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Production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) from excess activated sludge as a promising substitute of pure culture

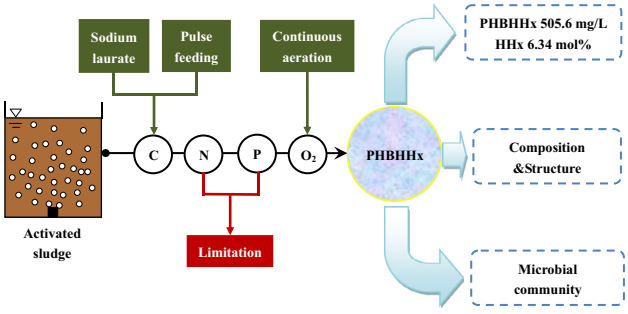
Liang Shen [a], Hongyou Hu [b], Hongfang Ji [b], Chuanpan Zhang [a], Ning He [a], Qingbiao Li [a], Yuanpeng Wang [a,*]

[a] Department of Chemical and Biochemical Engineering, College of Chemistry and Chemical Engineering, The Key Lab for Synthetic Biotechnology of Xiamen City, Xiamen University, Xiamen 361005, China
[b] College of the Environment and Ecology, Xiamen University, Xiamen 361005, China

HIGHLIGHTS

- Production of PHBHHx from activated sludge was proven feasible.
- Sodium laurate was an appropriate sole carbon substrate for sludge to produce PHBHHx.
- Feeding mode and amount of C, N, P and O_2 were optimized in PHBHHx producing process.
- Composition and structure of PHBHHx from sludge were close to that from pure culture.
- High-throughput sequencing characterized the community of PHBHHx producing sludge.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed to investigate the feasibility and technology to harvest poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) by mixed culture. Copolymer PHBHHx, usually fermented by pure strains, was reported to be synthesized from activated sludge for the first time. Sodium laurate was used as the sole carbon substrate for sludge acclimation and PHBHHx accumulation. Batch experiments were designed to look into the impact of the carbon, nitrogen, phosphorus and oxygen supply on PHBHHx production. The results showed that the acclimated excess sludge was able to produce PHBHHx, and the maximum output (505.6 mg/L PHBHHx containing 6.34 mol% HHx) was achieved with conditions of the continuous aeration, nitrogen and phosphorus limitation, and adequate carbon source implemented by pulse feeding 0.5 g/L sodium laurate every 4 h. Moreover, composition and structure of the PHBHHx from sludge were found similar to that from pure culture, according to literature, FTIR and NMR spectra. Finally, high-throughput sequencing technique characterized that phylum *Chlorobi* and genus *Leadbetterella* should be critical groups for PHBHHx synthesis in the sludge community.

1. Introduction

Polyhydroxyalkanoates (PHAs) are intracellular polyesters synthesized by a wide variety of bacteria as carbon and energy storage material in a stressful environment. This biopolymer has drawn increasing attention because of their similar material properties to conventional petrochemicals-based plastics and complete

* Corresponding author at: Department of Chemical and Biochemical Engineering, College of Chemistry and Chemical Engineering, Xiamen University, No. 422, Siming South Road, Xiamen 361005, China. Tel.: +86 592 2183751; fax: +86 592 2184822.
E-mail address: wypp@xmu.edu.cn (Y. Wang).

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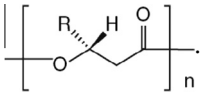
biodegradability. Generally, the molecular structure of PHAs is composed of 3-hydroxy fatty acids as shown as  . Three most common PHA monomers are hydroxybutyrate (HB), hydroxyvalerate (HV) and hydroxyhexanoate (HHx). Homopolymer of poly(3-hydroxybutyrate) (PHB) is a highly crystalline, stiff, but brittle material. Whereas, copolymers, like poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBHV), poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (PHB4HB) and particularly poly (3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), are discovered to have better biocompatibility and physical properties for mechanical processing (Table S1). PHBHHx was thus entitled as "the third-generation commercial PHA product", developed on the basis of PHB and PHBHV (Wei et al., 2013).

However, the market of PHA was severely restrained by the high cost from either the raw material or the complicate production process. The conventional way to produce PHAs is the fermentation using pure culture microorganisms in form of wild type or genetic recombinant. In the mean time, mixed culture process has rapidly developed to accumulate PHA in recent years. When compared with a pure culture, the merits of PHA production in open mixed culture would be a simpler process control, no requirement of sterilization, a reduced cost and an improved use of wastes (Salehizadeh and Van Loosdrecht, 2004). So far, activated sludge has been recognized a well-known mixed culture able to store PHA as carbon and energy storage under unsteady conditions such as a feast-famine regime or variation in the presence of an electron acceptor, which enlightened a promising reuse of the excess sludge in wastewater treatment plants (Satoh et al., 1998; Morgan-Sagastume et al., 2014). A number of studies have been carried out with activated sludge to produce PHB (Cavaillé et al., 2013; Dai et al., 2015) and PHBHV (Salehizadeh and Van Loosdrecht, 2004; Wang et al., 2013), successfully. In contrast, there is no report on the PHBHHx production by activated sludge yet, to our best knowledge.

