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Effects of sludge retention time, carbon and initial biomass concentrations on selection process: From activated sludge to polyhydroxyalkanoate accumulating cultures

Zhiqiang Chen, Long Huang, Qinxue Wen*, Huichao Zhang, Zirui Guo

State Key Laboratory of Urban Water Resource and Environment, Harbin Institute of Technology, Harbin 150090, China

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ABSTRACT

Four sequence batch reactors (SBRs) fed by fermented sugar cane wastewater were continuously operated under the aerobic dynamic feeding (ADF) mode with different configurations of sludge retention time (SRT), carbon and initial biomass concentrations to enrich polyhydroxyalkanoate (PHA) accumulating mixed microbial cultures (MMCs) from municipal activated sludge. The stability of SBRs was investigated besides the enrichment performance. The microbial community structures of the enriched MMCs were analyzed using terminal restriction fragment length polymorphism (T-RFLP). The optimum operating conditions for the enrichment process were: SRT of 5 days, carbon concentration of 2.52 g COD/L and initial biomass concentration of 3.65 g/L. The best enrichment performance in terms of both operating stability and PHA storage ability of enriched cultures (with the maximum PHA content and PHA storage yield ($Y_{PHA/S}$) of 61.26% and 0.68 mg COD/mg COD, respectively) was achieved under this condition. Effects of the SRT, carbon concentration and initial biomass concentration on the PHA accumulating MMCs selection process were discussed respectively. A new model including the segmentation of the enrichment process and the effects of SRT on each phase was proposed.

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Introduction

Polyhydroxyalkanoates (PHAs) have long been regarded as potential alternatives for conventional petroleum based plastics. These microbially synthesized polymers have similar physical properties to traditional plastics, but are completely biodegradable and biocompatible, making a closed loop carbon cycle possible (Albuquerque et al., 2013). PHAs can be accumulated as energy/carbon storage materials (Salehzadeh and Van Loosdrecht, 2004) in the cell cytoplasm by microbial cultures under stress conditions caused by a deficiency of electron donors or acceptors (e.g., carbon source or O_2) or external nutrients (e.g., nitrogen or phosphorus) (Anderson and Dawes, 1990; Majone et al., 1996). Microbial fermentation has become a

preferred method of large scale PHA production. However, the high production cost resulting from the use of sterile equipment and substrates (Choi and Lee, 1997) has blocked the further development of industrial PHAs production.

Alternative PHA production processes to reduce the production cost have been explored in the last decade. These include the utilization of mixed microbial cultures (MMCs) as biocatalyst and wasted organic material/biomass as substrate (Albuquerque et al., 2007, 2010; Dionisi et al., 2005; Satoh et al., 1998; Waller et al., 2012), which require lower investment and operating cost due to the use of open systems that do not need aseptic conditions, and bring remarkable environmental benefits such as carbon recovery from wastes. The key to the effectiveness of MMC PHA production processes relies on the

* Corresponding author. E-mail: wqxshelly@263.net (Qinxue Wen).

selection of PHA accumulating organisms (Albuquerque et al., 2010). Storage of PHA by MMCs occurs in a completely aerobic system under transient conditions of carbon supply (Majone et al., 1996). These conditions are commonly referred to as the “Feast-Famine” or aerobic dynamic feeding (ADF) process, where the MMCs are subjected to a selective pressure caused by consecutive periods of external substrate excess (feast) and limitation (famine). The SBRs operated with cycles of feeding, reaction, settling and dewatering were mostly utilized as selectors by studies related to the enrichment of PHA accumulating MMCs under ADF conditions (Dias et al., 2006). Effects of parameters such as organic loading rate (OLR) (Dionisi et al., 2006), influent substrate concentration (Albuquerque et al., 2010), sludge retention time (SRT) (Beun et al., 2000, 2002; Chua et al., 2003), reaction pH (Chua et al., 2003; Serafim et al., 2004) and temperature (Chinwetkitvanich et al., 2004; Johnson et al., 2010) on the PHA accumulation of MMCs have been investigated via evaluating either the accumulating dynamic response to specific factors under a steady state operation (stable PHA accumulation ability obtained) or along the enrichment process from original seed biomass (usually activated sludge) to PHA accumulating organisms-rich MMCs. During these studies, the PHA accumulating ability (*e.g.*, maximum PHA content, PHA yield) of the enriched cultures has always been the primary measures to evaluate the enrichment performance. However, the physical characteristics of the mixed cultures have barely been studied. The unstable physical state of MMCs may evolve into an irreversible deterioration, leading to the failure of selection, thus the stability control of the selection process is necessary.

Carbon concentration and SRT are the most important parameters because multiple effects will be involved when investigating the effect mechanism. For example, SRT would affect the enrichment process of PHA accumulating cultures by influencing the Feast/Famine (F/F) ratio (ecological selection and physiological adaptation) (Albuquerque et al., 2010; Beccari et al., 1998) and imposing a weeding out effect (physical selection) on the MMC. The physical selective pressure has been successfully used in PHA MMCs selection in Chen et al.’s (2015), however, its effect on the enrichment process needs further discussion. In addition, to our knowledge, no studies about the effects of initial (seed) biomass concentration of the selector on PHA MMC enrichment have been reported. On one hand, the biomass concentration tends to reach a steady state in batch cultivations when the carbon concentration and SRT are fixed. On the other hand, different initial biomass concentrations will significantly affect the physical status of MMCs before the steady state is achieved and consequently affects the enrichment process.

