g COD g^{-1} MLSS.day^{-1})	F/F (feast /famine)	Biomass yield* (xCmol $Cmol^{-1}$ Acetate)	PHB content* (wt%)	PHB yield * (PHB Cmol $Cmol^{-1}$ Acetate)	PHB productivity * (g PHB L^{-1} h^{-1})
3.0	296	0.98 ±0.12	1.06 ±0.10	0.72	3.07	0.32	0.18	26.7 ±1.0	0.27	0.11
6.0	366	1.70 ±0.09	1.74 ±0.06	0.76	1.77	0.42	0.23	31.2 ±0.9	0.24	0.14
7.5	415	2.10 ±0.05	2.26 ±0.05	0.73	1.43	0.62	0.19	33.8 ±1.0	0.28	0.16

* PHB content, PHB yield and PHB productivity represents the situation at the end of feast phase

3.5 Sludge PHB storage in SBRs and fed-batch reactors

To understand the effects of substrate and nutrient concentrations on sludge PHB accumulation in SBR cycles, the profiles of acetate, nutrients, DO and sludge PHB contents in batch cycles with different settling times at steady states were analysed as shown in Figure 5. Ammonium was consumed mainly for biomass synthesis, which could be used to represent new biomass growth. It can be seen from Figure 5 that ammonium consumption was much faster in the feast phase than in the famine phase when the settling time was 10 minutes with a biomass concentration of 4.3 g L^{-1} while equivalent ammonium consumption rates during feast and famine phases were found when setting time was 0 minute with biomass concertation of around 0.7 g L^{-1}. This is because with the biomass concentration of 0.7 g L^{-1}, the more PHB content accumulated in the feast phase could be used for biomass growth in the famine phase for a higher specific PHB degradation rate (i.e. 5% PHB g^{-1} MLSS·h^{-1}) while less biomass growth accompanied with a lower specific PHB degradation rate (i.e. 0.3%PHB g^{-1} MLSS·h^{-1}h) with the biomass concentration of 4.3 g L^{-1}. In addition, according to Table 2, it can be seen that at the end of the feast phase, PHB yields were equivalent while biomass yield with a biomass concentration of 0.98 g L^{-1} was 6 times higher than that with a biomass concentration of 4.3 g L^{-1}. This result is interesting because the amount of carbon that flowed to PHB (i.e. PHB yield) was not affected by biomass concentration and biomass loading rate in the reactors although biomass yield was influenced drastically. Sludge PHB content, however, was much higher at the lower biomass concertation due to higher biomass loading rate. From this perspective, lower biomass concentration is favourable due to an equivalent PHB yield and higher sludge PHB content.

The specific acetate uptake rate during the feast phase in Figure 5a was 1.06 acetate Cmol · $Cmol^{-1}$ biomass h^{-1} while 2.51 acetate Cmol · $Cmol^{-1}$ biomass h^{-1} in Figure 5b. Thus, low biomass concentration or high F/M encourages the microorganism to consume carbon faster. Similar results were found with a specific ammonium uptake rate, which was 0.0048 NH_4^+-N mol·$Cmol^{-1}$ biomass h^{-1} with 4.4 g L^{-1} biomass concentration while 0.021 NH_4^+-N mol · $Cmol^{-1}$ biomass h^{-1} with 0.7g L^{-1} biomass concentration. Thus, the high specific ammonium uptake rate indicates that biomass growth occurred in the feast phase as well although PHB was stored in this phase.