Actually, unlike many PHA producing microbes, only a few bacteria have been documented to synthesize PHBHHx. In the literature, the phototrophic *Rhodospirillum rubrum* was the first bacterium reported to accumulate PHBHHx anaerobically in the light, with butyrate or hexanoate as the sole carbon source (Brandl et al., 1989). Soon later, Shiotani and Kobayashi (1993) registered a patent on the *Aeromonas caviae* FA440 strain that could produce a random copolymer of 3HB and 3HHx by using a $\geqslant$6C fatty acid having an even number of carbon atoms or its lower alcohol ester or natural fats and oils as a carbon source. This species, with a synonym of *Aeromonas punctata*, was successful to synthesize the PHBHHx up to approximately 30 wt% of the cellular dry weight, with a 3HHx fraction ranging from 10 to 25 mol% (Shimamura et al., 1994; Doi et al., 1995). The other three best-studied wild strains for PHBHHx production belong to *Aeromonas hydrophila*, namely *Aeromonas hydrophila* 4AK4 (Chen et al., 2001; Zhang et al., 2002; Ouyang et al., 2003; Qiu et al., 2003), *Aeromonas hydrophila* WQ (Gan et al., 2003) and *Aeromonas hydrophila* CGMCC 0911 (Lu et al., 2004). Another *Aeromonas* sp. strain KC014 isolated from soil was also able to synthesize PHBHHx (Gong et al., 2008). Additionally, *Pseudomonas stutzeri* 1317 (He et al., 1998) and *Pseudomonas* sp. 61-3 (Abe et al., 1994) have been found to accumulate PHA containing HHx monomer in a glucose mineral medium, and so do *Pseudomonas oleovorans* ATCC 29347 (Lageveen et al., 1988) and *Pseudomonas putida* KT2442 (Lee et al., 2000) under nitrogen or phosphorus limitation, respectively. Apart from the above wild types, many recombinants harboring the synthesis genes from the above bacteria have been constructed and used to enhance the PHBHHx production to the industrial scale (Riedel et al., 2012; Wei et al., 2013). All these previous studies on pure culture inspire us to look into the feasibility of producing PHBHHx by mixed culture, although the information about this process and related microbiota is still scarce.

Therefore, in this study, excess activated sludge will be acclimated and cultivated to obtain PHBHHx, with sodium laurate that is commonly utilized in the pure strain fermentation as the solo carbon substrate. Strategies will be optimized to fulfill and improve PHBHHx accumulation, through adjusting the feeding quantity and mode of carbon source, nutrients and oxygen. PHBHHx produced in this study will be further compared with the standard PHBHHx fermented from the pure culture in terms of chemical composition and structure, as a verification of its quality. In the end, microbial community of this activated sludge will be characterized by use of the high-throughput sequencing technique, in connection with the production process. The results of this study point to the potential importance and impact of a new economic technology for PHBHHx synthesis.

2. Methods

2.1. Excess activated sludge acclimation

The excess activated sludge used in this work was from the secondary sedimentation tank of a municipal wastewater treatment plant in Xiamen, China. For the purpose of PHBHHx accumulating organisms' enrichment, acclimation was conducted in a 30-L sequencing biological reactor (SBR) that was operated in a sequential mode with a 24-h cycle comprised of 5-min feeding, 23.5-h aeration, 20-min settling and 5-min effluent withdrawal. The medium composition (/L) was modified according to a recipe for PHBHHx synthesis by both wild and recombinant pure cultures, i.e., sodium laurate 1 g; $MgSO_4{\cdot}7H_2O$ 0.6 g; NH_4Cl 016 g; EDTA 0.1 g; K_2HPO_4 0.092 g; KH_2PO_4 0.045 g; $CaCl_2{\cdot}2H_2O$ 0.07 g; peptone 0.334 g; yeast extract 0.124 g; trace element solution 2 mL (Qiu et al., 2003). Dissolved oxygen (DO) of approximately 80% was kept in the aeration phase by an air compressor with aeration rate of 2 L/min. The temperature of the SBR was controlled at 30 °C. COD, pH, MLSS and DO were monitored in each cycle. When these parameters approximately approached the stable values, the SBR stopped and the acclimated sludge was used for the next PHBHHx accumulation experiment.

