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Strategy to reduce the acclimation period for enrichment of PHA accumulating cultures

Zirui Guo[a], Zhiqiang Chen[a], Qinxue Wen[a,*], Long Huang[a], Rune Bakke[b], Maoan Du[a]

[a]*State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China, Tel. +86 18645105100; email: ziruiguo@126.com (Z. Guo), Tel. +86 15904603951; email: czqhit@126.com (Z. Chen), Tel. +86 13796077808; email: 356427522@qq.com (Q. Wen), Tel. +86 18345175529; email: hl2130@126.com (L. Huang), Tel. +86 15603601528; email: dmahit@126.com (M. Du)*
[b]*Faculty of Technology, Telemark University College, Porsgrunn 3901, Norway, Tel. +86 18686702230; email: rune.bakke@hit.no*

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ABSTRACT

A selective sludge discharge (SSD) process is proposed and evaluated in this study to accumulate the polyhydroxyalkanoate (PHA) accumulating bacteria in mixed microbial cultures (MMCs). Sequencing batch reactor (SBR) PHA production in the SSD mode was compared to the conventional aerobic dynamic feeding (ADF) mode. Aerobic granular sludge rich in PHA was cultivated in a SBR by seeding the MMCs. The granular sludge was formed on 14 and 8 d of the operation in SBR #1 and SBR #2, respectively, however, the PHA production performance of SBR #1 fluctuated during the 30 d. The MMCs in SBR #2 showed the best PHA accumulating ability in terms of the yield of PHA, specific substrate uptake rate and PHA storage rate, a maximum PHA content and average PHA storage rate of 58.2% and 0.33 Cmol PHA/(Cmol X/h), respectively, were achieved on the day 30 of enrichment operation in the batch assays.

Keywords: Polyhydroxyalkanoate; Acclimation period; Aerobic dynamic feeding; Selective sludge discharge; Mixed microbial cultures

1. Introduction

Polyhydroxyalkanoate (PHA) is a kind of biosynthetic polymer which has similar properties to conventional polyethylene plastic yet with the advantages of being biodegradable, biocompatible, and could be produced from renewable sources [1]. A greater diffusion of PHA has been hampered till now due to the high cost of the selection of the micro-organisms and of the substrate [2]. The combined use of mixed microbial cultures (MMCs) and waste organic carbon for the polymer production can be more easily carried out without the need for sterilization and the risk of culture contamination. Thus, the use of MMCs as the micro-organisms and organic wastes as carbon source for the polymer production has become one of the focuses in the PHA biosynthesis field, and many efforts have been made toward the study of efficient polymer production techniques [3–6]. There is conse-

*Corresponding author.

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quently a potential for a widening market to cut the high production cost [7].

In recent years, PHA accumulation with aerobic-activated sludge process has been reported by several researchers [8,9]. The feast and famine regimes are cultured under conditions of alternating excess and lack of external substrates give favorable conditions for micro-organisms capable of storing organic matter such as acetate, propionate, and lactic acids as PHA [10,11], so-called aerobic dynamic feeding (ADF). Under feast and famine conditions, the micro-organisms that are able to store the substrate during the initial feast phase have a competitive advantage over the other micro-organisms, as they can use the stored polymer as an internal carbon source in the famine phase. With acetate as the carbon source, the aerobic PHA content in the sludge can reach more than 70%.

A considerable amount of knowledge has been accumulated in the past decade, however, there are still some problems unsolved, like a long acclimation period was needed to select an effective PHA accumulating cultures [12], and the sludge bulking occurred from time to time during the cultures selection phase [13], which becomes the barrier for the commercialization of MMCs PHA production .The aerobic granular sludge cultivated in a SBR has been used for the PHB production due to it has dense structure and a very short phase of sludge sedimentation, compared with the conventional sludge flocs [14]. However, the PHB content was quite low and the filamentous bacteria outgrew on the granule surface [8].

In this research, the selective sludge discharge (SSD) mode in granular sludge cultivation was introduced in the enrichment process of PHA accumulating bacteria to investigate in terms of storage maximum rates and yields, growth yields, and polymer productivity. The SSD mode was derived from the ADF mode, the difference between them was the length of the settling phase in cycle. The aims of the study were (i) to shorten the acclimation period for the enrichment of PHA accumulating MMCs, (ii) to get the MMCs with a higher PHA production capacity.

