Research Article

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Polyhydroxyalkanoate production using two-stage continuous stirred tank activated sludge systems with glycerol as a carbon source

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Abstract

BACKGROUND: Polyhydroxyalkanoate (PHA) is a biopolymer that can be used as a biodegradable plastic. The incorporation of PHA production into wastewater treatment processes is a promising strategy for resource recovery. This study investigated PHA production in two-stage continuous stirred tank reactor (CSTR)-activated sludge (AS) systems fed with synthetic wastewater containing glycerol. The effects of the hydraulic retention time (HRT) ratios of the first and the second stages, which reflects the feast/famine ratio, at 0.04, 0.15, and 0.33 were examined.

RESULTS: The PHA accumulation and PHA consumption patterns under feast/famine conditions were successfully achieved in systems operated at HRT ratios of 0.04, 0.15, and 0.33 with influent glycerol concentrations of 3000 mg COD/L. Then, the sludge from these systems was tested for PHA accumulation in fed-batch reactors. Maximum PHA contents of 21.8 wt%, 13.1 wt%, and 8.7 wt% were obtained for the sludge from the systems operated at HRT ratios of 0.04, 0.15, and 0.33, respectively. From the microbial community analysis via 16S rRNA gene amplicon sequencing (MiSeq, Illumina), the relative abundance of betaproteobacteria decreased, whereas the relative abundances of alphaproteobacteria and gammaproteobacteria increased after cultivation in the two-stage CSTR AS systems.

CONCLUSION: The highest PHA accumulation was achieved at the lowest HRT ratio of the first and the second stages in the two-stage CSTR AS systems. This study serves as an example of integrating PHA production into wastewater treatment systems, which enables us to apply the concept of resource recovery from wastewater.

Keywords: polyhydroxyalkanoate; two-stage continuous stirred tank reactor; feast/famine feeding; mixed cultures; glycerol

INTRODUCTION

Polyhydroxyalkanoate (PHA) is a biopolymer that can be used as a biodegradable plastic.[1] The use of mixed microbial cultures for PHA production has recently gained substantial attention since it enables the process to be combined with wastewater treatment processes.[2] PHA production from organic wastewater has also been considered a promising strategy for resource recovery. Glycerol is the major component of wastewater from biodiesel production, an industry that has grown considerably over the last several years.[3] Unlike other substrates such as molasses,[4] glycerol can be used directly as a substrate for PHA production and does not require pre-fermentation processes,[5–7] making it an appealing choice as a substrate for PHA production.

Among several approaches for PHA production using mixed microbial cultures, including anaerobic/aerobic conditions, microaerophilic/aerobic conditions, and feast/famine conditions,[8] the highest PHA content (89 wt%) was achieved under feast/famine conditions.[9] The enrichment of PHA-accumulating microorganisms in mixed microbial cultures using a feast/famine feeding regime primarily relies on the competitive advantages of PHA-accumulating microorganisms over other microorganisms under these conditions since PHA-accumulating microorganisms can store PHA as an internal carbon source during the feast phase and subsequently use it during the famine phase; thus, they are highly resistant to starvation.[10,11] The repetitive cycles of feast/famine conditions tend to favor the proliferation of PHA-accumulating microorganisms.

Most of the previous research on PHA production using the feast/famine feeding regime has focused on the use of sequencing batch reactors (SBRs), whereas few studies have investigated PHA production under feast/famine conditions in continuous reactors,[4,12] despite its convenience of incorporation into conventional wastewater treatment systems, such as activated sludge (AS). Two-stage continuous stirred tank reactor (CSTR) systems have been developed for PHA production; in such systems, the

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first and the second stages of the CSTRs are expected to create the feast and famine conditions, respectively.[4,12]

This study aims to investigate PHA production in two-stage CSTR AS systems using glycerol as a carbon substrate, with a focus on the ratio of the hydraulic retention times (HRTs) of the two stages. This ratio reflects the feast/famine ratio, which is an important factor for PHA production under feast/famine conditions. In addition, microbial communities in the two-stage CSTR AS systems fed with glycerol were also examined using 16S rRNA gene amplicon sequencing (Illumina, MiSeq). The findings from this study can improve our understanding of the effects of the feast/famine ratio on PHA production in continuous systems, which can assist us in the development of a suitable PHA production approach that can be incorporated with existing wastewater treatment plants.

MATERIALS AND METHODS

Operation of two-stage CSTR AS systems

Figure 1 shows the diagram of a two-stage CSTR AS system. The system consists of two CSTRs and a sedimentation tank with sludge return to the first CSTR. Three sets of two-stage CSTR AS systems (F/F1, F/F2, and F/F3) were operated at room temperature (27–31.2 °C) to investigate the effects of the feast/famine ratio on PHA production. The feast/famine ratio, HRT, and volumes of the CSTRs of these systems are summarized in Table 1. The total HRT of each system was 48 h. In the F/F1 and F/F2 systems, the feast/famine ratio was initially set at 0.15 and 0.23. After 33 days of operation, we decreased the feast/famine ratio to 0.04 and 0.15, respectively. However, in F/F3, the feast/famine ratio was 0.33 throughout the operation. All systems were initially seeded with the sludge obtained from an aerobic SBR in the wastewater treatment plant of a fruit juice manufacturing factory. This seed sludge has previously been shown to have the capability to accumulate PHA after enrichment under feast/famine conditions in SBRs.[13,14] Synthetic wastewater was fed continuously into the systems at a flowrate of 10 L/d. Diaphragm pumps (CONCEPT^{PLUS}, ProMinent F.C., Thailand) were used for feeding the influent and returning the sludge. The solid retention time (SRT) was controlled at ten days in all of the systems. The sludge wastage rate, Q_w (in L/d), was calculated via $SRT = \frac{X_1V_1 + X_2V_2}{Q_wX_R + (Q - Q_w)X_e}$, where X_1, X_2, X_R, and X_e represent the mixed liquor suspended solid (MLSS) concentrations (in mg/L) in the first CSTR, in the second CSTR, at the bottom of the sedimentation tank, and in the effluent, respectively; V_1 and V_2 are the volumes (in liters) of the first and second CSTRs, respectively; and Q is the influent flowrate (10 L/d). The sludge was wasted once per day. The MLSS concentrations were measured every 2–3 days to calculate the corresponding Q_w.

The synthetic wastewater consisted of glycerol (Ajax Finechem, Taren point, NSW, Australia), 40.2 Cmmol/L (equivalent to 1500 mg COD/L); NH_4Cl (KEMAUS, Australia), 191.07 mg/L; KH_2PO_4), (Ajax Finechem), 43.87 mg/L; $MgSO_4$ (Ajax Finechem), 500 mg/L; $FeCl_3$ (Panreac applichem, Germany), 10 mg/L; $CaCl_2$ (Ajax Finechem), 10 mg/L; H_3BO_3 (Ajax Finechem), 4 mg/L; $CuSO_4{\cdot}5H_2O$ (Ajax Finechem), 2 mg/L; $MnCl_2{\cdot}2H_2O$ (Ajax Finechem), 0.3 mg/L; $NaMoO_4{\cdot}2H_2O$ (Sigma-Aldrich, Singapore), 2 mg/L; $ZnSO_4{\cdot}7H_2O$ (Ajax Finechem), 2 mg/L; $CoCl_2{\cdot}6H_2O$ (Ajax Finechem), 8 mg/L; $NiCl_2.6H_2O$ (Ajax Finechem), 2 mg/L; $NaHCO_3$ (Ajax Finechem), 50 mg/L as a pH buffer; and thiourea (Ajax Finechem), 20 mg/L as a nitrification inhibitor (modified from Kumar *et al.*[15] and Woraittinun and Suwannasilp[14]). After 72 days of operation, the concentration of glycerol was increased to 80.3 Cmmol/L (equivalent to 3000 mg COD/L). The pH values in the first CSTRs of all systems were maintained in the range 7.0–7.5 using automatic pH controllers (Alpha pH 560, Thermo Scientific, Waltham, MA, USA). All of the CSTRs were aerated and thoroughly mixed throughout the operation using air pumps (Magic 6600, Twin, China) with porous stone diffusers providing fine bubbles (~3 mm diameter). In all three two-stage CSTR AS systems, chemical oxygen demand (COD), MLSS, PHA contents, pH, dissolved oxygen (DO), temperature, and sludge volume index (SVI) were regularly monitored.