2.2. Batch experiments for PHBHHx accumulation

PHBHHx production was carried out in a fed-batch reactor with working volume of 1 L. Acclimated sludge was centrifuged at 3000 g and the pellet was washed with DI water and cold hexane. Then the reactor was inoculated with MLSS of 2 g/L. Sodium laurate, with concentration of 0.5, 1 and 1.5 g/L, was used as the solo carbon source for the experiments. Meanwhile the nitrogen and phosphorous sources were omitted in some batches to create an unbalanced nutritional condition. Sodium laurate was either loaded all in the beginning (namely single-dose feeding), or fed with equal quantity at intervals of four hours (namely pulse feeding). Similarly, aeration was set in a continuous or an intermittent mode; in the latter one the reactor was stirred at 200 rpm to disperse the mixed liquor during the non-aeration session. An air compressor was used for aeration with rate of 2 L/min. The temperature was maintained at 30 °C in the reactor. The details of each batch experiment were presented in Table 1. The solid- and liquid-phase samples were taken periodically from the reactor for further analysis of COD, PHBHHx content and microbial composition, etc.

Table 1
Conditions for batch experiments of PHBHHx accumulation from activated sludge.

Batch	Sodium laurate (g/L)	Feeding mode*	NH_4Cl (g/L)	$K_2HPO_4{\cdot}3H_2O$ (g/L)	Aeration mode**
1	1.5	I	0.16	0.092	a
2	1.5	I	–	0.092	a
3	1.5	I	0.16	–	a
4	1.5	I	–	–	a
5	1.0	I	–	–	a
6	0.8	I	–	–	a
7	0.5	I	–	–	a
8	1.5	II	0.16	0.092	a
9	1.5	II	–	0.092	a
10	1.5	II	0.16	–	a
11	1.5	II	–	–	a
12	1.5	II	–	–	b
13	1.5	II	–	–	c
14	1.5	II	–	–	d

* I: all sodium laurate was added at the zeroth hour; II: 0.5 g sodium laurate was added every 4 h since the zeroth hour.
** a: continuous aeration; b: cycle of 60-min aeration and 30-min anaerobic agitation; c: cycle of 60-min aeration and 60-min anaerobic agitation; d: cycle of 120-min aeration and 120-min anaerobic agitation.

2.3. Analytical methods

The PHBHHx content in the sludge was determined according to a methanolysis procedure described by Budde et al. (2011). The sludge was harvested, washed with cold deionized water and hexane, lyophilized, and then the cell dry weight (CDW) was measured. Dried sludge was in turn reacted with methanol and sulfuric acid in the presence of chloroform for 4 h at 90 °C in a screw-top glass test tube. This reaction converts PHA monomers into their related methyl esters. The concentrations of methyl esters were analyzed by gas chromatography (GC) (Agilent 7890, China) using a flame ionization detector and a 30 m × 0.32 mm capillary column. Nitrogen was used as the carrier gas. The injection port and detector were maintained at 200 and 220 °C, respectively. The GC oven was programmed to begin at 80 °C (1.5 min), increase to 80 °C (2 min) with a rate of 30 °C/min, and then arrive at 220 °C (9 min) with a rate of 30 °C/min. The sample injection volume was 1.0 μL with a split ratio of 50:1 (vent:column). Pure standards of methyl 3-HB and methyl 3-HHx were used to generate calibration curves. COD and MLSS were measured according to the standard methods. Analytic results were the average of three parallel samples. Fourier transform infrared (FTIR) spectra of samples were obtained from the Nicolet IR200 spectrometer with DTGS detector (Nicolet, USA), using a casting film technique. Solutions of biopolymers were prepared in chloroform. Solid films were cast from 1 wt% solution in chloroform. In this study, the FTIR spectrometer was set at resolution of 4 cm^{-1} and spectral range of 4000 cm^{-1} to 400 cm^{-1}. The ^1H nuclear magnetic resonance (NMR) spectra of the copolymers were recorded on a Bruker AV-400 NMR spectrometer (Bruker, Switzerland) at 400 MHz against trimethylsilane internal reference standard. Approximately 20 mg PHBHHx sample was dissolved in 0.6 mL deuterated chloroform ($CDCl_3$). For FTIR and NMR characterization, sample PHBHHx was extracted from the lyophilized sludge cells using hot chloroform in a Soxhlet apparatus, and purified by precipitation with ethanol (Brandl et al., 1989). Standard PHBHHx, fermented by pure culture, was supplied by Prof. Guoqiang Chen, Department of Biology, School of Life Sciences, Tsinghua University (Beijing, China).