2. Materials and methods

2.1. The substrate

A synthetic wastewater was used as the substrate in this work in both culture enrichment experiments and batch assays, in which propionate and acetate with the total chemical oxygen demand (COD) concentration of 1,000 mg/L, NH_4Cl and KH_2PO_4 were, respectively, used as the carbon, nitrogen, and phosphorous source, the C/N/P ratio (mass ratio) in each sequencing batch reactor (SBR) was kept at 100/6/1. The nutrient source was composed of (per liter): $MgSO_4$ 40 mg, $CaCl_2$ 30 mg, EDTA 20 mg, and 1 ml /L of trace elements which consisted of (per liter) H_3BO_3 0.15 g, $CoCl_2$ 0.15 g, $CuSO_4$ 0.03 g, $FeCl_3$ $6H_20$ 1.5 g, $ZnSO_4{\cdot}7H_2O$ 0.12 g, $MnCl_2{\cdot}H_2O$ 0.12 g, and $Na_2MoO_4{\cdot}2H_2O$ 0.06 g. In addition, 40 mg/L thiourea was added to prevent the nitrification. NH_4Cl was absent from the substrate used in batch assays. No attempt was made to control pH in the SBRs since pH fluctuation ranged from 8 to 9, which was suitable for the selection of PHA-producing cultures [13].

2.2. SBR operation

The enrichment of PHA accumulating cultures was conducted in three laboratory-scale SBRs that were inoculated with activated sludge from the aerobic tank of a local municipal wastewater treatment plant in Harbin, China. Two identical columns were used for SSD mode of operation (SBR #1 and SBR #2), each with a height–diameter ratio of 10:1 and a working volume of 8 L. The SSD mode (6 h per cycle) which contains feeding (15 min), aerobic reaction (305 min), settling (10 min), withdrawal and idle (30 min) (Fig. 1(a)). Propionate and acetate were used as the carbon source, respectively. The regime of one operating cycle is shown in Fig. 1(b). SBR #3 with the column diameter of 12 cm, height of 25 cm, volume of 4 L was operated with 12 h cycles which contain feeding (10 min), aerobic reaction (670 min), settling (30 min), withdrawal and idle (10 min) (shown in Fig. 1(b)) as the control, acetate was used as the carbon source. Fed-batch experiments were conducted to evaluate the potential PHA production ability of the MMCs. The mixed liquor suspended solids (MLSS), COD, sludge volume index (SVI), dissolved oxygen (DO), granular sludge size, and PHA content were measured during the experiment. Dynamic parameters like organic loading rate, PHA storage yield, average specific substrate uptake rate, and PHA storage rate were calculated according to Wen et al. [13].

SBR cycle was usually characterized by measurements of biomass concentration (at the end of the cycle), substrates, PHA, and DO (during the whole cycle). The aeration intensity is controlled in 200 L/h, the DO concentration in the reactor during the feast phase was about 3 mg/L, in the famine phase the DO concentration was 6–7 mg/L. The profile of DO during the cycles was used to determine the time of substrate depletion (corresponding to the end of the feast phase and the start of the famine phase and to the maximal PHA concentration during the cycle).