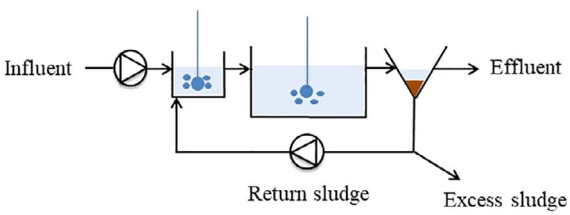

Figure 1. Diagram of a two-stage continuous stirred tank reactor (CSTR) activated sludge (AS) system.

PHA production in batch and fed-batch reactors

The sludge from two-stage CSTR AS systems was transferred to batch reactors and fed-batch reactors to further investigate PHA production. Fed-batch reactors have previously been used to examine the maximum PHA accumulation in mixed microbial cultures.[9,16] The sludge from the second CSTR of the F/F1, F/F2, and F/F3 systems was collected on Day 170, Day 177, and Day 183 of the operation and transferred to Batch F/F1 (9 L) and Fed-batch F/F1 (9 L), Batch F/F2 (8.5 L) and Fed-batch F/F2 (8.5 L), and Batch F/F3 (7.8 L) and Fed-batch F/F3 (7.8 L), respectively. To achieve concentrations of MLSS in the batch and fed-batch reactors similar to those in the two-stage CSTR AS systems, the sludge was settled, and the supernatant was removed. Then, the sludge was added to synthetic wastewater to achieve the initial volumes of sludge obtained. Synthetic wastewater identical to that fed to the two-stage CSTR AS reactors was prepared, except that nitrogen and phosphorus were not included to prevent the growth of microorganisms. For the batch reactors, synthetic wastewater was introduced only once at the beginning. However, in fed-batch reactors, synthetic wastewater was introduced at the beginning, and glycerol was also continuously fed at the rates estimated from the substrate removal rates (5.16 mg COD/L min, 4.15 mg COD/L min, and 4.25 mg COD/L min for the sludge from F/F1, F/F2, and F/F3, respectively) obtained from our preliminary batch experiments. The continuous feeding of glycerol was intended to maintain high substrate concentrations throughout the operation of the fed-batch reactors for maximum PHA accumulation. COD concentrations and PHA contents were monitored over 48 h for all of the batch and fed-batch reactors.

Analytical methods

PHA quantification was performed according to Woraittinun and Suwannasilp[14] and Chanprateep *et al.*[17] The collected sludge samples were centrifuged at 8000 rpm for 5 min and oven-dried at 80 °C for 24 h. Methyl esterification of PHA in the dried samples was performed in a 1:1 (*v/v*) mixture of chloroform (QRëC, New Zealand) and methanol (Honey Well, USA) containing 3% sulfuric acid (QRëC) by heating at 80 °C for 3 h.[17] Gas chromatography with a flame ionization detector (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-INNOWax capillary

Table 1. Feast/famine ratio of the two-stage continuous stirred tank reactor (CSTR) activated sludge (AS) systems and the hydraulic retention time (HRT) and volume of each CSTR

	Days 1–33					Days 34–190				
		HRT (h)		Volume (L)			HRT (h)		Volume (L)	
Two-stage CSTR AS systems	Feast/famine ratio	First CSTR	Second CSTR	First CSTR	Second CSTR	Feast/famine ratio	First CSTR	Second CSTR	First CSTR	Second CSTR
F/F1	0.15	6	42	2.70	17.50	0.04	2	48	0.77	19.38
F/F2	0.23	9	39	3.83	16.88	0.15	6	42	2.70	17.50
F/F3	0.33	12	36	5.17	15.63	0.33	12	36	5.17	15.63

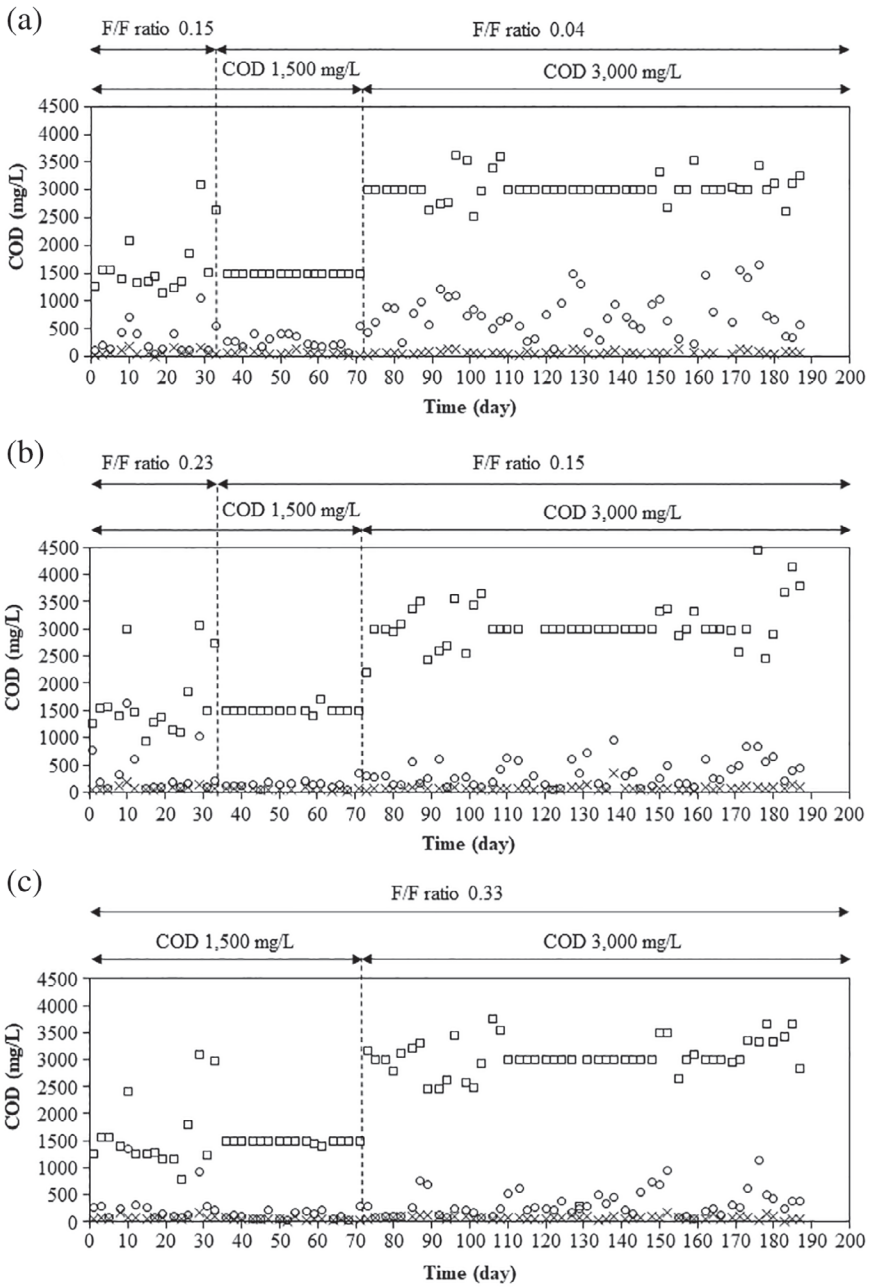

Figure 2. Chemical oxygen demand (COD) concentrations in the influent (□) and effluent of the first continuous stirred tank reactors (CSTRs) (○) and effluent of the second CSTRs (×) in the two-stage CSTR activated sludge (AS) systems: (a) F/F1, (b) F/F2, and (c) F/F3.