2.4. Calculations

The mass contents of HB, HHx and PHBHHx in the sludge were defined in Eqs. (1)–(3), respectively.

$$M_{HB}\ (\%\ \text{of CDW}) = \text{mass of HB in the dry sludge/CDW of the dry sludge} \quad (1)$$

$$M_{HHx}\ (\%\ \text{of CDW}) = \text{mass of HHx in the dry sludge/CDW of the dry sludge} \quad (2)$$

$$M_{PHBHHx}\ (\%\ \text{of CDW}) = M_{HB}\ (\%\ \text{of CDW}) + M_{HHx}(\%\ \text{of CDW}) \quad (3)$$

The mole fraction of HHx among PHBHHx was defined in Eq. (4).

$$F_{HHx}\ (\text{mol}\%) = \frac{\text{mass of HHx in the dry sludge}/114}{\text{mass of HB in the dry sludge}/86 + \text{mass of HHx in the dry sludge}/114} \quad (4)$$

The concentrations of HB, HHx and PHBHHx in the reactor were expressed as Eqs. (5)–(7), respectively.

$$C_{HB}\ (\text{mg/L}) = M_{HB}\ (\%\ \text{of CDW}) \times \text{MLSS} \quad (5)$$

$$C_{HHx}\ (\text{mg/L}) = M_{HHx}\ (\%\ \text{of CDW}) \times \text{MLSS} \quad (6)$$

$$C_{PHBHHx}\ (\text{mg/L}) = C_{HB}\ (\text{mg/L}) + C_{HHx}\ (\text{mg/L}) \quad (7)$$

The PHBHHx yield and synthesis rate were calculated using Y_{PHBHHx} and q_{PHBHHx}, given by Eqs. (8) and (9), respectively.

$$Y_{PHBHHx}\ (\text{g/g COD}) = \frac{\text{amount of PHBHHx produced}}{\text{amount of carbon sources consumed}} \quad (8)$$

$$q_{PHBHHx}\ (\text{g/L/h}) = \frac{\text{amount of PHBHHx produced}}{\text{volume} \times \text{reaction time}} \quad (9)$$

The specific utilization rate of substrate was defined as Eq. (10),

$$u = \frac{dS}{S \cdot dt} \quad (10)$$

in which S was the concentration of carbon source (g COD).

2.5. DNA extraction and Illumina MiSeq sequencing of 16S rRNA gene amplicons

The sludge samples were collected by centrifugation and stored at −20 °C before DNA extraction using a 3S DNA Isolation Kit v2.2 (Shenneng Bocai, China). Approximately 1 g biomass was weighed into each DNA extraction tube. All of the extraction steps were carried out according to the manufacturer's protocol.

Universal primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with 12 nt unique barcode was used to amplify the V4 hypervariable region of 16S rRNA gene for high-throughput sequencing using MiSeq sequencer (Caporaso et al., 2012). The PCR mixture (25 μL) contained 1× PCR buffer, 1.5 mM $MgCl_2$, each deoxynucleoside triphosphate at 0.4 μM, each primer at 1.0 μM and 0.5 U of Ex Taq (TaKaRa, China) and 10 ng sludge genomic DNA. The PCR amplification program included initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 40 s, 56 °C for 60 s, and 72 °C for 60 s, and a final extension at 72 °C for 10 min. Conduct two PCR reactions for each sample, and combine them together after PCR amplification. PCR products were subjected to electrophoresis using 1.0% agarose gel. The band with a correct size was excised and purified using SanPrep DNA Gel Extraction Kit (Sangon Biotech, China) and quantified by a UV spectrophotometer (Quawell Q6000, USA). All samples were pooled together with equal molar amount from each sample. The sequencing samples were prepared using TruSeq DNA kit (Illumina, USA) according to manufacturer's instruction. The purified library was diluted, denatured, re-diluted, mixed with PhiX (equal to 30% of final DNA amount) as described in the Illumina library preparation protocols, and then applied to

an Illumina MiSeq system for sequencing with the Reagent Kit v2 2 × 250 bp as described in the manufacture manual.

2.6. Data analysis

The sequence data were processed using QIIME Pipeline-Version 1.7.0 (http://qiime.org/). All sequencing reads were trimmed and assigned to each sample based on their barcodes. The sequences with high quality (length > 150 bp, without ambiguous base 'N', and average base quality score > 30) were used for downstream analysis. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold. The aligned ITS gene sequences were used for chimera check using the Uchime algorithm (Edgar et al., 2011). All the samples were randomly-resampled to 6500 reads. We calculated alpha-diversity (phylogenetic distance whole tree, chao1 estimator of richness, observed species and Shannon's diversity index) and beta-diversity (Principal coordinates analysis (PCoA), UniFrac) analyses, for which the rarefaction curves were generated from the observed species. Taxonomy was assigned using the Ribosomal Database Project classifier (Wang et al., 2007). All data were analyzed using Microsoft Excel, Origin and SPSS. Treatment effects were analyzed using one-way ANOVA, and the LSD multiple range test was used to determine the statistical significance ($P < 0.05$) between pairs with SPSS.