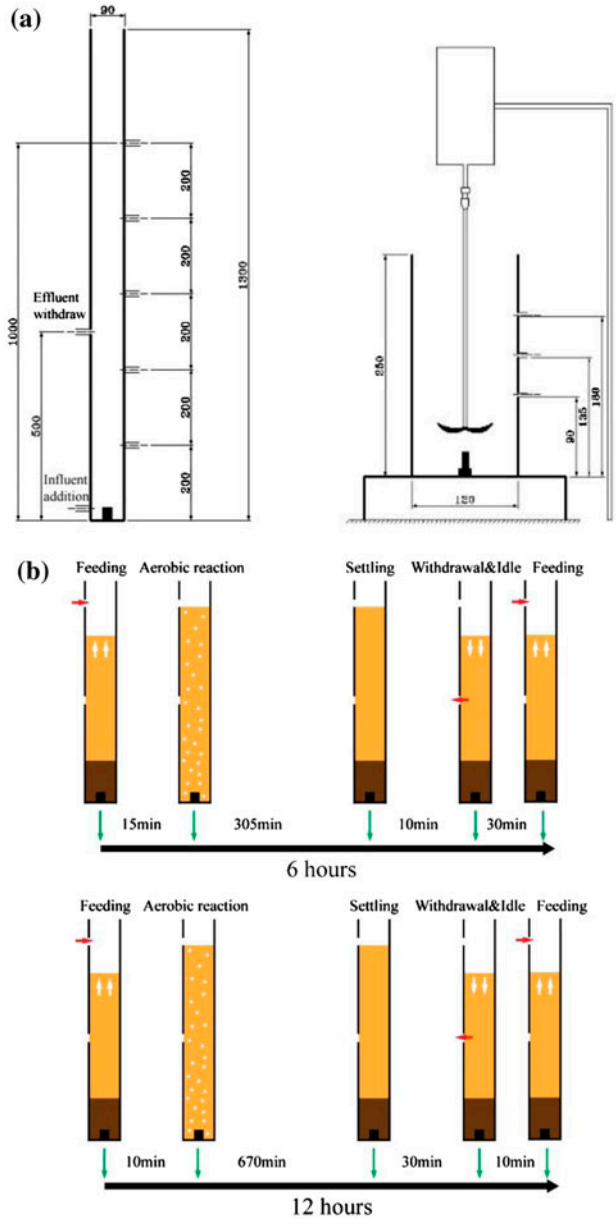

Fig. 1. The regime of one operating cycle for different feeding mode (a) SDD mode used in SBR #1 and SBR #2 and (b) ADF mode used in SBR #3.

2.3. Batch assays for PHA production

Biomass from the stable SBR was used for the PHA production in fed-batch assays. They were carried out for PHA accumulation to assess the maximum PHA accumulation capacity, the PHA storage yield on the substrate, and the production rate of the selected cultures enriched in the SBRs. The accumulation assays were carried out in beakers with a working volume of 500 ml. For each beaker, 500 ml of the mixed liquor was taken from the SBRs at the end of an operating cycle. The mixed liquor was then settled and 250 ml of the supernatant was removed. The aeration began at least 1 h before feeding to exhaust the residual nutrients in the beakers. To investigate the maximum PHA storage capacity, the substrate was fed in several steps. Each time when substrate was exhausted (indicated by the increase of DO), 250 ml of supernatant was withdrawn after settling and then 250 ml of the substrate (acetate and nutrients without nitrogen) was added. The step-feed assays lasted 10–12 h. Such a procedure was chosen to achieve a high organic loading rate while maintaining a substrate concentration [15]. The acetate concentration and PHA content were measured immediately before and after each spike, and the depletion of the substrate was indicated by a rapid increase in the DO concentration. Air was supplied through a ceramic diffuser from a compressed-air pump and the temperature was maintained at 20 ± 0.5°C. The pH was not controlled.

2.4. Analytical methods

The MLSS, COD, and sludge index (SVI) was measured according to the Standard Methods [16]. DO was measured by a dissolved oxygen meter (YSI DO200) as percentage of air saturation. The settling speed of the sludge was measured in a 100 ml cylinder when settling height was divided by settling time.

2.5. Calculation of kinetic and stoichiometric parameters

The activated biomass concentration was converted from g/L into carbon moles per liter (C mol/L) under the assumption that the biomass has empirical formula of $CH_{1.8}O_{0.5}N_{0.2}$ with a molecular weight of 25.1 g/C mol [17]. PHA concentration was also converted from g/L into C mol/L according to its monomer formula (mainly composed from polyhydroxybutyrate (PHB)), $C_4H_6O_2$, with a molecular weight of 21.5 g/C mol. The PHA content of the sludge (% PHA), specific substrate uptake rate ($-r_s$, C mol Ac/C mol/X/h), PHA storage rate (r_p, C mol PHA/(C mol/X/h) and activated biomass yieid ($Y_{X/S}$, C mol PHA/C mol/X/h), and yields of PHA ($Y_{PHA/S}$, C mol PHA/C mol Ac) were calculated according to Eqs. (1)–(5):

$$\%\mathrm{PHA} = \frac{\mathrm{PHA_e} - \mathrm{PHA_0}}{\mathrm{MLSS}} \times 100\% \quad (1)$$

$$-r_s = \frac{S_0 - S_e}{X_a \cdot t} \quad (2)$$

$$r_p = \frac{PHA_e - PHA_0}{X_a \cdot t} \quad (3)$$

$$Y_{X/S} = \frac{NH_{4,0} - NH_{4,e}}{11.4\%} \cdot \frac{1.42}{S_0 - S_e} \quad (4)$$