column (30 m × 0.25 mm id 0.25 μm, Agilent Technologies) was used to measure the methyl esters of hydroxybutyrate (HB) and hydroxyvalerate (HV). Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing a 12 wt% 3-HV monomer (Sigma-Aldrich, St Louis, MO, USA) was used as a PHA standard, whereas benzoic acid was used as an internal standard. The PHA content in the sludge was reported as wt%, and calculated based on the PHA content (in g PHA) as a percentage of the sludge dry mass (in g MLSS). In addition, the structure of the obtained PHA was analyzed using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. Sludge samples from Fed-batch F/F1 and Fed-batch F/F3 were used to represent the PHA obtained from feast/famine ratios of 0.04 and 0.33, respectively. Due to the low amount of sludge samples, combined sludge samples from Batch F/F2 and Fed-batch F/F2 were used to represent the PHA obtained from the feast/famine ratio of 0.15. PHA was extracted from the dried sludge samples using a Soxhlet extractor with Whatman™ cellulose extraction thimbles (GE Healthcare Life Sciences, USA), and chloroform (VWR Chemicals BDH®, USA) was used as the solvent. The extraction was performed at 80°C for 48 h. Then, the chloroform was vaporized and the remaining PHA film was re-dissolved with chloroform and precipitated in hexane (Macron Fine Chemicals™, USA). These PHA samples were then used subjected to a ^{1}H-NMR analysis (Avance III HD, Bruker, USA) at 500 MHz with deuterated chloroform at 25°C. COD, MLSS, SVI, and ammonia were measured according to the standard method numbers 5200C, 2540D, 2710D, and 4500D in AHPA *et al.*,[18] respectively. The pH values were measured using pH probes in pH controller systems or a pH meter (InLab Expert Pro-ISM electrode, Mettler Toledo, USA). DO was measured by a DO probe (Orion 083010MD, Thermo Scientific).

Microbial community analysis using 16S rRNA gene amplicon sequencing

The seed sludge and sludge samples from all of the two-stage CSTR AS systems at the end of operation were collected to analyze the microbial communities using 16S rRNA gene amplicon sequencing. The sludge from the first and the second CSTR of each system was collected and mixed and served as a representative of the microbial community of the system. Microbial community analysis using 16S rRNA gene amplicon sequencing was performed. DNA was extracted using a DNA extraction kit (FastDNA® Spin Kit for Soil, MP Biomedicals, USA). Polymerase chain reactions (PCRs) were performed with the universal primers for bacteria and archaea, F515 (5′-GTGYCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) (Sigma-Aldrich, Singapore).[19] The PCR products were purified using the GF-1 Ambi-Clean Kit (Gel & PCR, Vivantis Technologies, Selangor Darul Ehsan, Malaysia) and indexed using the Nextera XT index kit. The indexed 16S rRNA gene amplicons were purified using AMPure XP beads (Beckman Coulter, USA), pooled, and diluted to 4 pmol/L for final loading. The sample was then sequenced on an Illumina MiSeq Sequencer at Omics Sciences and Bioinformatics Center (Chulalongkorn University, Bangkok, Thailand). FASTQC software was used to check the quality of sequences, and paired-end reads were merged using PEAR.[20] FASTX-Toolkit was used to remove the assembled reads with 90% of the bases having quality scores less than 30 or the reads shorter than 200 bp. Chimeric sequences were removed by the UCHIME method with vsearch1.1.1 and uchime_ref option against the chimera-free Gold RDP database.[21,22] Operational taxonomic unit (OTU) picking was conducted with the SortMeRNA method in QIIME 1.9.0. The Greengenes database was used to assign

Table 2. Average chemical oxygen demand (COD) concentrations, mixed liquor suspended solid (MLSS) concentrations, polyhydroxyalkanoate (PHA) contents, dissolved oxygen (DO), pH, and temperature in the first and second continuous stirred tank reactors (CSTRs) of the two-stage CSTR activated sludge (AS) systems

	F/F1		F/F2		F/F3	
Two-stage CSTR AS systems	First CSTR	Second CSTR	First CSTR	Second CSTR	First CSTR	Second CSTR
Days 1–33, feast/famine ratio of 0.15, 0.23, and 0.33 with influent COD of ~1500 mg/L						
COD (mg/L)	316 ± 283	81 ± 50	373 ± 457	79 ± 40	319 ± 350	86 ± 34
MLSS (mg/L)	2098 ± 787	1525 ± 292	1752 ± 599	1462 ± 305	1331 ± 350	1047 ± 376
PHA (% gPHA/gMLSS)	1.52 ± 0.59	0.53 ± 0.45	1.42 ± 0.49	0.79 ± 0.48	1.11 ± 0.30	0.66 ± 0.39
DO (mg/L)	4.45 ± 0.76	6.63 ± 0.53	5.69 ± 0.85	6.88 ± 1.16	5.88 ± 2.23	6.89 ± 0.46
pH	7.17 ± 0.17	7.43 ± 0.25	7.20 ± 0.20	7.40 ± 0.25	7.18 ± 0.10	7.42 ± 0.22
Temperature (°C)	28.3 ± 1.2	28.0 ± 1.0	28.5 ± 1.2	28.1 ± 0.9	28.3 ± 1.2	27.9 ± 0.9
Days 34–72, feast/famine ratio of 0.04, 0.15, and 0.33 with influent COD of ~1500 mg/L						
COD (mg/L)	278 ± 121	68 ± 34	143 ± 72	53 ± 19	121 ± 82	50 ± 23
MLSS (mg/L)	2106 ± 138	869 ± 259	2143 ± 714	1556 ± 270	1335 ± 580	1310 ± 410
PHA (% gPHA/gMLSS)	5.79 ± 5.64	1.59 ± 0.58	1.35 ± 0.55	0.80 ± 0.33	0.49 ± 0.29	0.35 ± 0.24
DO (mg/L)	4.06 ± 1.12	5.66 ± 0.91	3.38 ± 0.88	5.90 ± 0.48	4.71 ± 1.03	6.07 ± 0.86
pH	7.05 ± 0.08	7.57 ± 0.17	7.03 ± 0.05	7.65 ± 0.16	7.04 ± 0.05	7.61 ± 0.16
Temperature (°C)	29.2 ± 1.1	28.3 ± 1.0	29.4 ± 1.1	28.4 ± 1.0	29.3 ± 1.0	28.5 ± 0.9
From Day 73, feast/famine ratio of 0.04, 0.15, and 0.33 with influent COD of ~3000 mg/L						
COD (mg/L)	759 ± 373	76 ± 29	337 ± 230	77 ± 49	301 ± 242	80 ± 32
MLSS (mg/L)	3100 ± 1058	2080 ± 689	4161 ± 1286	2206 ± 504	2383 ± 583	1905 ± 525
PHA (% gPHA/gMLSS)	5.84 ± 5.53	2.74 ± 4.44	3.33 ± 3.19	1.16 ± 1.03	2.13 ± 2.37	0.97 ± 1.67
DO (mg/L)	1.25 ± 0.96	3.97 ± 0.94	1.62 ± 1.38	5.04 ± 1.09	1.15 ± 1.00	4.98 ± 1.03
pH	7.06 ± 0.05	7.56 ± 0.23	7.07 ± 0.10	7.52 ± 0.26	7.06 ± 0.06	7.62 ± 0.32
Temperature (°C)	28.5 ± 0.9	27.6 ± 0.7	28.2 ± 0.7	27.6 ± 0.7	28.6 ± 0.7	27.7 ± 0.7

taxonomy. The unmatched sequences were clustered *de novo* using SUMACLUST, and the OTUs with less than 0.1% of reads were removed.