In the present work, four SBRs using fermented sugar cane wastewater as the substrate were operated under the ADF mode to select PHA accumulating mixed cultures from municipal activated sludge. Physical characteristics, PHA content, kinetic and stoichiometric parameters of the MMCs in the SBRs were monitored during the selection period (from activated sludge to PHA accumulating organisms-rich MMCs) to determine the suitable SRT, carbon concentration and initial biomass concentration for the enrichment process. The multiple effects of the three parameters on the enrichment process were investigated as well. The terminal restriction fragment length polymorphism

(T-RFLP) analysis was performed, aiming to relate the microbial community structure and PHA accumulating ability of the enriched cultures. A model to further understand the affecting mechanisms of SRT was proposed.

1. Material and methods

1.1. Experimental setup

1.1.1. Fermentation reactor

The fermented sugar cane wastewater was obtained from a modified anaerobic continuous stirred tank reactor (CSTR) with a working volume of 19.6 L. The hydraulic retention time (HRT) was 9.6 hr, and pH in the reactor was maintained at 4.8 (± 0.2) by adding NaHCO₃ in the substrate. Temperature was maintained at 35°C through a temperature control system. Sugar cane solution (chemical oxygen demand (COD) of 336 g/L, according to the oxidation stoichiometry: 1.12 g COD/g sugar) with nutrients and trace elements (1 mL/L) was used as the influent, and an empirical optimal C/N/P ratio (mass ratio) of 1000/5/1 was adopted to keep the steady state of the CSTR. The composition of the nutrients is shown in Table 1. The trace elements stock solution consisted of (in g/L): 0.06 KI, 0.24 MnCl₂·4H₂O, 0.12 Na₂MoO₄·2H₂O, 3 FeCl₃·6H₂O, 0.3 H₃BO₄, 0.06 CuSO₄·5H₂O, 0.24 ZnSO₄·7H₂O, 0.3 CoCl₂·6H₂O and 3 EDTA. The fermented effluent was filtered through a hollow fiber membrane system (2 × 10⁴ MW cut-off). The filtered effluent consisted mainly of volatile fatty acids (VFAs) (acetate, propionate, butyrate and valerate, accounting for 71.2% of the total soluble chemical oxygen demand (SCOD)), saccharides (glucose and undefined polysaccharose, accounting for 20.6% of the total SCOD) and undefined inert substance (accounting for 9.2% of the total SCOD).

1.1.2. Enrichment selectors

Four SBRs, each with a working volume of 4 L, were operated for the enrichment of PHA accumulating cultures. The SBRs were inoculated with the activated sludge taken from a local wastewater treatment plant in Harbin, China. The main operating parameters are shown in Table 2. The filtered effluent from the CSTR with additional nutrients and trace elements was used as the influent for the SBRs. The composition of nutrients is listed in Table 1. In order to ensure the existence of ammonia throughout the operating cycle, the C/N/P ratio (mass ratio) of each selector was maintained at 100/6/1. 1 mL/L of concentrated trace elements solution was added into the influent of each selector, of which composition was determined according to the early study of Vishniac and Santer (1957). In

Table 1 – The composition of the continuous stirred tank reactor (CSTR) influent and each sequencing batch reactor (SBR) influent in the experiment.

Components (unit)	NH ₄ Cl (g/L)	KH ₂ PO ₄ (g/L)	MgSO ₄ (g/L)	CaCl ₂ (g/L)	EDTA (g/L)
CSTR	0.14	0.03	0.05	0.05	–
SBR #1	0.25	0.05	0.10	0.10	0.10
SBR #2	0.54	0.11	0.10	0.10	0.10
SBR #3	0.54	0.11	0.10	0.10	0.10
SBR #4	0.54	0.11	0.10	0.10	0.10

Table 2 – The operating parameters of the selectors under aerobic dynamic feeding (ADF) mode.

Operating parameters	SBR #1	SBR #2	SBR #3	SBR #4
Hydraulic retention time (HRT) (day)	1	1	1	1
Operating cycle length (hr)	12	12	12	12
SRT (day)	10	10	5	5
Carbon concentration (g COD/L)	1.41 (± 0.15)	2.48 (± 0.33)	2.52 (± 0.20)	2.48 (± 0.25)
Initial biomass concentration (g MLSS/L)	3.52 (± 0.12)	3.61 (± 0.14)	3.65 (± 0.25)	5.91 (± 0.16)

Carbon and initial biomass concentration were measured values;
Values in the brackets are the standard deviation;
SRT: sludge retention time.

addition, thiourea was added to inhibit nitrification in the enrichment systems. Initial pH was adjusted to 7 (± 0.1) by using a NaOH solution (2 mol/L) and left uncontrolled during the operating cycle. All three selectors were operated at room temperature ($20 \pm 3^\circ\text{C}$). The selectors were operated with 12 hr cycles which consisted of four discrete periods: feeding (0 to 10 min); aerobic reaction (10 min to 11 hr); sedimentation (11 hr to 11 hr 50 min); withdrawal (11 hr 50 min to 12 hr). In the aerobic reaction phase, MMCs entered into the famine phase when the substrate (carbon source, the same below) was exhausted. Air was supplied by an air pump through ceramic diffusers and the dissolved oxygen (DO) concentration in the feast phase was above 3 mg/L.