$$Y_{PHA/S} = \frac{PHA_e - PHA_0}{S_0 - S_e} = \frac{r_p}{-r_S} \quad (5)$$

where PHA_e and PHA_0 represent PHA content at the time substrate is exhausted and right after feeding, respectively; S_0, $NH_{4,0}$ are the ammonia concentration after feeding and the S_e and $NH_{4,e}$ are the residual substrate and ammonia concentration when the substrate is exhausted in one operating cycle. Activated biomass (X_a) is average activated biomass concentration, it is calculated as the difference between volatile suspended solids (VSS) and PHAs storage; t represents the length of the feast phase in one SBR operating cycle, as well as the duration of one batch assay.

3. Results and discussion

3.1. Formation of aerobic PHA-rich granules in the SBR experiment

The total initial sludge concentration was around 5,000 ± 150 mg/L, it is reasonable to believe that there were more loose flocs than dense flocs in the initial inoculating activated sludge. The loose flocs have a better substrate uptake capability than dense flocs and granules. Loose flocs normally have slower settling velocities than denser flocs. This means that with the selective discharge method, relatively slow-settling sludge and the loose sludge flocs were discharged from the bioreactor after a short sludge sedimentation phase in SBR #1 and SBR #2, which screened out the dense granules with rapid growth rate in the bioreactor. SBR #3 with ADF mode, the sedimentation phase was 30 min, means loose flocs survived in the bioreactor, and then the aerobic granulation is impossible.

Under the SSD mode, the loose sludge flocs were washed out in the early stage of the cultivation in both SBR #1 and SBR #2. Granular sludge was formed on 14 d in SBR #1 and 8 d in SBR #2. The average size of the granular sludge was over 200 μm in SBR #1 and SBR #2 (Fig. 2). The MLSS in the two reactors decreased initially and increased after the granular sludge becoming dominating. The SVI in both of the reactors kept below 120. SBR #3 was operated under the ADF mode, the MLSS increased gradually and the SVI was much higher (Fig. 3). The result suggests that the SSD mode had better performance in cultivating the aerobic PHA-rich granules with good stability.

3.2. SBR performance

Fig. 4 shows the variation in the operational parameters of the reaction regime in one typical cycle of the three SBRs on day 30 after the inoculation. The substrate was quickly consumed when oxygen was supplied to the three SBRs. At the end of the feast phase, the COD and ammonia concentration were consumed and indicated by a rapid increase in DO. The remainder of the reaction regime was regarded as the famine phase. Although different operating conditions were employed in the three SBRs, the performance of the SBR #1 and SBR #2 showed similar characteristics. The acetate supplied at the commencement of the cycle was rapidly taken up and used both for cell growth and PHB accumulation while the SBR #3 was slow uptake rate, comparatively.

The parameter of Feast/Famine (F/F) ratio defined as the length of feast time divided by the length of famine time, it is a key factor that will affect the polymer storage response vs. the growth response in culture selection on the MMCs PHA production. The bounds of the feast and famine phase were clearly shown by the rapid increase in DO at the remainder of the feast phase. Once the Feast/Famine ratio was decreased to around 0.05, the DO concentration grew rapidly and stabilized. This allowed the SBR to reach a "pseudo-steady state condition." Immediately after the start of the feeding phase, the DO concentration in SBR #1 and SBR #2 showed a sharp drop due to the fast increase in the metabolic activity of the biomass in the presence of external substrates. The DO concentration remained constant at 3 mg/L for about 0.25 h in SBR #1 and 0.3 h in SBR #2; then, it increased rapidly to the initial value. The fast increase in the DO concentration corresponded to the substrate depletion and was applied to monitor the maximum concentration of PHAs in the biomass. Once the selection process is done, the F/F ratio in SBR #1 and SBR #2 was much lower than in SBR #3. The low F/F ratio apparently imposes the SSD pressure through a longer famine phase where more PHA in the cells at the end of the feast phase is needed for the bacteria to survive the longer famine phase.