RESULTS AND DISCUSSION

Operation of two-stage CSTR AS systems

The three two-stage CSTR AS systems, F/F1, F/F2, and F/F3, were initially operated at the HRT ratios of the first and the second CSTRs of 0.15, 0.23, and 0.33, respectively. The first and the second stages of CSTRs were expected to create the feast and famine conditions, respectively, which are suitable for the enrichment of PHA-accumulating microorganisms. The synthetic wastewater containing glycerol was initially fed at 1500 mg COD/L. Figure 2 shows the COD concentrations in the influent and effluent of the first CSTRs and the effluent of the second CSTRs in all three two-stage CSTR AS systems. The average COD concentrations, MLSS concentrations, PHA contents, DO, pH, and temperature in the first and second CSTRs of the two-stage CSTR AS systems are summarized in Table 2.

Since the COD concentrations in the first CSTRs (feast phase) of the three systems were rather similar, 316 ± 283 mg/L, 373 ± 457 mg/L, and 319 ± 350 mg/L in F/F1, F/F2, and F/F3, respectively, we are not certain whether the feast/famine ratio chosen to investigate (0.15, 0.23, and 0.33) was in a wide enough range to clearly see its effects on the systems. Therefore, after 33 days of operation, we changed the HRT ratio of the first and second CSTRs (feast/famine ratio) from 0.15 to 0.04 in F/F1 and from 0.23 to 0.15 in F/F2, whereas the HRT ratio in F/F3 remained the same at 0.33.

After adjusting the feast/famine ratio (Days 34–72), the COD concentrations in the first CSTR (feast phase) of F/F1 (278 ± 121 mg/L) were higher than those in F/F2 (143 ± 72 mg/L) and F/F3 (121 ± 82 mg/L). According to Fig. 3, COD removal primarily occurred in the first CSTRs in all of the systems (F1, F2, and

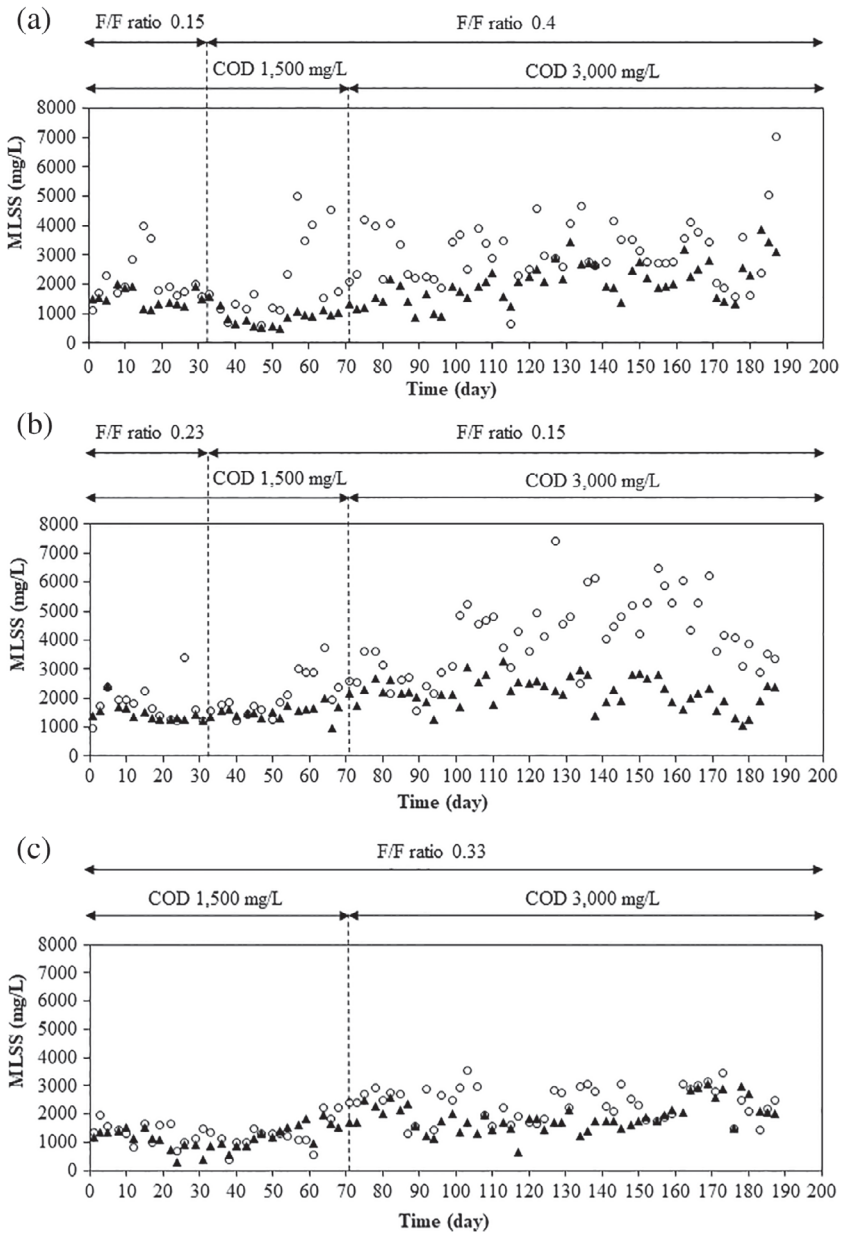

Figure 3. Mixed liquor suspended solid (MLSS) concentrations in the first continuous stirred tank reactors (CSTRs) (○) and the second CSTRs (▲) in the two-stage CSTR activated sludge (AS) systems: (a) F/F1, (b) F/F2, and (c) F/F3.

F3). Figure 3 shows the MLSS concentrations in the influent and effluent of the first CSTRs and the effluent of the second CSTRs in all three two-stage CSTR AS systems, which can reflect biomass growth and/or decay in the systems. The reduced amount of biomass in the second CSTRs compared to the first CSTRs indicates biomass decay in the second CSTRs of the systems, which conformed to the famine phase.

The PHA contents in the sludge obtained from the first and second CSTRs are shown in Fig. 4. Despite the high fluctuation, the PHA contents in the first CSTR were greater than those in the second CSTR in the F/F1 system operated at the feast/famine ratio of 0.04. The results reveal the PHA accumulation and PHA consumption patterns under feast/famine conditions in the F/F1 system. In contrast, this pattern was still unclear in the F/F2 and F/F3 systems operated at feast/famine ratios of 0.15 and 0.33 when the influent COD was 1500 mg/L. We hypothesized that the differences in COD concentrations in the first and the second CSTRs were not large enough for microorganisms to perceive the distinct shifts between the feast and famine phases. Therefore, we increased the influent glycerol concentrations from 1500 mg COD/L to 3000 mg COD/L on Day 73 to examine this hypothesis.