3. Results and discussion

So far, all the wild type PHBHHx producing strains, with only one exception as phototrophic *R. rubrum*, were found able to use laurate or lauric acid to synthesize PHBHHx. Hence, sodium laurate was selected as the solo carbon source throughout this study, including the acclimation phase. Meanwhile, PHBHHx was not observed in the sludge at the end of the acclimation stage. It suggests that the feeding quantity and mode of substrates in terms of C, N, P and O_2 should be the limiting factors to harvest PHBHHx, after the PHBHHx producing bacteria have been enriched in the sludge. Several batch experiments were thereby conducted to assess the impact of these factors on the formation and composition of PHBHHx from activated sludge.

3.1. Effect of the nitrogen and phosphorus source on PHBHHx production

Since pure culture microorganisms have been reported to obtain PHAs containing HHx monomer when triggered by nitrogen or phosphorus limitation (Lageveen et al., 1988; Lee et al., 2000; Chen et al., 2001; Riedel et al., 2012), we hope to utilize this nutrient limitation strategy in the mixed culture scenario likewise. The PHBHHx production performances of batch experiments 1 to 4 with the single-dose feeding of carbon source were plotted in Fig. 1, among which some runs were limited to nutritious elements of nitrogen and phosphorus. The curves of HB and HHx contents exhibited a peak-like pattern in the batches 1 and 3 with nitrogen source, whereas these monomers remained relatively constant in cells till the end of the batches 2 and 4 without nitrogen source (Fig. 1b). Combining Fig. 1a, it was found that the monomer peaks concurred with the COD fast depletion period in the batches 1 and 3. Çiğgin et al. (2011) have reported similar observation for the PHA decay in the acclimated activated sludge feeding with acetate. They explained this behavior as the specific substrate uptake rate is high during the feast phase as well as PHA consumption is fast during the famine phase for growth metabolism. For batches 2 and 4, the absence of nitrogen source seems to retard the metabolic activity and in turn contribute the monomers accumulation in the sludge. It is noteworthy that HHx was not detected in batch 2 with phosphorus. In other words, a strict oligotrophic condition in the acclimated sludge would favor the PHBHHx production.

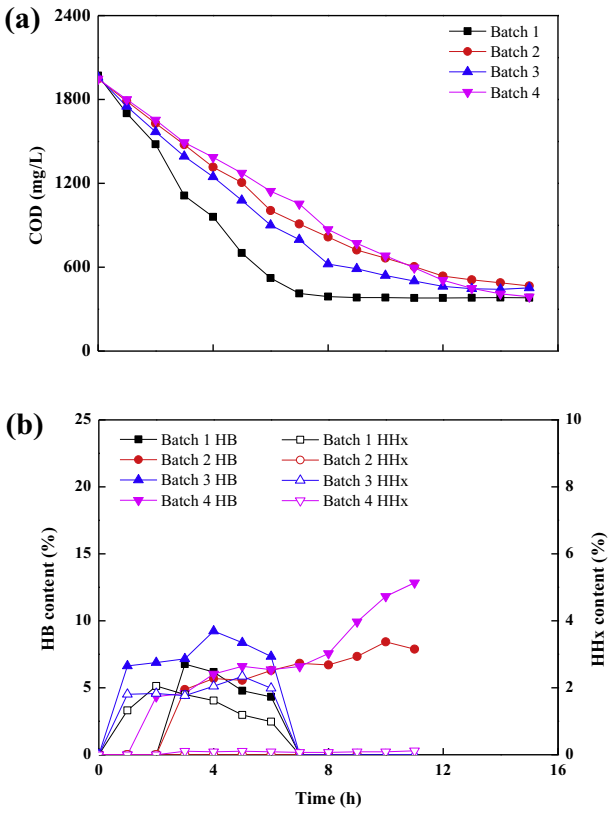


Fig. 1. Time-course record of (a) COD concentration and (b) PHBHHx monomer content in batches 1 to 4.

3.2. Effect of the carbon source on PHBHHx production

Initial concentration and feeding mode of sodium laurate were changed to investigate the influence of this solo carbon source on PHBHHx production. Batches 4 to 7 were all carried out without nutrients addition, the real-time results demonstrated that HB and HHx contents also followed a peak-like tendency when the initial carbon source was below 1.5 g/L in the batches 5 to 7 (Fig. S1). Although feast-famine has been commonly recognized as a strategy to enforce microorganisms producing PHA as intracellular carbon and/or energy substitute, it was seen that this secondary carbon source would be reused when the external carbon source was hardly available. The PHA should be the outcome of concurrent synthesis and degradation. Therefore, in order to maintain the PHBHHx accumulation process in the presence of adequate carbon source, pulse feeding of total 1.5 g/L sodium laurate into the reactor was performed in batches 8 to 11 (Fig. 2), with other conditions the same as those in batches 1 to 4, respectively. With comparison of batches 11 and 4, pulse feeding was found to result in a small variation for the values of substrate specific utilization rate ($u = 0.12–0.26\ h^{-1}$) (Fig. 2a), which were close to that in the fast depletion period of the corresponding single-dose feeding batch ($u = 0.18\ h^{-1}$) (Fig. 1a). Consequently, HB and HHx monomers could steadily accumulate till the end of each pulse feeding batch (Fig. 2b). Similar to the observation in the above section, the maximum PHBHHx was obtained under both nitrogen and phosphorus source limitation (i.e., batch 11), in which HB, HHx content and