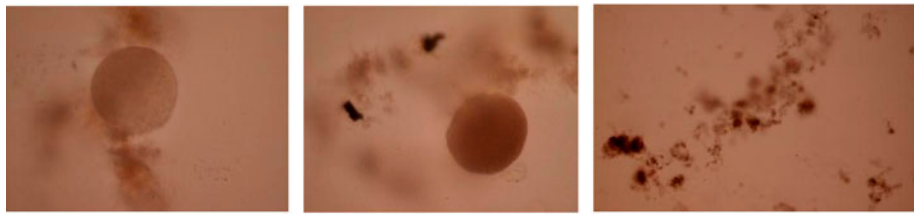

Fig. 2. The photography of microscopic in SBRs at day 15 acclimation: (a) SBR #1, (b) SBR #2, and (c) SBR #3.

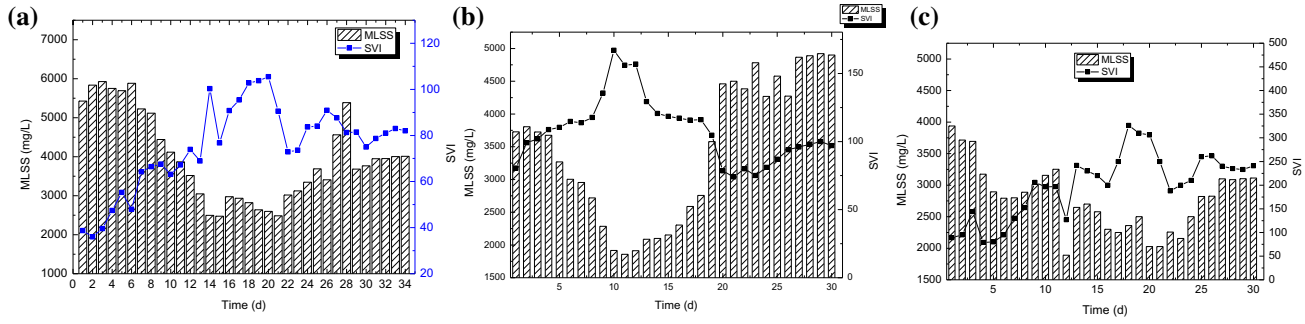

Fig. 3. The profile of the concentration of MLSS and SVI in the SBRs throughout the experiment: (a) SBR #1, (b) SBR #2, and (c) SBR #3.

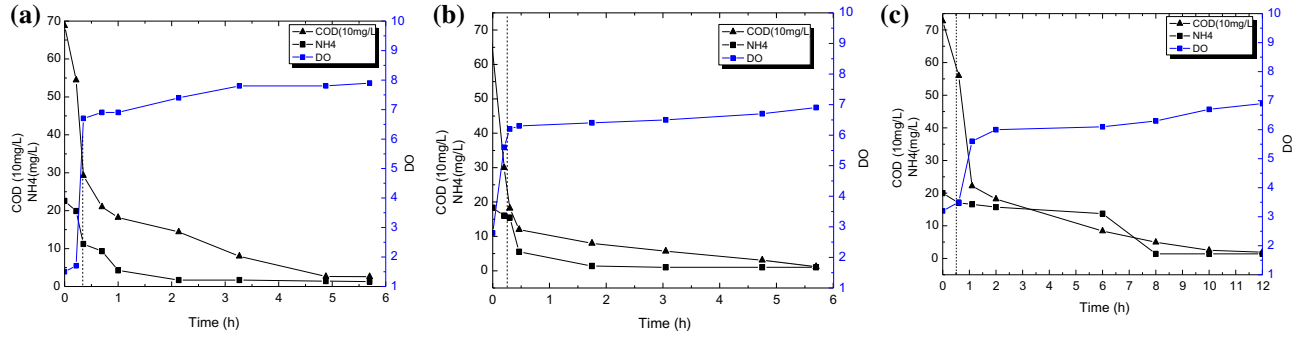

Fig. 4. Time profiles of the different parameters in a cycle of the: SBR #1 (a), SBR #2 (b), and SBR #3 (c) on day 15. Dashed lines mark the end of the feast phase.