After the influent glycerol concentrations were increased, the differences in the COD concentrations in the first and the second CSTRs were larger in all of the systems (F/F1, F/F2, and F/F3). The PHA contents in the first CSTRs (feast phase) were generally greater than those in the second CSTRs (famine phase) in all of the systems. The PHA accumulation and PHA consumption patterns under feast/famine conditions were achieved in all of the

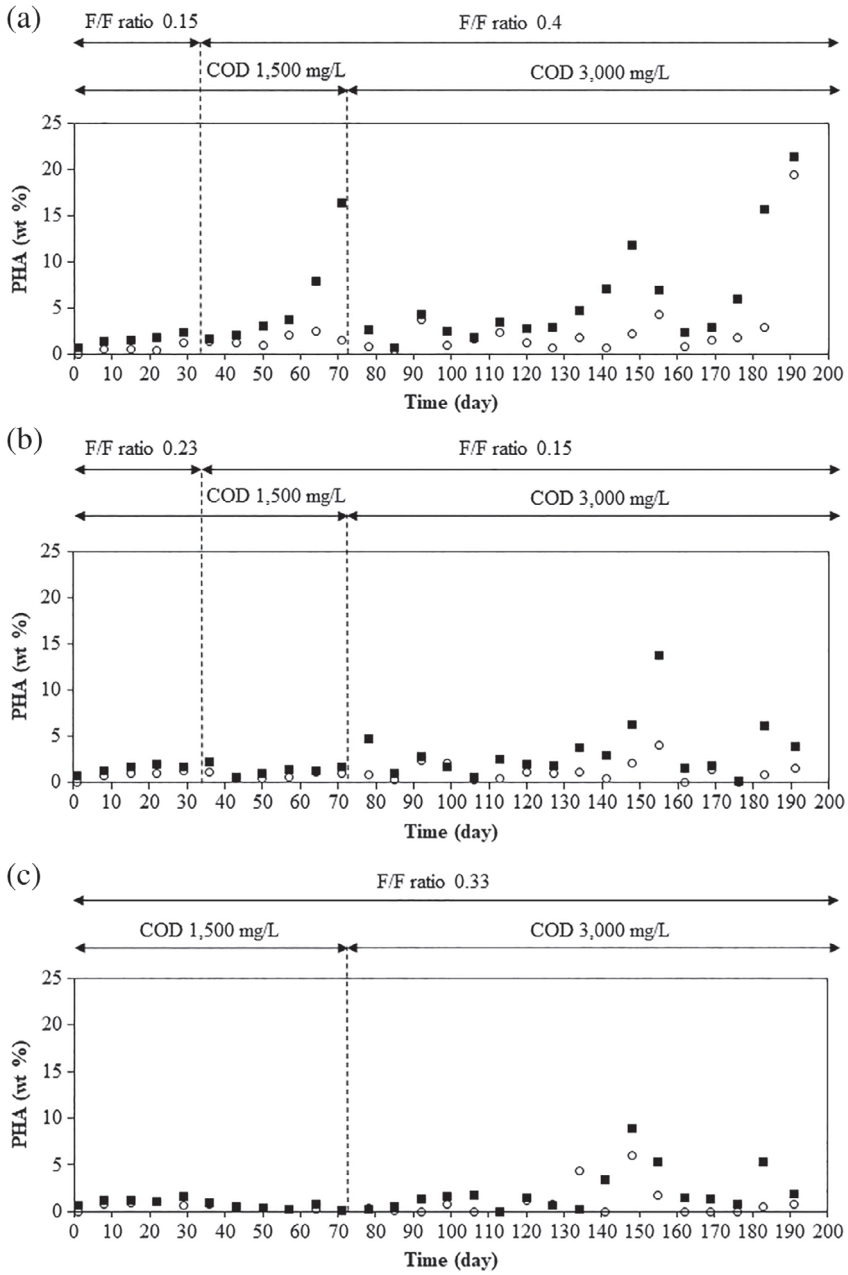

Figure 4. Polyhydroxyalkanoate (PHA) contents in the first continuous stirred tank reactors (CSTRs) (■) and the second CSTRs (○) in the two-stage CSTR activated sludge (AS) systems: (a) F/F1, (b) F/F2, and (c) F/F3.

two-stage CSTR AS systems operated at feast/famine ratios of 0.04, 0.15, and 0.33 at influent glycerol concentrations of 3000 mg COD/L. A previous study by Albuquerque *et al.*[4] also demonstrated that an increase in influent substrate concentration could create excess substrate availability, which is suitable for the feast phase in a two-stage CSTR system.

The results from this study are consistent with previous studies that investigated the effects of feast/famine ratios on PHA production in SBRs and reported that a low feast/famine ratio (≤ 0.28) allowed PHA-accumulating microorganisms to outcompete non-PHA-accumulating microorganisms.[7,9,23,24] In this study, in which continuous systems were used, we are able to obtain PHA accumulation and PHA consumption patterns under feast/famine conditions at feast/famine ratios of 0.04 to 0.33, which are similar to the ranges for SBR observation. For the lowest feast/famine ratio (0.04), feast/famine conditions can be achieved at a lower influent COD concentration (1500 mg COD/L), whereas higher influent COD concentrations (3000 mg COD/L) were required for higher feast/famine ratios (0.15 and 0.33).

It should be noted that sporadic increases in the PHA contents in the two-stage CSTR AS systems were observed (Fig. 4), thus revealing certain instabilities of the systems in terms of PHA production. Further research on improving the stability of PHA production systems using mixed microbial cultures under continuous operation is still required.

In brief, the treatment of wastewater containing glycerol was successfully achieved in these two-stage CSTR AS systems at the influent COD of 3000 mg/L, and the COD removal efficiencies were 97.6 ± 1.1%, 97.6 ± 1.6%, and 96.9 ± 3.9% in F/F1, F/F2, and F/F3, respectively, while the effluent COD concentrations were 76 ± 29 mg COD/L, 76 ± 48 mg COD/L, and 80 ± 32 mg COD/L in F/F1, F/F2, and F/F3, respectively. Simultaneously, the feast/famine conditions were created in these systems as a selective pressure for the enrichment of PHA-accumulating microorganisms. The PHA-accumulation capacities of the sludge were further investigated in the following batch and fed-batch reactors.

PHA production in batch and fed-batch reactors

The sludge from the two-stage CSTR AS systems operated at feast/famine ratios of 0.04, 0.15, and 0.33 was transferred to batch reactors, Batch F/F1, Batch F/F2, and Batch F/F3, respectively, to further investigate the PHA accumulation and consumption patterns of the sludge. Figure 5 shows the COD concentrations and PHA contents in Batch F/F1, Batch F/F2, and Batch F/F3. The COD concentrations tended to decrease over time. However, the PHA contents increased and reached maximum contents of 8.32 wt%, 8.20 wt %, and 4.71 wt% at 6 h, 24 h, and 24 h in Batch F/F1, Batch F/F2, and Batch F/F3, respectively. Then, the PHA contents decreased, suggesting internal PHA consumption. It should be noted that the sludge obtained from the system operated at the lowest feast/famine ratio (0.04) accumulated the highest PHA contents (8.32 wt%) within the shortest period of time (6 h) compared to the other sludge.

