Reduce the Degradation of Polyhydroxyalkanoates: Feeding in the Oxygen-limiting Section Increases the Accumulation of PHAs

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Abstract

To solve the problem that the content of polyhydroxyalkanoates (PHAs) in the aerobic section of the traditional anaerobic-aerobic process continues to decrease, two different feeding modes in the oxygen-limiting section were explored under the anaerobic-oxygen limiting process(for reducing the energy consumption of aeration). Based on 16SrRNA sequence analyses, the results show that the abundance of *Hydrogenovora*(9.57%), *Zoogloea*(6.73%), *Thaurea*(1.49%) and other bacterial genera in the microbial community had increased after enrichment. PHAs were synthesized in the oxygen-limiting stage of both one-time feeding and batch feeding mode. The maximum content of PHA reached 52.17% when C/N increased to 150 in the one-time feeding mode, and that reached 42.04% in batch feeding mode when C/N was 100. The one-time feeding mode shows a more significant accumulation potential of PHAs than the batch feeding mode. The content of PHAs shows an increasing trend with the increase of C/N ratio, and a high C/N ratio may lead to sludge bulking and other problems, making the sludge system unsteady.

1. Introduction

Polyhydroxyalkanoates (PHAs) are one of the bioplastics with good performance and development prospects. It can be degraded into CO_2 and H_2O by microorganisms in nature, which can significantly reduce pollution. When nutrients such as nitrogen, phosphorus, and oxygen are limited, and carbon is excessive in the growth environment of microorganisms, PHAs can act as intracellular storage when the microorganisms grow out of balance [1]. However, the cost of pure culture of bacteria [2] and chemical synthesis methods is too high to hinder the commercialization of PHAs. Mixed microbial cultures and volatile fatty acids produced by organic waste fermentation are used to synthesize PHAs[3, 4]. Currently, the production of PHAs can be mainly divided into two- and three-segment synthesis[5]. The three-step process of substrate fermentation-enrichment-accumulation synthesis proved to be an effective method for obtaining high PHAs content culture.

The aerobic dynamic feeding process(ADF) was proposed in 1996 to produce PHAs[6]. The cycles of "Feast and Famine" during the operation enable the mixed bacteria with the ability of PHAs-synthesis in the activated sludge system to rapidly proliferate under environmental screening and become the absolute dominant bacteria in the system. Anaerobic-aerobic(AA) process, known as enhanced biological phosphorus removal process (EBPR), is common in wastewater treatment and runs through the phosphorus removal process commonly used in wastewater treatment plants. The energy generated by the process of hydrolysis of polyphosphates to orthophosphates is used to absorb an external carbon source to synthesize PHAs under the anaerobic section[7]. Then PHAs were degraded in the aerobic section, and more polyphosphates return to the cell. The activated sludge from a non-EBPR wastewater treatment system was used for 90 days of acclimation enrichment under the anaerobic-aerobic process[8]. It was found that the microbial community in sludge showed good phosphorus accumulation and polyhydroxyalkanoate synthesis effect, and the dominant species was authenticated as phosphate

9] compared the content of PHAs synthesized by mixed

bacteria in the ADF process and anaerobic-aerobic process. Under the condition of acetate as the carbon source, the maximum content of PHAs in the two methods was 16% and 44.7%, respectively. The anaerobic-aerobic process was significantly better, and its energy consumption was lower than that of the complete aerobic process.

In the study to further improve the production of PHAs by the mixed bacteria, different feeding methods and nutritional restrictions were used to promote the content of PHAs. In the study of Francesco Valentino[10], fermented whey permeate and acetate as substrates were explored for synthesizing PHAs by "feed on demand" mode under the condition of restricted nutrition. "Feeding on demand" limits the supply rate of COD and makes it close to the demand of microorganisms, and this is a crucial method to realize the selective growth of PHA-accumulating bacteria in the biomass. Liu[11] obtained a maximum PHB accumulation of 64% when nitrogen limiting conditions are C/N = 125 under the anaerobic-aerobic process. An imbalance in the nutrients or oxygen supplied increased PHAs production during batch fermentation profile, and low aeration significantly influenced PHA production in B.endophyticus [12].