1.1.3. Batch tests

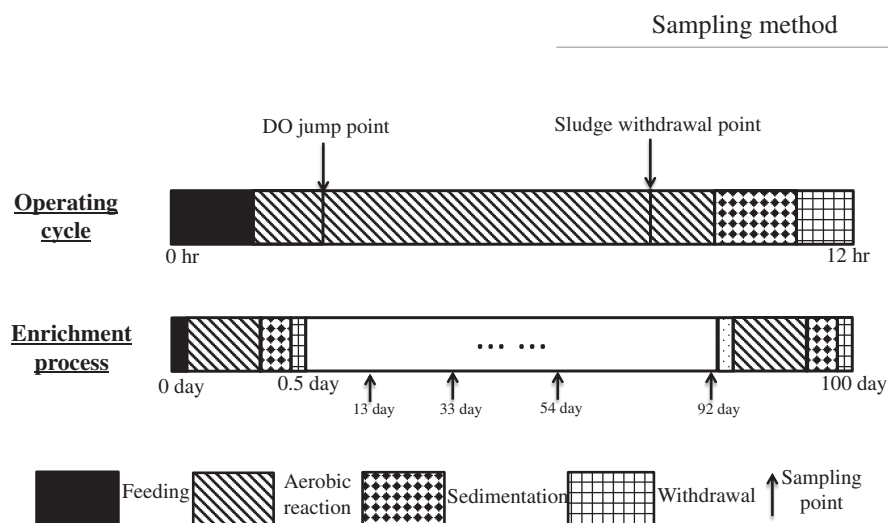
Batch tests were carried out to determine the maximum PHA accumulation ability and relevant kinetic performance of the enriched cultures. The excess sludge (inoculum) collected from the SBRs was aerated for about an hour before the inoculation to remove residual ammonia, and then concentrated to a final concentration of around 4500 mg/L. The batch reactors, each with a working volume of 500 mL, were inoculated with 250 mL of concentrated excess sludge from the SBRs. The batch reactors were then filled with 250 mL of the filtered effluent from the fermentation CSTR and neither ammonia nor phosphate was added to ensure the nutrients limiting condition. To avoid the

substrate limitation, pulse wise feeding mode was applied. Each time when the substrate was exhausted (indicated by a rapid increase in the DO concentration), 250 mL of the supernatant was withdrawn after settling and then 250 mL of the substrate (the filtered effluent of the fermentation reactor with pH adjusted to 7.0 ± 0.1) was added. The batch tests lasted 10–12 hr. Samples were taken before and after each spike to measure the substrate concentration and PHA content. Air was supplied through a ceramic diffuser from a compressed-air pump. The tests were conducted at room temperature ($20 \pm 3^\circ\text{C}$) with pH uncontrolled.

1.2. Sampling and analytical methods

The definition of the “operating cycle” and the “enrichment process” are shown in Fig. 1. The stability of the enrichment process, which can be reflected by the mixed liquor suspended solids (MLSS) and the sludge volume index (SVI), was monitored every three days. The PHA accumulation performance of the enrichment cultures was evaluated after 13, 33, 54 and 92 days of operation. These sampling points are marked in Fig. 1.

SCOD (potassium dichromate method), ammonia (Nessler reagent spectrophotometry), volatile suspended solids (VSS), MLSS, sludge volume (SV_{30}) were measured according to the standard analytical methods (Chinese SEPAC, 2002). DO concentration and pH were measured using a DO meter (Oxi 330i, WTW, Germany) and a pH meter (HI8424, HANNA, Italy), respectively.

**Fig. 1 – The distribution of sampling points during the operating cycle and the enrichment process.**

The quantification of VFAs and PHAs were carried out using a gas chromatograph (GC7890N, Agilent, United States) with a flame ionization detector (FID) according to the methodology described by Wen et al. (2012), hydroxybutyrate and hydroxyvalerate concentrations were calculated using P(HB-HV) (95%/5%, mass fraction) (Sigma, USA) standards. The total carbohydrates concentration was detected using anthrone agent colorimetry (Morris, 1948).

The total genomic DNA was extracted from the activated sludge samples taken from the three selectors by the end of operating cycle in triplicate using the E.Z.N.A. bacterial DNA kit (OMEGA, United States) and then mixed well for the next analysis. The universal primers of 27f (5'-AGAGTTTGATCCT GGCTCAG-3') (labeled with the phosphoramidite dye 6-FAM at the 5' end) and 1387R (5'-GGGCGWGTGTACAAG GC-3') (Marchesi et al., 1998) were used for Polymerase chain reaction (PCR) and 4-bp cutting enzymes *MspI* were used for endonuclease digestion. The terminal restriction fragments (TRFs) were sized by Sangon Biotech (Shanghai, China). The abundance of the individual TRF in a given sample was calculated based on the total peak area of the TRFs. TRFs with a peak area no more than 3% of the total area of the sample were excluded from further analysis. Microbial Community Analysis (MiCA), accessed at <http://mica.ibest.uidaho.edu/>, was utilized to perform the phylogenetic assignment of TRFs (Shyu et al., 2007; Xin et al., 2015). The typical tolerance for the ISPaR method was set to 1 bp. The data base used during the analysis was SILVA (R106) 512, 037 SSU (16S/18S) Ref.