In addition, the sludge from the two-stage CSTR AS systems was also tested for its maximum capacity to accumulate PHA in fed-batch reactors in which the substrate was continuously fed to maintain high substrate availability. Figure 6 shows the COD concentrations and PHA contents in Fed-batch F/F1, Fed-batch F/F2, and Fed-batch F/F3, in which the sludge was obtained from the two-stage CSTR AS systems operated at the feast/famine ratio of 0.04, 0.15, and 0.33, respectively. Maximum PHA contents of 21.8 wt%, 13.1 wt%, and 8.7 wt% were achieved for the sludge from the systems operated at feast/famine ratios of 0.04, 0.15, and 0.33, respectively. The results clearly suggest that the sludge enriched at the lowest feast/famine ratio (0.04) had the maximum capacity to accumulate PHA up to 21.8 wt%. Nonetheless, the maximum PHA content achieved in this study is still low compared to that achieved in SBRs with glycerol as a substrate (37.2–80 wt%)[5,7,25] and in two-stage CSTR AS systems fed with fermented molasses (61 wt%)[4] or fermented paper mill wastewater (48 wt%)[12] containing mixed volatile fatty acids, which are favored substrates for PHA production.

The PHA compositions obtained in the fed-batch reactors using the sludge from the systems operated at feast/famine ratios of 0.04, 0.15, and 0.33 were 77:23, 98:2, and 100:0 as % HB:HV by mole, respectively. The obtained PHA was dominated by HB, which is a common monomer of PHA when glycerol is used as a carbon source.[5–7] Nevertheless, HV might be produced through other substrates formed during glycerol degradation, which could be possible in mixed microbial cultures. The structure of the obtained PHA was also analyzed using ^{1}H-NMR. The results are shown in Fig. 7. The ^{1}H-NMR spectra of the PHA suggested the copolymer of HB and HV with the dominant signals of HB and low signals of HV. The HV signals observed from the PHA obtained from the feast/famine ratio of 0.04 were the highest, whereas the lowest signals of HV were observed in the PHA obtained from the feast/famine ratio of 0.33. The trends appear to be consistent with

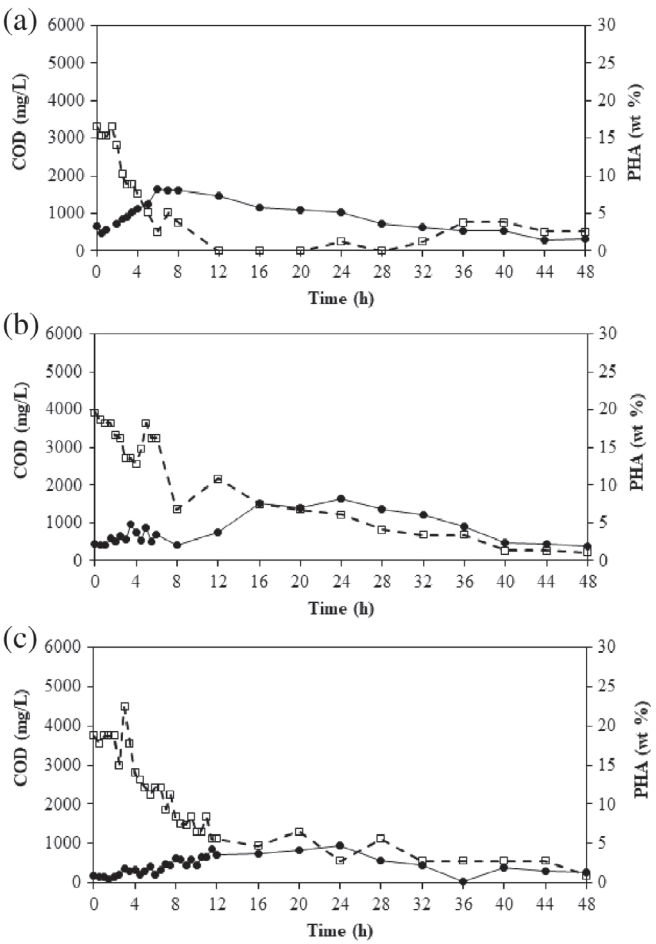

Figure 5. Chemical oxygen demand (COD) concentrations (-⊟-) and polyhydroxyalkanoate (PHA) contents (—●—) in (a) Batch F/F1, (b) Batch F/F2, and (c) Batch F/F3, in which the sludge was obtained from the two-stage continuous stirred tank reactor (CSTR) activated sludge (AS) systems operated at the feast/famine ratio of 0.04, 0.15, and 0.33, respectively.

the composition analysis performed by gas chromatography although HV was not detected by gas chromatography for the PHA obtained from the feast/famine ratio of 0.33.

Microbial community analysis using 16S rRNA gene amplicon sequencing

Microbial communities of the seed sludge and the sludge samples from the two-stage CSTR AS systems were examined using 16S rRNA gene amplicon sequencing (MiSeq). Figure 8 shows the percent relative abundance of microbial phyla in the seed sludge and the sludge samples from the two-stage CSTR AS systems F/F1 (feast/famine ratio 0.04), F/F2 (feast/famine ratio 0.15), and F/F3 (feast/famine ratio 0.33) at the end of the operation. The microbial community in the seed sludge was clearly different from those in all of the two-stage CSTR AS systems enriched under feast/famine conditions. The relative abundance of proteobacteria increased after cultivation in the two-stage CSTR AS systems, with a decrease in the relative abundance of betaproteobacteria but an increase in the relative abundance of alphaproteobacteria and gammaproteobacteria. It should be noted that these three classes of proteobacteria (alphaproteobacteria, betaproteobacteria, and gammaproteobacteria) generally play important roles in PHA production in mixed microbial cultures, as diverse groups of microorganisms in these classes are known to have PHA-accumulating capabilities.[26–29]

Nevertheless, in addition to the enrichment under feast/famine conditions, substrate types, operating conditions of reactors, and environmental conditions could be other factors that influence microbial communities. The fact that this sludge was previously in a full-scale SBR fed real fruit juice wastewater and was then switched to glycerol-fed continuous reactors could also result in shifts in microbial communities.