3.3. *Aerobic PHA-rich granules accumulating performance in the selectors and in batch assays*

The effect of the different carbon sources on the stage of the proposed process was studied in different SBR operations. Cultures selected under the different modes were also compared in terms of PHA accumulation efficiency by carrying out both in SBRs and batch assays. The SBR #1 and SBR #2 have been operated under the SSD mode and the different between them is the carbon source, the former was propionate-fed and the latter was acetate-fed. Within the acclimation period, granules formed faster in SBR #2 than in SBR #1, and the acclimation time of SBR #1 and SBR #2 are shorter than that of SBR #3 (the control experiment), by feeding the same carbon source, the acclimation phase of SBR #2 is superior to SBR #3 due to the higher PHA content. The increase in the PHA production in SBR #2 and SBR #1 was observed with the operation, however, the decrease in the PHA production was observed after day 15 due to the start-up of famine condition where the stored PHA granules gets utilized for maintaining the normal cell activity in the absence

of substrate [10]. PHA content in the SBR can be used as the indicators to reflect the proportion of the PHA-enriched mixed bacteria percentage in the biomass, the PHA synthesis ability was reflected by the maximum PHA content in PHA-enriched bacteria in the batch assays. As can be seen from Fig. 5, the PHA content in SBR #1 and SBR #2 are increased rapidly at first time, then growth steady. Nevertheless, the PHA content was declined in SBR #1 after 15 d, at that place is no remained stable state in the residue of the SBR operations. From the day 15, the day 15, the PHA content in SBR #2 was much more that in SBR #1in both SBR operation and the batch assays, indicated that the acetate can be more easily utilized than propionate-fed as the substrate to converse into the storage cells [18], the biomass in SBR #2 had stronger substrate uptake capacity that that in SBR #1. PHA content achieved by acetate-fed bacteria was higher than those from propionate-fed bacteria. With respect to propionate-fed, the acetate-fed can shortened the acclimation time to achieve a high value in PHA content.

The same carbon source was feeding in both SBR #2 and SBR #3 which operated in different modes. In the traditional ADF mode, the PHA-enriched bacteria were accumulated under the conditions of alternating excess and lack of external substrates (so-called the ecological-selection). However, in addition to the traditional feast–famine mechanism, SSD mode shortens the settling time to wash out the activated sludge with poor settling property that means a "physical selection" was introduced in the process. Surveys have shown [19] that the PHA synthesized bacteria have the good settling ability. By the end of the famine phase, the bacteria with no or low PHA synthesis capability have a slower settling rate were screened out from the reactors. Thus, the combination of the ecological-selection and the physical-selection reserved the strong PHA-enrich bacteria, and so as to achieve the purpose of accelerating the acclimation phase. When the reactor ran to day 30, the maximum PHA production content in batch #2 was 59%, which was 37% higher than batch #3. It showed the SSD mode has more advantage over the ADF mode in PHA-rich bacteria accumulation

3.4. Kinetics and stoichiometric coefficients of the PHAs production

PHA storage yield ($Y_{PHA/S}$) and substrate absorption rate reflect the microbial synthesis ability and the biomass storage ability. The activated biomass yield ($Y_{X/S}$) represented the population characteristics of the activated sludge. In order to evaluate the PHA accumulating performance of the selectors during the enrichment process, the kinetic parameters in the feast phase of each SBR were calculated, results are presented in Table 1. The PHA storage yield ($Y_{PHA/S}$), specific substrate uptake rate ($-r_s$), PHA storage rate (r_p), and the activated biomass yield ($Y_{X/S}$) in SBRs were calculated. The SBR #2 performed well in terms of PHA content, furthermore, the kinetic parameters also presented better than the other two SBRs.

The activated biomass yield ($Y_{X/S}$) of SBR #3 (ADF mode) was fluctuated during the enrichment phase that indicated the undesired non-PHA accumulating bacteria were existed in the reactor [20]. In order to screen out these bacteria from the environment, a long-term feast–famine alternation is needed, so the acclimation phase prolonged that has an adverse effect on the PHA production cycle.