1.3. Calculation of kinetic and stoichiometric parameters

SVI was calculated by dividing SV_{30} (mL/L) by MLSS (g/L). PHA content is defined as a fraction of MLSS on a mass basis (% PHA = g PHA/g MLSS \times 100). Conversion rates, including PHA storage yield ($Y_{PHA/S}$ in mg COD/mg COD) and biomass yield ($Y_{X/S}$ in mg COD/mg COD), were calculated by dividing the amount of PHA formed or the increased biomass by the amount of substrate consumed (S), respectively. The calculation of biomass increment was referred to the description in Wen et al. (2012). Biomass specific conversion rates, including specific PHA storage rate (q_{PHA} in mg COD/mg X/hr) and specific substrate uptake rate ($-q_s$ in mg COD/mg X/hr) were calculated by dividing the amount of PHA formed or the substrate consumed by the average active biomass concentration (X) between two sampling points and the reaction time. The active biomass concentration was calculated by subtracting the amount of PHA from MLSS. The unit of the active biomass was converted from mg/L to mg COD/L with a conversion factor of 1.42 mg COD/mg X and PHAs concentration (mg/L) was converted to mg COD/L according to the oxidation stoichiometry: 1.67 mg COD/mg polyhydroxybutyrate (PHB) and 1.92 mg COD/mg polyhydroxyvalerate (PHV) (Beccari et al., 2009).

2. Results and discussions

2.1. Stability of the enrichment systems

The stability of the enrichment system was evaluated by monitoring physical characteristics of the MMCs (reflected by

MLSS and SVI) along the enrichment process, the results are shown in Fig. 2. All the four SBRs were operated over 100 days under the ADF mode. The variation of MLSS in the SBRs showed a consistent trend during the early period of the enrichment process. MLSS concentration decreased (remarkably for #1, #3 and #4) during the first 20 days then it began to increase. The biomass loss during this period may be due to that bacteria with poor PHA accumulating capacity in the seed sludge were weeded out from the enrichment systems. Therefore, the initial 20 days can be designated as "selection period", the boundary of which is indicated by the rather steady amount of the biomass.

The steady MLSS concentration in SBR #3 was close to that in SBR #4 due to the similar influent substrate concentration (2.52 g COD/L vs. 2.48 g COD/L) and SRT (5 days) employed. Theoretically, SBR #2 with a high influent concentration (2.48 g/L) and a long SRT (10 days) should have the maximum biomass concentration, however, the biomass concentration in SBR #2 decreased steeply after reaching a high value (above 4500 mg/L). The poor settleability of the sludge in SBR #2 caused the biomass loss in the withdrawal. SVI in SBR #1 reached the maximum value on day 20 then recovered to a stable state. The maximum value of SVI in SBR #4 also occurred on day 20 before it fell to a lower level, but it rose again in the later stage of the operation. SVI in SBR #2 maintained a relative high value (around 200) and increased to more than 500 after 40 days of operation. Mixed cultures in such a state cannot be used in the following batch accumulating assays. Microscopic examination ruled out the possibility of filamentous bulking, indicating that the fluctuation of the sludge physical characteristics was caused by the unstable extracellular polymer metabolism (Guo et al., 2014; Shin et al., 2000). SVI in SBR #3 (influent concentration of 2.52 g COD/L; SRT of 5 days; food to microorganism ratio (F/M) of 0.69) was steady below 100 during the whole enrichment process, indicating that these operating conditions resulted in a stable selection process.

2.2. PHA accumulation along the enrichment process

2.2.1. PHA accumulation performance in the selectors (nutrients available)

In order to evaluate the PHA accumulation performance of the MMCs in the selectors during the enrichment process, the PHA content, kinetic and stoichiometric parameters in the feast phase of each SBR were calculated. The results are presented in Table 3. The maximum PHA content (PHA_{max}), $Y_{PHA/S}$ and q_{PHA} obtained in the feast phase of all the SBRs increased with the operation time except for those of SBR #2, which declined significantly on day 54. The poor PHA accumulating performance in SBR#2 was caused by the deteriorated physical characteristics of the MMCs. The $Y_{PHA/S}$ of the cultures in the three selectors (#1, #3 and #4) kept increasing with the operation time, while the growth yield ($Y_{X/S}$) showed an opposite trend, indicating a shift of the carbon partitioning (from growth dominating to PHA accumulation dominating) of the MMCs under the ADF mode. The selectors (#3 and #4) with high influent concentration (2.52 g COD/L and 2.48 g COD/L, respectively) and short SRT (5 days) enriched the MMCs with better PHA accumulating performance. The maximum PHA_{max} (30.75%) and $Y_{PHA/S}$ (0.80 mg COD/mg COD) were achieved in SBR #3 with a relatively lower biomass concentration.