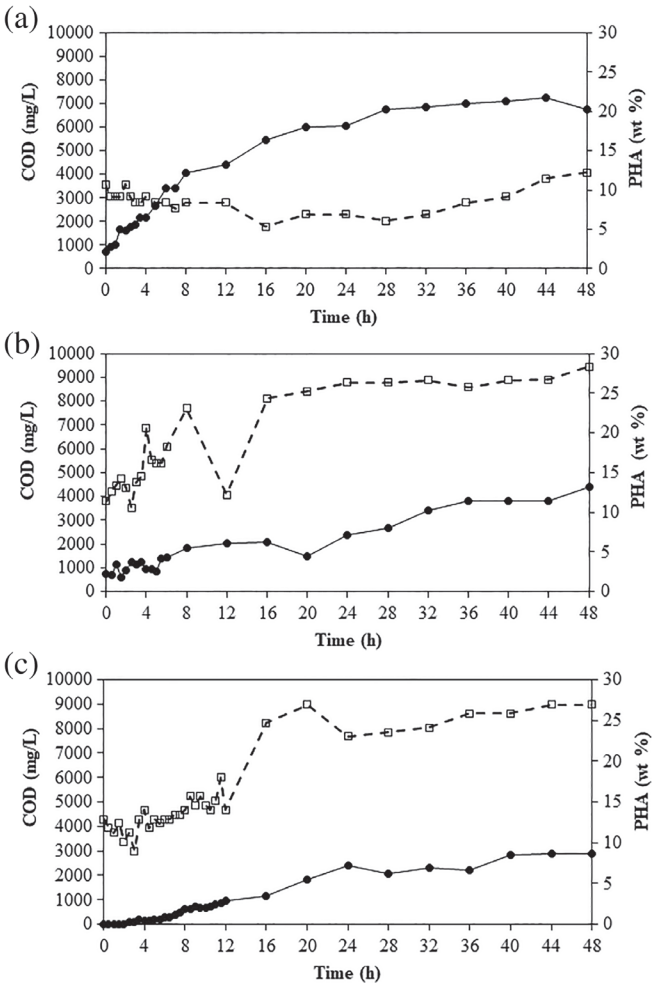

Figure 6. Chemical oxygen demand (COD) concentrations (--⊟--) and polyhydroxyalkanoate (PHA) contents (—●—) in (a) Fed-batch F/F1, (b) Fed-batch F/F2, and (c) Fed-batch F/F3, in which the sludge was obtained from the two-stage continuous stirred tank reactor (CSTR) activated sludge (AS) systems operated at the feast/famine ratio of 0.04, 0.15, and 0.33, respectively.

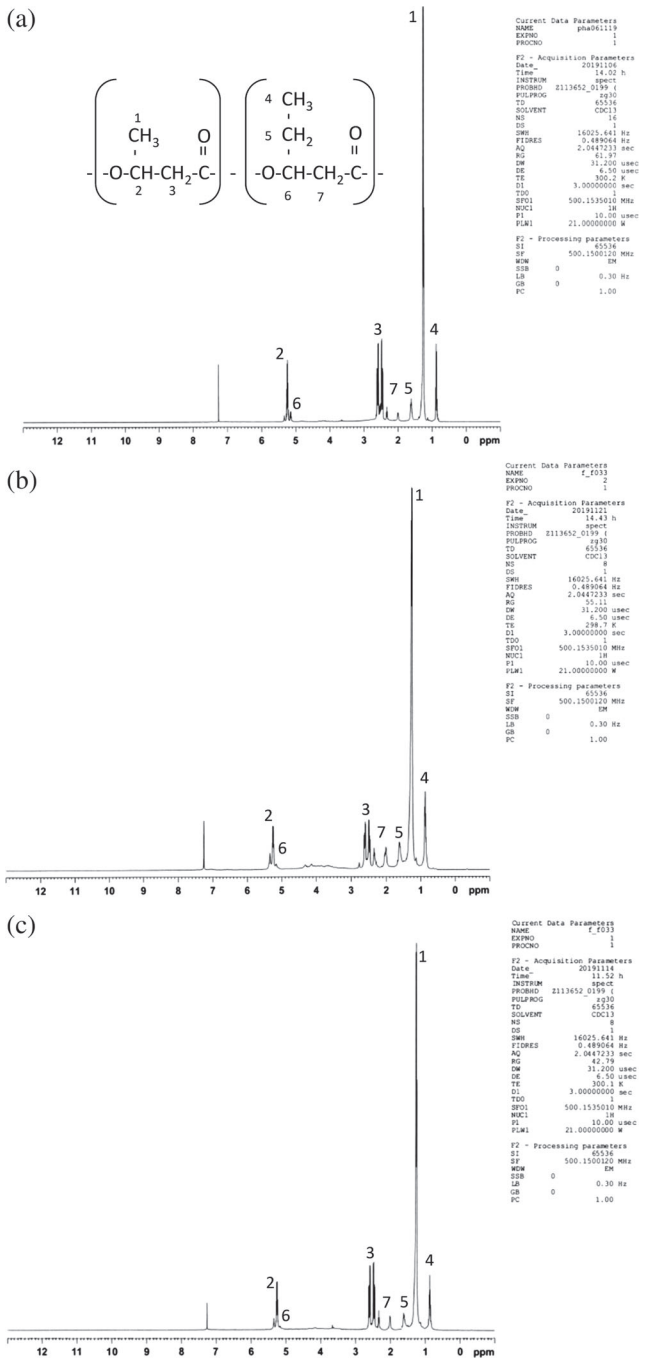

Figure 7. Proton nuclear magnetic resonance (^{1}H-NMR) spectra of the polyhydroxyalkanoate (PHA) samples obtained from the feast/famine ratios of 0.04 (a), 0.15 (b), and 0.33 (c).

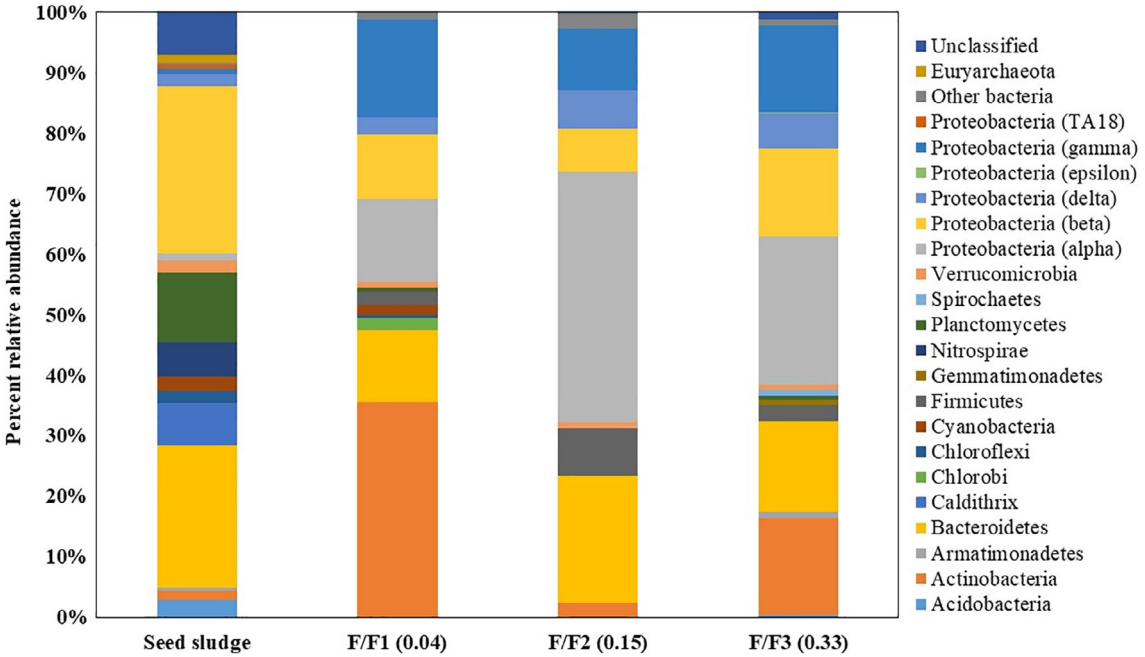

Figure 8. Microbial community analysis using 16S rRNA gene amplicon sequencing, shown as percent relative abundance of microbial phyla, in the seed sludge and the sludge samples from two-stage continuous stirred tank reactor (CSTR) activated sludge (AS) systems F/F1 (feast/famine ratio 0.04), F/F2 (feast/famine ratio 0.15), and F/F3 (feast/famine ratio 0.33).

The top five most relatively abundant genera/families in the seed sludge and the sludge samples from the two-stage CSTR AS systems are summarized in Table 3. Among these genera/families, *Rhodobacter* spp. and bacteria in the families Rhodocyclaceae, Comamonadaceae, Xanthobacteraceae, Enterobacteriaceae, and Bradyrhizobiaceae have been reported to accumulate PHA.[30–34] All of these groups of microorganisms belong to proteobacteria in the class alphaproteobacteria, betaproteobacteria, or gammaproteobacteria.