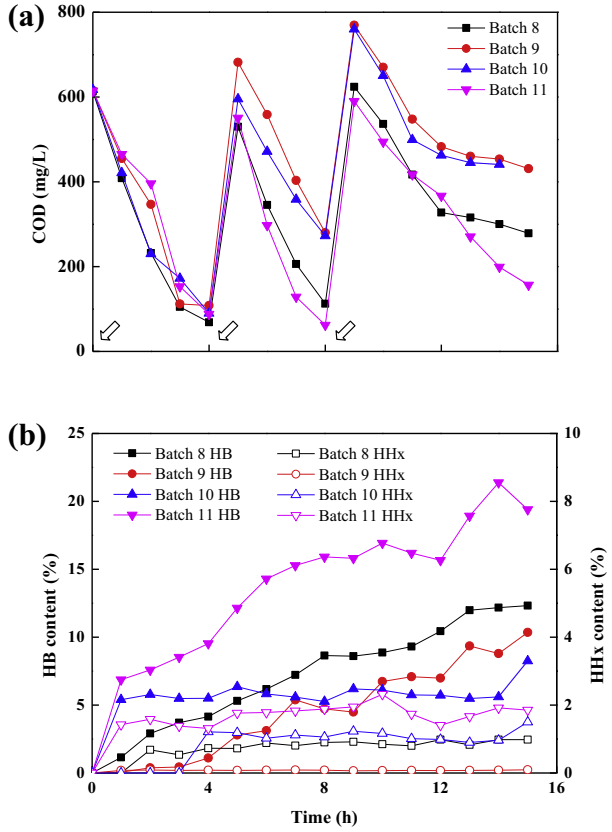


Fig. 2. Time-course record of (a) COD concentration and (b) PHBHHx monomer content in batches 8 to 11, hollow arrows indicating the pulse feeding points.

PHBHHx concentration were 21.4%, 1.9% and 505.6 mg/L, respectively.

3.3. Effect of the aeration mode on PHBHHx production

PHA has been known to be temporarily stored by microorganisms in activated sludge in the anaerobic–aerobic processes. Activated sludge could accumulate PHA of around 20% under anaerobic conditions and up to 33% under aerobic conditions (Satoh et al., 1998). In order to further clarify the role of oxygen, PHBHHx production behaviors of batches 11 to 14 were studied under both the pulse feeding and nutrient limitation conditions, but in various aeration modes (Fig. S2). As indicated in Fig. S2b, the accumulation of PHBHHx was much higher with continuous aeration (batch 11) than those with alternative aerobic-anaerobic sessions (batches 12 to 14). When the proportion of anaeration among the whole cycle rose to 1/2, or the period of anaeration extended to 60 min, the monomer HHx was detected little in the sludge. Although the influence of oxygen for PHA accumulation in activated sludge is still debatable (Chang et al., 2011), most wild microorganisms producing PHBHHx, except *R. rubrum*, are found aerobic strains (Brandl et al., 1989). Thus, it can be postulated that the microorganisms in the acclimated sludge prefer the constant aerobic situation for PHBHHx accumulation.