In contrast, the reactors used the SSD model performed well that was due to the rapid drainage in the famine approach, making the poor settleability, the low activity discharged from the reactors, and then promoted the dual pressures (physical and biological) on the reactors which enhanced the acclimation effects. The kinetic parameters were better than those under the ADF mode, however, the data in SBR #2 were still better that those in SBR #1. The results showed that the

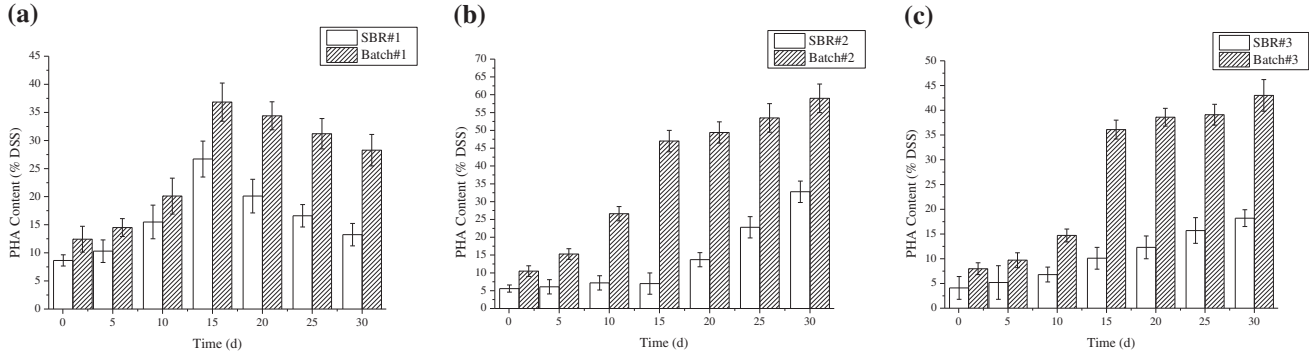

Fig. 5. Maximum PHA content achieved in the SBRs and in the batch tests: (a) SBR #1, (b) SBR #2, and (c) SBR #3.

Table 1
The PHA accumulating performance of MMCs in the three SBRs

Operation timeafter theinoculation (d)		$Y_{PHA/S}$ (C mol PHA/C mol Ac)	$Y_{X/S}$ (C mol PHA/C mol X h)	$-r_S$ (C mol Ac/(C mol X h))	r_P (C mol PHA/(C mol X h)
SBR #1	5	0.29 (±0.03)	0.18 (±0.06)	0.21 (±0.04)	0.06 (±0.02)
	15	0.30 (±0.04)	0.21 (±0.01)	0.37 (±0.01)	0.11 (±0.02)
	30	0.32 (±0.05)	0.23 (±0.01)	0.44 (±0.02)	0.14 (±0.03)
SBR #2	5	0.39 (±0.05)	0.17 (±0.02)	0.23 (±0.04)	0.09 (±0.02)
	15	0.38 (±0.01)	0.24 (±0.04)	0.42 (±0.03)	0.16 (±0.04)
	30	0.40 (±0.07)	0.27 (±0.05)	0.48 (±0.05)	0.19 (±0.05)
SBR #3	5	0.29 (±0.03)	0.21 (±0.01	0.24 (±0.04)	0.07 (±0.02)
	15	0.30 (±0.08)	0.24 (±0.04)	0.43 (±0.05)	0.13 (±0.03)
	30	0.34 (±0.05)	0.23 (±0.03)	0.44 (±0.07)	0.15 (±0.02)

Note: Values in the brackets are the standard deviation.

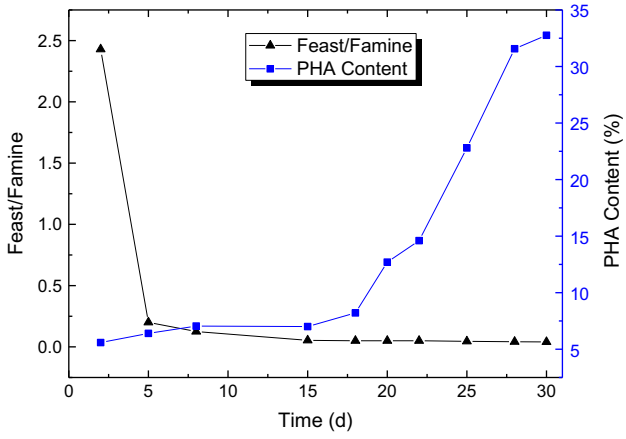

Fig. 6. PHA content and Feast/Famine value in SBR #2.

sodium acetate as the carbon source has a higher activated sludge substrate absorption rate and PHA synthesis efficiency. Compared with the sodium propionate, sodium acetate is more easily assimilated by the micro-organisms, and superior in cultivation and acclimation of the activated sludge [18].