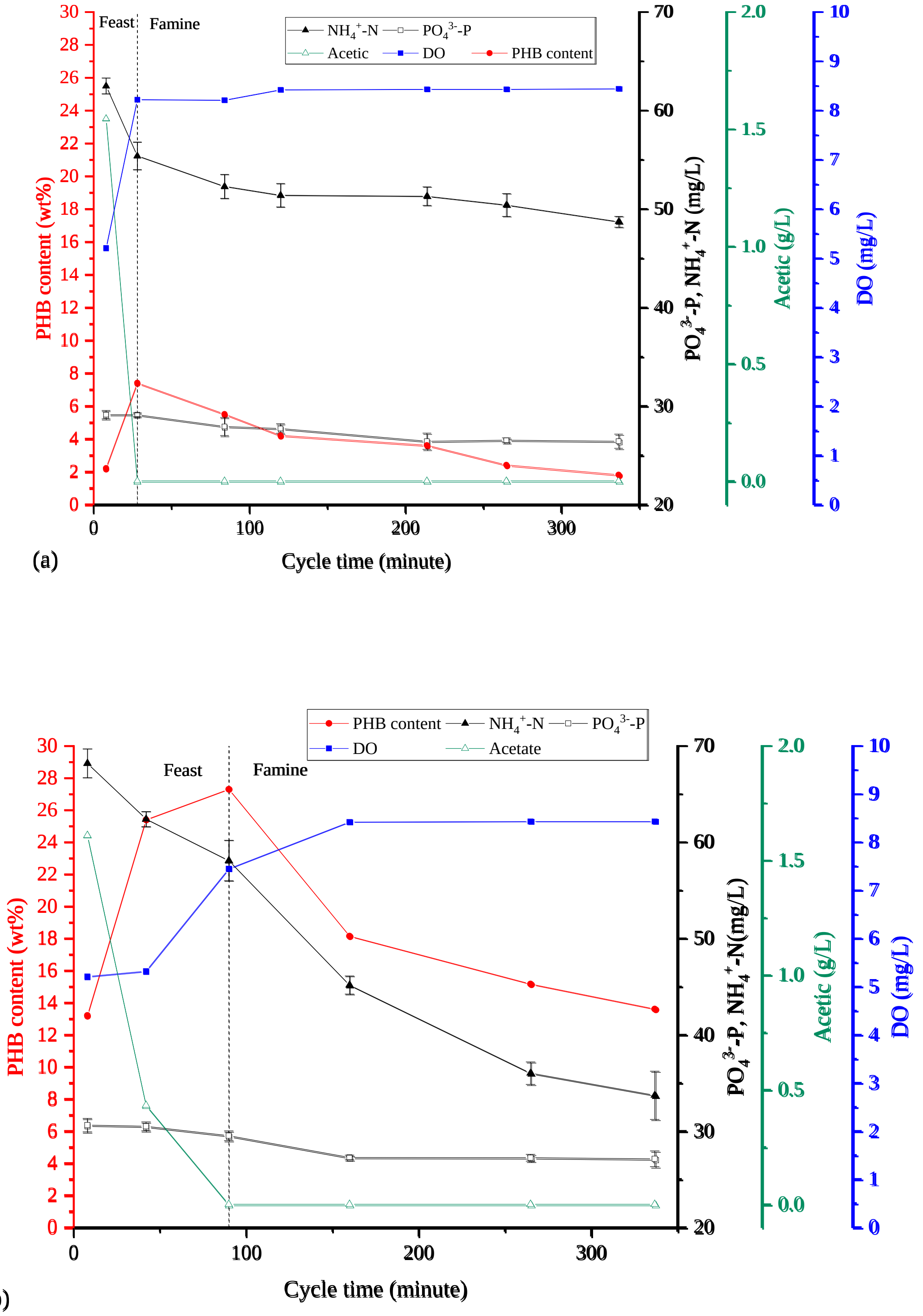

Figure 6. Profiles of acetate, nutrients, DO and sludge PHB contents at the end of the feast phases in batch cycles of SBRs at steady states with (a) settling time of 10 minutes and a biomass concentration of 4.3 g L^{-1}, (b) settling time of 0 minute and a biomass concentration of 0.7 g L^{-1}

Furthermore, PHB contents at the end of the feast phase in SBRs and fed-batch reactors based on the feed-on-demand method were compared as shown in Table 4. For both batch and bed-batch experiments, no nutrient limitation was used to study the effects of biomass and substrate concentrations on PHB accumulation. The initial acetate concentration was reduced to 3.7 Cmmol acetate L^{-1} in the fed-batch experiment from 24 Cmmol acetate L^{-1} in the batch experiment to study the possible inhibition of acetate on PHB accumulation with two different biomass concentrations at a settling time of 1 min and 0 min, respectively. It was found that when biomass concentrations were the same at around 1.3 g L^{-1} in the batch and fed-batch experiments, PHB contents in batch and fed-batch experiments were almost the same, suggesting there was no inhibition from the substrate at the concentration of 24 Cmmol acetate L^{-1}. When the biomass concentration in the fed-batch experiment was reduced to 0.8 g L^{-1}, PHB content reached 25.7%, an increase by 70% compared with the biomass concentration at 2.4 g L^{-1} in the batch experiment although the same sludge was used. This again indicates that lower biomass concentration has a positive impact on PHB accumulation in cells as the effects from substrate inhibition or different microbial population were ruled out. (Chua et al., 2003) also found out that PHB contents of 31% and 21% with biomass concentrations of 0.7 and 2.5 g L^{-1}, respectively, in the batch experiments with the same sludge. This finding is beneficial to the downstream PHB extraction as adjusting biomass concentration could be conducted to maximise sludge PHB content apart from the enrichment of PHB accumulating organisms.