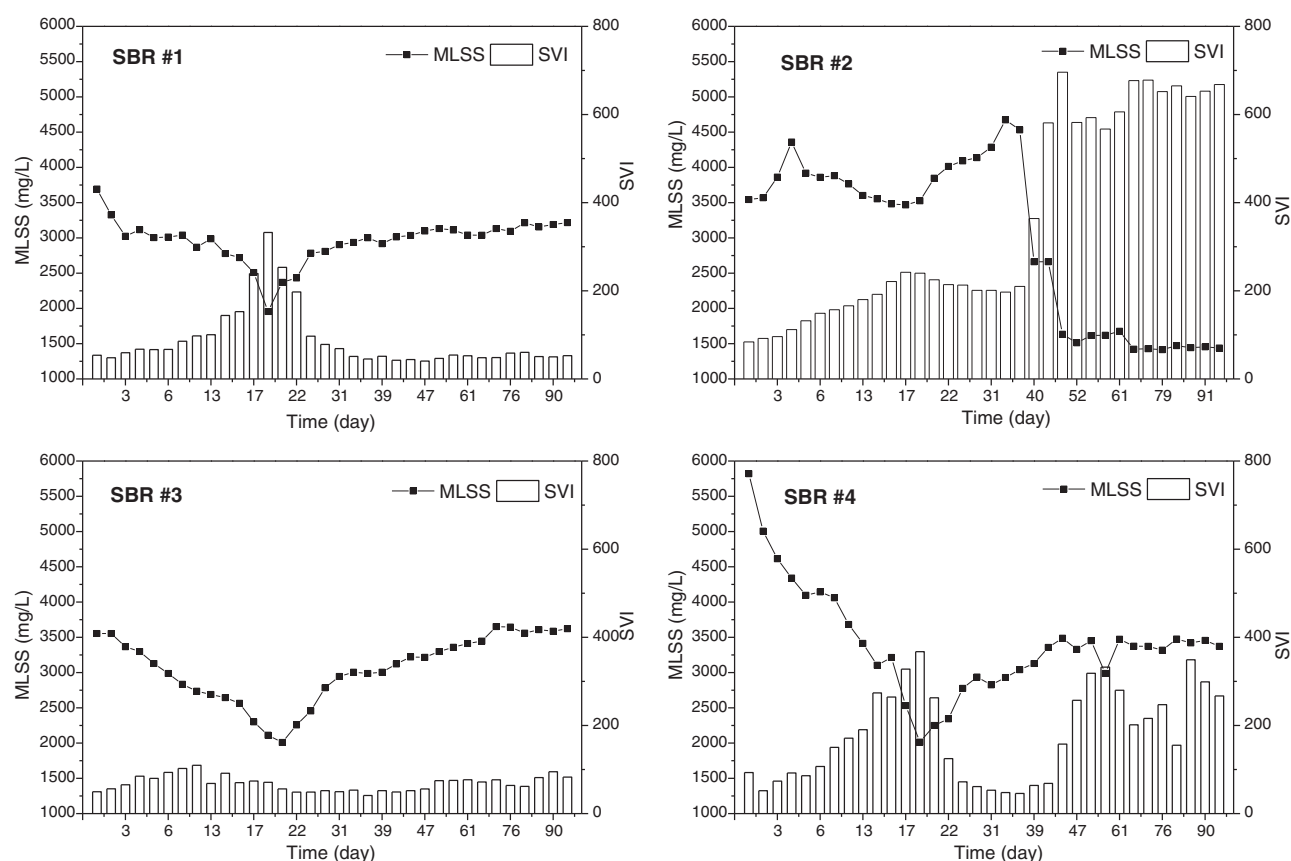


Fig. 2 – Mixed liquor suspended solids (MLSS) and sludge volume index (SVI) of the mixed cultures in four sequence batch reactors (SBR) along the enrichment process.

2.2.2. PHA accumulation performance in batch assays (nutrients limiting)

The PHA production capacities of the enriched MMCs cultivated in the four selectors were evaluated in batch assays

under nutrients limiting conditions. Parameters including PHA_{max} , $Y_{PHA/S}$ and q_{PHA} were calculated and summarized in Fig. 3. No data of SBR #2 at day 92 was gotten due to the serious biomass loss at the final stage of the enrichment

Table 3 – The comprehensive polyhydroxyalkanoate (PHA) accumulation performance of mixed microbial cultures (MMCs) in the three selectors under aerobic dynamic feeding (ADF) mode during the enrichment process.

Selector	Sample time point (operation days)	F/F ratio	PHA_{max}^a (%)	$Y_{PHA/S}$ (mg COD/mg COD)	$Y_{X/S}$ (mg COD/mg COD)	q_{PHA} (mg COD/mg X/hr)	$-q_s$ (mg COD/mg X/hr)
SBR #1	13	0.03	9.55 (0.62)	0.49 (0.05)	0.15 (0.04)	0.40 (0.01)	0.81 (0.05)
	33	0.04	15.10 (0.78)	0.50 (0.02)	0.14 (0.06)	0.31 (0.03)	0.62 (0.02)
	54	0.03	16.45 (0.45)	0.63 (0.04)	0.14 (0.02)	0.35 (0.02)	0.56 (0.04)
	92	0.03	18.90 (0.96)	0.71 (0.02)	0.11 (0.03)	0.42 (0.05)	0.60 (0.05)
SBR #2	13	0.02	17.65 (0.55)	0.59 (0.04)	0.21 (0.03)	0.42 (0.02)	0.84 (0.04)
	33	0.02	24.52 (0.43)	0.68 (0.02)	0.18 (0.01)	0.45 (0.02)	0.70 (0.07)
	54	0.16	19.32 (0.92)	0.32 (0.02)	0.28 (0.05)	0.18 (0.05)	0.51 (0.11)
SBR #3	13	0.11	20.94 (0.79)	0.68 (0.04)	0.18 (0.05)	0.31 (0.02)	0.45 (0.02)
	33	0.06	29.03 (0.75)	0.76 (0.02)	0.15 (0.04)	0.51 (0.04)	0.67 (0.02)
	54	0.04	28.20 (1.29)	0.75 (0.08)	0.12 (0.02)	0.53 (0.02)	0.70 (0.05)
	92	0.04	30.75 (0.64)	0.80 (0.02)	0.08 (0.01)	0.58 (0.01)	0.73 (0.03)
SBR #4	13	0.07	16.72 (0.68)	0.47 (0.06)	0.16 (0.08)	0.26 (0.04)	0.57 (0.01)
	33	0.08	20.31 (0.93)	0.70 (0.05)	0.14 (0.05)	0.42 (0.03)	0.62 (0.05)
	54	0.09	22.65 (0.85)	0.72 (0.09)	0.16 (0.03)	0.49 (0.05)	0.68 (0.04)
	92	0.05	28.94 (0.53)	0.73 (0.05)	0.15 (0.05)	0.54 (0.03)	0.78 (0.06)

Values in the brackets are the standard deviation.