The relationship between PHA content by the end of the feast phase and the F/F ratio is described in Fig. 6. In the early stage, the feast period is longer than a famine period. That mainly attributed to the adoption of the mixed culture with the carbon source, yet, with the acclimation, culture has adjusted to the substrate gradually. The mixed microbial population has fast substrate uptake rate, which made PHA-rich bacteria easier to survive. Furthermore, when the substrate uptake rate of the MMCs became higher, the value of F/F became smaller. Small F/F value prolonged the period of the famine phase relatively, forming a higher selective pressure toward PHA accumulating MMCs.

That means microenvironment in SBRs is suitable for the PHA-rich culture. Uptake the equivalent carbon source, the better of PHA accumulation, the more energy stored inside the cells would remain at the end

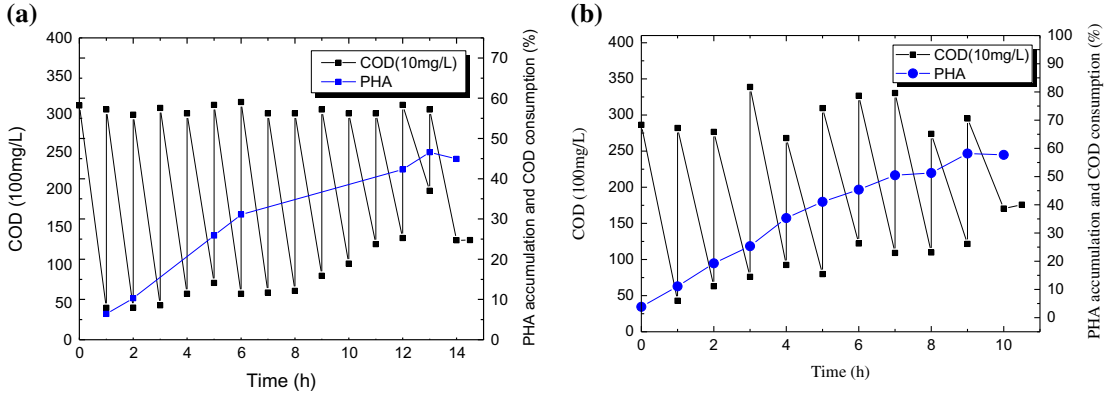

Fig. 7. COD consumption and PHA accumulation in Batch #2: (a) 15 d and (b) 30 d.

of famine, and the heavier of the biomass would be. That is the advantage of SSD mode for PHA accumulation. Select pressure method was utilized for weeding out the bacteria of none and little PHA accumulation ability. The method is suitable for the growth of PHA-rich culture, to achieve the purpose of accelerating the PHA enrichment. After approximately one month operation, mixed culture with high PHA enriching ability is dominant.

The maximum PHA production capacity enriched by the acetate-fed under SSD mode was investigated in fed-batch experiments with nitrogen limitation in order to obtain the maximum PHA content. During the fed-batch experiments, COD consumption and PHA accumulation during the fed-batch experiments, sampled from the SBR #2 at day 15 and day 30, batch tests show the relationship between COD consumption and PHA accumulation, are shown in Fig. 7. The maximum PHA content achieved was 58.2 wt % (on a dry-weight cell basis), and the highest productivity was 0.13 kg Cmol PHA/Cmol/X/h (15 d) and 0.33 kg Cmol PHA/Cmol/X/h (30 d) under SSD mode.

4. Conclusions

A SSD mode was proposed in this study to select PHA-rich cultures. The selective discharge of relatively loose sludge flocs was the crucial operating factor for an SBR to shorten the acclimation phase. In this mode, the entire process of the PHA production cycle was reduced in order to save the time and costs at the same time. The fast storage response of the MMCs under the SSD mode is attributed to the quick settling and withdrawal at the end of the famine phase.

A stable microenvironment with high yield in PHA-rich culture was screened out. Discharge of loose sludge flocs removes these competitors in suspended-growth mode and makes the substrate more available for uptake by the PHA-rich biomass, the highest PHA production in SBR #2 was 59%, the whole process exhibited a potential to produce 0.13 kg Cmol PHA/Cmol/X/h (15 d) and 0.33 kg Cmol PHA/Cmol/X/h (30 d), higher than the traditional ADF mode. The results also showed that acetate-fed sludge was superior to propionate-fed sludge under the SSD mode in terms of both sludge characteristics and the PHA production rate.

Acknowledgment

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