When acetate concentration was doubled in SBRs and increased to 47 Cmmol L^{-1}, it was found that sludge PHB content in the fed-batch experiment was 38.7%, 18% higher than 32.8% in the batch experiments with the same sludge under the same biomass concentrations. (Serafim et al., 2004) also reported that 67.5% of the maximum PHB content was obtained with 180 Cmmol/l of acetate supplied in one pulse of a batch test while it reached 78.5% when 180 Cmmol L^{-1} of acetate was fed in three pulses of 60 Cmmol L^{-1} each. This might suggest that the higher acetate concentration used in this study, i.e. 47 Cmmol L^{-1}, slightly inhibited PHB accumulation in the batch experiment. Reducing the acetate concentration to 7.4 Cmmol L^{-1} in the fed-batch was advantageous regarding the alleviation of substrate inhibition for the maximum accumulation with the same sludge, but the increased percentage is not as high as that by reducing biomass concentration. From the comparison results in this study, it could be known that controlling both substrate and biomass concentrations could enhance PHB accumulation in cells. This could provide guidance for the operation of SBRs by controlling settling time (to control biomass concentration), and exchange ratio (to control substrate concentration) to achieve sludge PHB content as high as possible in SBRs, which has a practical significance for harvesting sludge at the end of the feast phase instead of using an extra fed-batch reactor for PHB accumulation to reduce capital and operating costs when only wastewater streams containing excessive nutrients are available for PHB accumulation. Furthermore, it has been reported that higher PHB content in sludge could lead to high biogas yield from digestion due to higher biodegradability of PHB than cells (SATOH and MINO, 2013), thus, the treatment of VFA-rich wastewater containing excessive nutrients in SBRs could be combined with sludge PHB production in a single reactor, from which sludge harvested from the end of feast phase could be digested for biogas production to increase energy recovery efficiency from wastewater when PHB content is not high enough for extraction.

Table 4. Sludge PHB contents in SBRs and fed-batch reactors

OLR (g L^{-1} day^{-1})	Settling time (minute)	MLSS in SBRs (g L^{-1})	The highest acetate concentration in the batch cycle (Cmmol L^{-1})	PHB content (wt%) at the end of feast phase in SBRs	MLSS in fed-batch reactors (g L^{-1})	The highest acetate concentration in the fed-batch experiment (Cmmol L^{-1})	PHB content (wt%) at the end of fed-batch experiment
3.0	1	2.4	24	15.1	0.8	3.7	25.7
3.0	0	1.3	24	25.7	1.2	3.7	26.3
6.0	0	2.0	47	32.8	1.9	7.4	38.7

4. Conclusions

This study revealed the relationships between sludge morphologies and biomass concentration in SBRs with sludge PHB storage at varied settling times and organic loading rateswithout nutrient limitations. The conclusions are summarised as follows:

- Under OLR of 3 g L^{-1} d^{-1}, sludge PHB content was highly dependent on biomass concentration, which could be described by a well-established correlation equation. Sludge morphologies (i.e. granular sludge, suspended sludge, bulking sludge) have no impact on PHB accumulation in cells.
- Under OLR of 3 g L^{-1} d^{-1}, the sludge experienced biomass loading shock induced a surge of PHB content in sludge within 1 day from a physiological response.

- With the increased OLRs from 3 to 7.5 g L^{-1} d^{-1} under the same settling time of 0 minutes, the corresponding increase in biomass concentration led to slightly increased PHB content in sludge, which is inverse dependence on biomass concentration compared with the constant OLR of 3 g L^{-1} d^{-1}.

The different dependence of sludge PHB content on biomass concentration under different operational conditions indicated the intricate nature of operating conditions with parameters interactedg each other. No universal rule could be achieved and the pattern obtained is applicable to a restricted range of operational conditions. But the dependence of sludge PHB content on biomass concentration implies a practical significance for the operation of combined PHB accumulation and wastewater treatment in SBRs by adjusting biomass concentration to maximize PHB content in sludge.

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