^a PHA_{max} refers to the maximum PHA content in the sludge (as a percentage of MLSS) at the end of the feast phase;

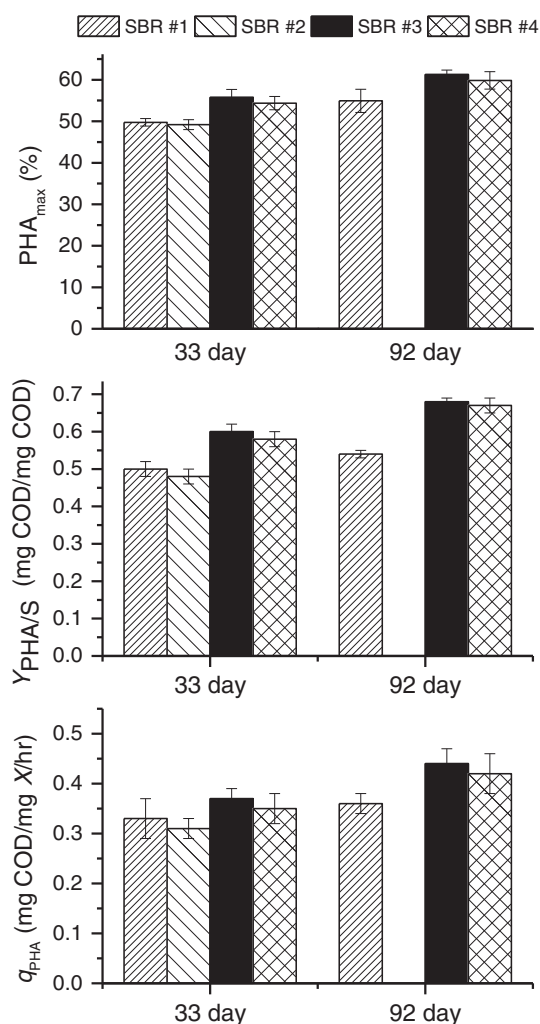


Fig. 3 – PHA accumulating performance obtained from the batch assays inoculated with the enriched mixed microbial culture (MMC) from the selectors after 33 and 92 days of operation.

period. The value of the three parameters for enriched cultures from SBR #1, #3 and #4 increased with the operation time, and those of SBR #3 changed remarkably.

Under the same operating conditions (Section 1.1.3), the cultures enriched in SBR #3 showed the best PHA production ability after 92 days of operation, the maximum PHA content of 61.26%, $Y_{PHA/S}$ of 0.68 mg COD/mg COD and q_{PHA} of 0.44 mg COD/mg X/hr were achieved in the batch assays. PHA accumulating performance of SBR #4 with the similar SRT and carbon concentration were close to that of SBR #3. Such results from the batch assays indicated that enriched cultures from selectors with short SRT (5 days of SBR #3 and #4) showed an advantage in PHA accumulation over the cultures enriched from selectors with longer SRT (10 days of SBR #1 and #2).

2.3. Microbial community analysis via T-RFLP

The changes of the microbial community structures under selective pressures in the three selectors (#1, #3 and #4) after 92 days of operation as well as the seed biomass were analyzed using 16S rRNA targeted T-RFLP. The distributions

and relative abundance of the distinct TRFs are shown in Fig. 4. The results showed that the diversity and dominant strains of the MMCs changed significantly after the enrichment process with different operating conditions. In T-RFLP analysis, a certain distinct TRF can represent an operational taxonomic unit (OTU) (Dunbar et al., 2001), therefore, the number of the TRFs can reflect the diversity of the MMCs. There were less distinct TRFs in the samples from the three selectors after the enrichment compared to the seed sludge, indicating that the microbial community diversity in the selectors decreased when they were subjected to selective pressures. The diversity of the MMCs in SBR #3 and SBR #4 was similar, as shown in Fig. 4, and was less than that in SBR #1. This indicated that a short SRT will impose a stronger ecological selective pressure on the MMCs.

Three distinct fragments, 81, 441 and 487 bp, presented in all the samples, this indicated that there were microorganisms with PHA accumulating ability in the municipal wastewater treatment sludge that can be enriched under proper conditions. The relative abundance of each presented fragment in samples taken from different enrichment systems was not consistent. For example, the relative abundance of T[441] (TRFs of 441 bp) and T[430] in SBR #3 and SBR #4 was lower than those in SBR #1 (the relative abundance of T[430] in SBR #4 was too small to show in the figure). This indicated that the bacteria represented by T[441] and T[430] have generation time between 5 and 10 days. The relative abundance of T[81] in SBR #3 was higher than that in SBR #4, while the abundance of T[487] presented in the opposite way. Given the PHA accumulation performance of the two selectors, it suggested that the bacterium represented by T[81] has higher PHA accumulating ability.

The conclusions for the microbial community structure in the previous research works (Albuquerque et al., 2007, 2010, 2013) on MMC PHA production using sugar molasses as the substrate were consulted. The details of the dominant bacteria in the three selectors are showed in Table 4. The dominant bacteria in SBR #3 were *Bacillus* and *Thauera*. *Pseudomonas* dominated in both SBR #1 and SBR #4.