3.4. Composition and characterization of PHBHHx obtained from activated sludge

The above discussion demonstrated the feasibility of PHBHHx production using activated sludge. An optimal operation strategy was herein proposed as batch 11 described, briefly, continuous aeration, nitrogen and phosphorus limitation, and adequate carbon source implemented by pulse feeding 0.5 g/L sodium laurate every 4 h. In such way, the maximum output in this study was obtained, as 505.6 mg/L PHBHHx with mass content of 23.3% and containing 6.34 mol% HHx. To compare the PHBHHx from activated sludge with those from the wild type fermentation, Table 2 compiled the best achievements of each wild strain reported able to synthesize PHBHHx in history, in which the highest values of PHBHHx content (M_{PHBHHx}), concentration (C_{PHBHHx}) and HHx molar content (F_{HHx}) were 68%, 25096.5 mg/L and 25 mol%, respectively (Gan et al., 2003; Chen et al., 2001; Doi et al., 1995). It reflects that activated sludge could not compete with pure cultures in view of PHBHHx productivity, yet. Meanwhile, the composition of PHBHHx from sludge was close to that from pure strains (Table 2). Considering chemical structure, FTIR spectra revealed that the PHBHHx produced in batch 11 of this study was in accord with the standard PHBHHx (Fig. S3). Six distinct absorption peaks appeared in both two spectra around the same regions, indicating the existence of the same functional groups. Specifically, the peak at around 3438 cm^{-1} represents the O—H stretching vibration, implying the presence of interlamellar water molecules in these polymers; the peaks at around 2975, 2935 and 2877 cm^{-1} are due to the C—H stretching vibration in —CH_3, —CH_2— and —CH—, respectively; intense absorption peaks around 1726 cm^{-1} are indicative of the C=O stretching vibration in —COO—; and the peak observed at 1454 cm^{-1} can be assigned to the C—H bending vibration in —CH_2—. The intermediate portion between wavenumber 1300 and 900 cm^{-1} is usually referred to as the "fingerprint" region of FTIR spectrum, because the absorption pattern in this region is rather complex caused by the stretching vibration of all single bonds and vibration of the molecular skeleton. Through comparing the patterns of "fingerprint" between the sample and the standard (Fig. S3), it suggests that the PHBHHx from activated sludge coincided with that from pure culture. Besides, NMR spectra further confirmed the structure of PHBHHx as shown in Fig. S4. The observed chemical shifts a to e are assigned to methyl, methylene and methine protons in the PHA monomers according to literature (Gumel et al., 2012). And the NMR peaks obtained from activated sludge were in good agreement with those of the PHBHHx standard. In all, the above results state an acceptable quality of the PHBHHx from the activated sludge referring to that from the pure culture.

3.5. Analysis of microbial community in PHBHHx producing sludge

The microbial community diversities and phylogenetic structures of the activated sludge after the batch experiments 8 to 11 completed were analyzed by Illumina MiSeq sequencing, as well as the acclimated seed sludge. Over 4794 sequences were obtained for each sample. Bacterial diversity was measured through OTU (defined at 97% sequence similarity), Chao1 richness, Shannon diversity index (Table 3) and rarefaction curves (Fig. S5). It can be seen that the diversity and richness of the activated sludge producing low PHBHHx (batches 8 to 10) were approximate to those of the seed sludge. Meanwhile, the sludge sample from batch 11, which accumulated the maximum PHBHHx in this study, exhibited an apparent decrease in the microbial community diversity and richness. This could be ascribed to the deficiency of nutrients (Ince et al., 2012; Cavaillé et al., 2013). The differences in microbial community structures between the PHBHHx producing sludge and the seed sludge were also demonstrated by PCoA (Fig. S6). Although the spatial distances of sample points in *x* and *y* axes were not huge, sample of the sludge producing the maximum PHBHHx, i.e. of batch 11, was clearly separated from the others, indicating a distinctive microbial community structure.

Table 2
Composition of PHBHHx produced by activated sludge and pure wild strains, the bold marking the highest value in the column.

Microbes	M_{PHBHHx} (wt%)	M_{HHx} (wt%)	F_{HHx} (mol%)	C_{PHBHHx} (mg/L)	C_{HHx} (mg/L)	Incubation time (h)	Y_{PHBHHx} (g/g COD)	q_{PHBHHx} (g/L/h)	Reference
Rhodospirillum rubrum	13.1	1.5	8.9	76.5	6.8	300	–	–	Brandl et al. (1989)
Aeromonas hydrophila CGMCC 0911	49	3.7	5.9	1097.6	84.2	48	–	–	Lu et al. (2004)
Aeromonas sp. KC014	29.84	2.14	5.5	2650	190.8	95	0.06	0.08	Gong et al. (2008)
Aeromonas hydrophila WQ	**68**	**11.1**	12.8	1754.4	224.6	72	–	–	Gan et al. (2003)
Aeromonas caviae	27	8.3	**25**	999	249.75	48	–	–	Doi et al. (1995)
Aeromonas hydrophila 4AK4	50.7	7.4	11.4	**25096.5**	**2861**	25	–	0.540	Chen et al. (2001)
Activated sludge	23.3	1.9	6.34	505.6	41.7	14	0.46	0.334	Batch 11, this study

Table 3
Diversity indices calculated based on a cutoff of 97% similarity of 16S rRNA sequences of 4794 reads per sample.

Sample	Chao1	Observed OTU	Shannon index
Batch 8	1521 ± 154	647 ± 70	6.190 ± 0.269
Batch 9	1452 ± 103	678 ± 78	6.425 ± 0.233
Batch 10	1617 ± 195	718 ± 45	6.990 ± 0.156
Batch 11	1246 ± 302	585 ± 118	6.645 ± 0.445
Seed sludge	1349 ± 295	684 ± 165	7.235 ± 0.445

Note: all data are presented as mean ± SD.