Although a correlation between the HRT ratios and particular changes in the microbial communities among the two-stage CSTR AS systems was not clearly observed at the phylum level (Fig. 8), the most relatively abundant genera/families in these systems greatly varied with different PHA-accumulating microorganisms observed in the systems (Table 3). Different HRT ratios can affect the production of PHA and the types of PHA-accumulating microorganisms; however, this factor did not appear to change the overall microbial communities at the phylum level, in which co-existence of other groups of microorganisms in addition to PHA-accumulating microorganisms are required for wastewater treatment.

Information on the microbial communities obtained from 16S rRNA gene amplicon sequencing (MiSeq) cannot pinpoint the groups of microorganisms that actually contributed to PHA accumulation. Thus, definite mechanisms/pathways for PHA synthesis cannot be identified in this current study. Nevertheless, in general, the major bacterial PHA synthesis pathway from glycerol occurs via the formation of dihydroxyacetone-P, glyceraldehyde-3P, pyruvate, and acetyl-CoA to PHA.[5]

Implications for incorporating PHA production into wastewater treatment processes

This study demonstrated a prospective approach for incorporating PHA production into wastewater treatment processes using two-stage CSTR AS systems with glycerol as a carbon substrate.

Table 3. Top five most relatively abundant genera/families (percent of total sequences) in the seed sludge and sludge samples from the two-stage continuous stirred tank reactor (CSTR) activated sludge (AS) systems F/F1 (feast/famine ratio 0.04), F/F2 (feast/famine ratio 0.15), and F/F3 (feast/famine ratio 0.33)

	Two-stage CSTR AS systems		
Seed sludge	F/F1 (feast/famine ratio 0.04)	F/F2 (feast/famine ratio 0.15)	F/F3 (feast/famine ratio 0.33)
Family Cryomorphaceae (14.3%)	Genus *Mycobacterium* (26.3%)	Genus *Desulfovibrio* (5.9%)	Genus *Gordonia* (14.0%)
Family Rhodocyclaceae (11.1%)a[25]	Genus *Dokdonella* (12.2%)	Genus *Dysgonomonas* (5.5%)	Genus *Dokdonella* (9.8%)
Genus *Nitrospira* (5.7%)	Genus *Gordonia* (7.6%)	Family Xanthobacteraceae (5.4%)a[26]	Genus *Opitutus* (9.8%)
Family Pirellulaceae (5.0%)	Genus *Rhodobacter* (5.5%)a[26]	Family Porphyromonadaceae (5.1%)	Genus *Devosia* (9.2%)
Family Cytophagaceae (4.8%)	Family Comamonadaceae (5.1%)a[26]	Family Enterobacteriaceae (4.4%)a[27,28]	Family Bradyrhizobiaceae (9.2%)a[29]

[a] Bacteria in these genera/families have been reported to accumulate polyhydroxyalkanoate (PHA).

The results suggest that the treatment of wastewater containing glycerol can effectively be achieved using two-stage CSTR AS systems while PHA-accumulating microorganisms can simultaneously be enriched. The excess sludge in the systems can then be transferred to fed-batch reactors for PHA production. This model has a strong potential to be applied to actual wastewater treatment plants.

Potential PHA production (in g PHA/d) from these two-stage CSTR AS systems together with fed-batch reactors can be estimated by multiplying the sludge production (in g MLSS/d) from the two-stage CSTR AS systems by the PHA contents in the sludge achieved from the fed-batch reactors. The sludge production (in g MLSS/d) from the two-stage CSTR AS systems can be estimated from $\frac{X_1V_1+X_2V_2}{SRT}$, where X_1 and X_2 are the MLSS (in mg/L) in the first and second CSTRs, respectively; and V_1 and V_2 are the volumes (in liters) of the first and second CSTRs, respectively. After Day 73, in which the systems were fed with COD ~3000 mg/L, the sludge production was 4.27 ± 1.4 g MLSS/d, 4.98 ± 1.0 g MLSS/d, and 4.21 ± 0.94 g MLSS/d in the systems operated at HRT ratios of 0.04, 0.15, and 0.33, respectively. Thus, the estimated PHA production was 0.93 g PHA/d, 0.65 g PHA/d, and 0.37 g PHA/d from the systems operated at HRT ratios of 0.04, 0.15, and 0.33, respectively.

Although previous studies that used a short SRT (1–4 days) achieved high PHA contents of up to 89 wt%,[5,9,27,35] the biomass concentrations (MLSS) associated with low SRTs tend to be very low, which can cause problems of biomass settling in AS systems. Therefore, an SRT of ten days was selected in this study, and this value is in the range for conventional AS systems (SRT 5–20 days), thus allowing this concept to be practical for the modification of existing conventional AS systems. Nevertheless, further research on the effects of SRT on PHA production in two-stage CSTR AS systems with glycerol as a carbon source is still required.

The two-stage CSTR AS systems used in this study were able to achieve COD removal efficiencies of ~97%, which is comparable to that of conventional AS systems. However, these two-stage CSTR AS systems require an HRT of 48 h, which is considered long for an SRT of ten days. This long HRT corresponds to larger volumes and construction costs compared with that of conventional AS systems.

In addition, Fra-Vazquez *et al.*[36] has recently shown that the PHA-storage capacity of a mixed microbial culture did not decrease under ammonia oxidation. This finding by Fra-Vazquez *et al.*[36] suggests that (1) a nitrification inhibitor (thiourea or allylthiourea) does not need to be added to enrich PHA-accumulating microorganisms, which will lead to more economical and practical PHA production processes by mixed microbial cultures; and (2) it is possible to achieve nitrification together with PHA production under feast/famine conditions, which is beneficial in terms of nutrient removal.

From an economic perspective, this PHA production system using mixed microbial cultures with continuous reactors, which has a great potential for incorporation into wastewater treatment plants, can eliminate the energy demand associated with the sterile process required in industrial PHA production using pure cultures and reduce the cost of carbon sources, which account for approximately 50% of the PHA production cost.[37] Nevertheless, the low PHA contents in the biomass obtained from our proposed process could result in higher PHA extraction costs compared with processes that obtain higher PHA contents.[4,5,7,12,25] Thus, additional research is required on the enhancement of PHA contents obtained from systems that use mixed microbial cultures with continuous reactors.

CONCLUSIONS

PHA production in two-stage CSTR AS systems using glycerol as a carbon substrate was investigated at the HRT ratios of the first and the second stages of 0.04, 0.15, and 0.33, which reflects the feast/famine ratio. The sludge enriched in these systems fed with a glycerol concentration of 3000 mg COD/L revealed PHA accumulation and consumption patterns commonly observed under feast/famine conditions. Maximum PHA contents of 21.8 wt%, 13.1 wt% and 8.7 wt% were achieved in the fed-batch reactors with the sludge from the systems operated at the feast/famine feeding ratio of 0.04, 0.15, and 0.33, respectively. The results suggest that the highest PHA content was achieved at the lowest feast/famine ratio (0.04). The results of 16S rRNA gene amplicon sequencing (MiSeq, Illumina) revealed the presence of *Rhodobacter* spp. and bacteria in the families Comamonadaceae, Xanthobacteraceae, Bradyrhizobiaceae, and Enterobacteriaceae in the two-stage CSTR AS systems, which have previously been reported to accumulate PHA.

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