2.4. Multiple effects of parameters on the selection process

2.4.1. Initial biomass concentration

Experimental data obtained from SBR #3 and #4 with the same SRT and carbon concentration was compared to investigate the effect of initial (seed) biomass concentration (3.65 vs. 5.91 g/L) on the enrichment process. The PHA accumulating performance of the enriched MMCs from SBR #4 in the batch assays was similar to that of SBR #3, but physical properties of the MMCs in SBR #4 fluctuated during the enrichment process. Therefore, it is suggested that higher initial biomass concentration would lead to an unstable physical status (reflected by SVI values) of the MMCs. The PHA_{max} , $Y_{PHA/S}$ and q_{PHA} of the enriched cultures from SBR #4 in batch assays were a little bit lower than those of SBR #3 although the differences were inconspicuous, as shown in Table 3. Therefore, it can be concluded that the initial biomass concentration has no significant influence on the PHA accumulating performance of the enriched cultures regardless of the change in physical characteristics. Unlike pure-culture PHA fermentation, the normal operation of the open system for MMCs PHA production relies on the good settleability of the MMCs, therefore, choosing a suitable initial biomass

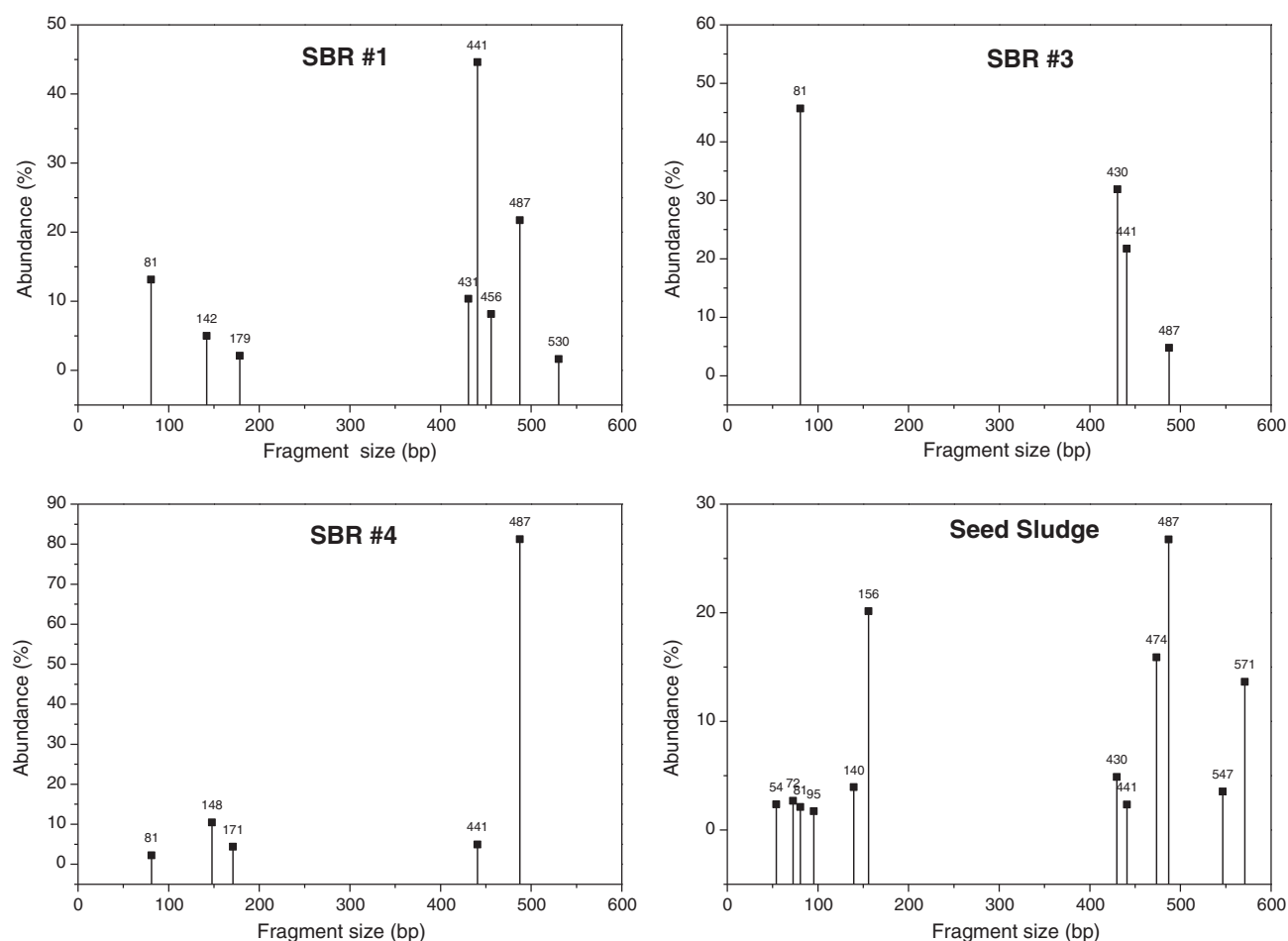


Fig. 4 – T-RFLP results for sludge samples from three selectors and the seed sludge.

concentration for seeding is necessary. In other words, results described above indicated that the PHA accumulating performance of MMCs have no substantial relationship with their physical characteristics, made the discussions of effects of carbon concentration and SRT on the PHA accumulation without considering the physical state of the MMCs viable.