Additionally, based on the high-throughput 16S rRNA gene sequencing data, taxonomic assays for bacterial communities of the PHBHHx producing sludge and the seed sludge were performed at the phylum to genus levels. Fig. 3 showed the total 14 phyla identified with abundance above 0.1% in the sludge samples from batches 8 to 11 and the seed sludge, respectively. *Proteobacteria* and *Bacteroidetes* dominated in the bacterial community in all sludge samples, which is in agreement with the observation of most activated sludge systems (Hu et al., 2012; Ranasinghe et al., 2012). Substantial PHB-storing microorganisms in activated sludge have been characterized affiliating to *Proteobacteria* (Liu et al., 2011; Dai et al., 2015). And so far the best known wild types able to synthesize HHx, such as the genera *Aeromonas* (Shiotani and Kobayashi, 1993; Shimamura et al., 1994; Chen et al., 2001; Gan et al., 2003; Lu et al., 2004; Gong et al., 2008) and *Pseudomonas* (Abe et al., 1994; He et al., 1998; Lageveen et al., 1988; Lee et al., 2000), both fell into the phylum *Proteobacteria*, implying that sodium laurate should act as an effective selective substrate to enrich the PHBHHx accumulating bacteria. Meanwhile, there is an evident difference regarding the phylum *Chlorobi*, which had the highest abundance (10.560%) in the maximum PHBHHx producing sludge (batch 11), followed by the medium values (1.302–3.432%) in the low PHBHHx producing sludge (batches 8 to 10), but was detected just 0.643% abundance in the seed sludge (Fig. 3). *Chlorobi* is in turn supposed to be a key group of bacteria exerting some special function for PHBHHx accumulation, whereas there is scarce information about it till now, let alone its role in PHA synthesis. Similarly, among the total 598 genera recognized (Table S2), *Leadbetterella*, belonging to phylum *Bacteroidetes*, was the dominant genus in the maximum PHBHHx producing sludge (14.128% abundance, batch 11), but had <2.150% abundance in the other samples. Recently, only one *Leadbetterella* strain has been isolated, from cotton-waste composts, which was found strictly aerobic, G^-, non-motile, non-gliding, rod-shaped, catalase- and oxidase-positive cell (Weon et al., 2005). This is in line with the observation of Section 3.4, i.e., PHBHHx accumulated more in a continuous aeration mode than an intermittent one. And this abundant *Leadbetterella* genus could be assumed as a critical group involved in PHBHHx synthesis.

4. Conclusions

The production of PHBHHx from activated sludge was demonstrated feasible for the first time. Sodium laurate was appropriate as the sole carbon substrate for both sludge acclimation and

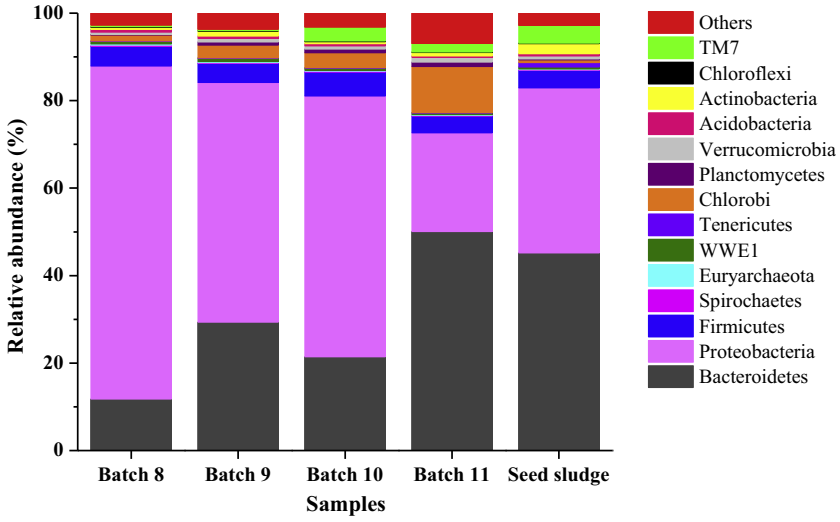


Fig. 3. Taxonomic classification of 16S rRNA gene sequences from bacterial communities of the PHBHHx producing sludge and the seed sludge at the phylum level. Relative abundance of each taxon was defined as the percentage of the same taxon to the corresponding total sequences for each sample. Some phyla were unknown or <0.1% abundances were summarized as others.

PHBHHx accumulation. Through adjusting the feeding mode as well as quantity of carbon source, nutrients and oxygen, the maximum 505.6 mg/L PHBHHx containing 6.34 mol% HHx was obtained in this study, which had similar structure to PHBHHx from pure culture as FTIR and NMR indicated. High-throughput sequencing showed that the community diversity decreased in the maximum-productivity sludge. Phylum *Chlorobi* and genus *Leadbetterella* were inferred as key functional members for PHBHHx synthesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.04.007.

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