2.4.2. Carbon concentration

Two carbon concentration levels were used in this study: 1400 (for SBR #1) and 2500 (for SBR #2, #3 and #4) mg COD/L. The continuous deteriorated physical characteristics of SBR #2 after 40 days of the operation made PHA accumulation unfeasible, however, the experimental data before the failure were available to compare with those of SBR #1 to evaluate the effects of carbon concentration on PHA accumulation. SBR #2 with higher carbon concentration showed better PHA accumulating performance in

terms of $Y_{PHA/S}$ and q_{PHA} on the day 13 and 33. However, the slightly lower maximum PHA content (51.75% vs. 51.2%), $Y_{PHA/S}$ (0.48 vs. 0.50 mg COD/mg COD) and q_{PHA} (0.31 vs. 0.33 mg COD/mg X/hr) of enriched cultures from SBR #2 compared to those of #1 were observed in the following batch assays. The possible reason for the better PHA accumulating performance of SBR #2 was the high kinetic response under higher carbon concentration. The deteriorated physical characteristic of the MMCs in SBR #2 may be related to high biomass concentration caused by high carbon concentration. Further study needs to be conducted to reveal the details. It can be suggested that the two carbon concentration levels used in this study did not influence the selective pressure significantly.

2.4.3. SRT

It is worth noting that sludge washout occurred after 40 days operation in SBR #2 caused by the deteriorated settleability, made the actual SRT lower than the set value (10 days). Therefore, the discussion of SRT affecting mechanism involving SBR #2 was restricted to the period before sludge washout.

The PHA accumulating performance of the enriched MMCs of SBR #3 and #4 (SRT = 5 days) in batch assays were better than those of SBR #2 and #1 (SRT = 10 days) in this study. In addition, the T-RFLP analysis indicated that the MMCs in SBR #3 and #4 were subject to a stronger selective pressure. These results are consistent with the previous reported study on ADF process with

Table 4 – Identification of the dominant bacteria obtained from the three selectors.

Fragment size (bp)	Enrichment system	Relative abundance (%)	The maximum possible organism
81	SBR #2	45.7	<i>Bacillus</i>
441	SBR #1	44.6	<i>Pseudomonas</i>
430(431)	SBR #2	31.9	<i>Thauera</i>
487	SBR #3	81.2	<i>Pseudomonas</i>

SRT of 2 days (Johnson et al., 2009), which demonstrated that a lower SRT favored PHA accumulation, but does not conform to other works that believed a longer SRT favored PHA accumulation (Beun et al., 2000, 2002; Pagni et al., 1992). Therefore, effects of SRT on the ADF enrichment process need to be further discussed to understand its role in selection processes.

Two factors were suggested to be involved in the SRT influencing mechanism: (1) Feast/Famine ratio, which affects both the physiological pressure (i.e., the internal growth inhibition) (Albuquerque et al., 2010; Majone et al., 1996) and ecological selective pressure on the mixed cultures. It is worth noting that although higher F/F ratios of SBR #3 and #4 were observed compared to those of SBR #1 and #2 on the day 13 and 33 (Table 3), the enriched MMCs in SBR #3 and #4 still showed better PHA accumulating performance. A low biomass concentration at the beginning of the feast phase, resulting from a short SRT (SBR #3 and #4), may become the restrictive factor of the volumetric substrate uptake rate, leading to a relatively longer feast phase and a shorter famine phase, which was believed to have negative effects on the enrichment of PHA accumulating organisms (Beccari et al., 1998; Dias et al., 2006; Dionisi et al., 2006). (2) Weeding out effect. It is widely accepted that the PHA storing organisms can maintain a kinetic advantage to outgrown non-storing heterotrophs due to the rapid kinetics of carbon storage during the feast phase. Therefore, the stronger weeding out effect caused by a short SRT would enhance the growth advantage of PHA-storing organisms, consequently lead higher observed specific reaction rates. On the other hand, more inert biomass (most of which are in the endogenous respiration period) would be accumulated in the selectors under a longer SRT and probably would lead to a decreased $-q_s$ in the feast phase along the enrichment process. The above assumptions were supported by the increased $-q_s$ in SBR #3 and #4 and the decreased $-q_s$ in SBR #1 and #2 along the enrichment process.

In this study, a new model was proposed to explain the multiple effects of the above two factors caused by SRT on the enrichment process. In this model, the enrichment process is divided into two phases: the selection phase, in which the weeding out effect would impose a physical selective pressure on the system and play the dominant role; and the enhancement phase, in which the physiological adaptation caused by F/F ratio plays the dominant role, and a stable microbial community is formed. Using SBR #3 (SRT = 5 days) for example, the enriched MMCs in SBR #3 showed a better PHA accumulation performance compared to SBR #1 and #2 (SRT = 10 days) in the batch assays (maximum PHA content and $Y_{PHA/S}$) although the F/F ratio was higher (0.16 and 0.07 on day 13 and 33, respectively) in the selection phase. This indicated that the weeding out pressure played a dominant role in this phase. When the process went into the enhancement phase, PHA accumulating bacteria has become the dominant organisms in the MMC, their PHA storing ability strongly depended on the metabolic status (i.e., internal growth limitation) of the bacteria, which mainly affected by the F/F value (Albuquerque et al., 2010).

3. Conclusions

Effects of SRT, carbon and initial biomass concentrations on PHA producing MMCs enrichment systems were investigated in

this study. The selector with the SRT of 5 days, initial carbon concentration of 2.52 g COD/L and initial biomass concentration of 3.65 g/L gave the best enrichment performance in terms of both PHA accumulating ability and operating stability. The carbon concentration level (1.41–2.52 g COD/L) used in this study showed no significant influence on the process. A higher initial biomass concentration resulted in an unstable physical status of the MMCs, but had little effect on the PHA accumulating ability of the enriched cultures. SRT affects the selection process through either the F/F ratio or the weeding out effect. These two effects exist simultaneously and competing with each other to affect the selection performance throughout the enrichment process.

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