Compared with the anaerobic-aerobic process, phosphorus removal is similar to the synthesis process of PHAs under the anaerobic-anoxic process with lower energy consumption[13]. In the anaerobic-aerobic process, the content of PHAs reaches its maximum at the end of the anaerobic phase. Then it would be consumed in the aerobic section for the growth of microorganisms, leading to the decrease of the accumulation of PHAs, thus affecting the subsequent PHAs recovery.

For PHAs to be recycled and accumulated, it is undesirable for PHAs to be consumed during the aerobic section. The traditional anaerobic-aerobic operation mode was improved in this study to synthesize PHAs from activated sludge. The optimal operating parameters of the anaerobic-oxygen limiting process were investigated under acetate with different concentrations, different nutrient conditions, and carbon source feeding mode (feeding in oxygen-limiting section).

2. Materials And Methods

2.1 Enrichment of PHAs-accumulating bacteria

A Sequencing Batch Reactor(SBR) (Fig. 1(left)) with practical volume of 7.5L was used to enrich the PHAs-accumulating bacteria in anaerobic-oxygen limiting screening mode. The sludge used for the experiment was taken from the A/O process of the Nancun Wastewater Treatment Plant. COD was controlled at 200 mg/L and was stably operated on for three days to recover activity. The carbon source concentration then continued to be added until the COD was 800 mg/L. And it was added to 100 mg/l and ran steadily for two days each time. The initial C/N/P ratio was controlled as 100/6/2(mol/mol/mol), and it had been shown[14] that increasing the phosphorus source could stimulate an increase in PAOs. Then C/N and C/P were increased stepwise in the daily influent water so that it reached C/N P = 100/5/1 after five days of operation and reached the nutrient equilibrium condition of the EBPR system[15].

d, one-time feeding and batch feeding

In the enrichment reactor(Fig. 2(up)), it takes 15min for each cycle to fill 5L water, 2h for the anaerobic section, and 3h for the oxygen-limiting section(DO remains below 2mg/L[16]). The following 30min was for sedimentation, and the final 15min was for drainage and resting. This process is cycled four times every day. The MLSS of the initial sludge is about 3500 mg/L, and the actual MLSS of the reactor is 3800 mg/L. Part of the sludge-water mixture is discharged at the end of the anaerobic section every day to keep the MLSS in the range of 4000–5500 mg/L. The anaerobic phosphorus release, effluent phosphorus content, MLSS, SVI, and the state of the sludge were measured to judge the sludge activity and the acclimation of PAOs.

The microbial community in sludge before and after enrichment were sampled and 16SrRNA sequencing was performed. PE reads obtained by second-generation sequencing are firstly spliced according to overlap relationship. The sequence quality is controlled and filtered after distinguishing samples, and then OTU clustering (ASV denoising) analysis and species taxonomy analysis were performed.Based on OTU clustering (ASV denoising) analysis results, diversity index analysis of OTU (ASV) and sequencing depth detection can be performed.Based on taxonomic information, statistical analysis of community structure can be carried out at various taxonomic levels.

2.2 Accumulation of PHAs

A 1L beaker was used as a reactor(Fig. 1(right)) in the PHAs-accumulating unit. The sludge was taken from the enrichment reactor and put into this beaker. In the PHAs accumulation reactor, the MLSS was controlled at about 4500 mg/L, the COD of the inflow was maintained at 800mg/L, and the nutrient ratio of the inflow was C/N/P = 100/5/1. Based on anaerobic-oxygen limiting operation, a peristaltic pump was used to control adding carbon-only substrate in the oxygen-limiting section (Fig. 2(middle and down)). One-time feeding mode ran in 1h after oxygen-limitation, and the concentration of COD was 200 mg/L. Batch feeding was also performed after 1h of oxygen-limitation, and substrate was added every 30min (without adding at the end of the reaction) to make the COD return to 50 mg/L.

To investigate the influence of the C/N ratio of 60, 100, and 150 on the synthesis of PAHs with two different feeding methods, the influent nitrogen source concentration was adjusted successively based on the normal nutrient ratio (C/N/P = 100/5/1) in the oxygen-limiting feeding process, and all nutrient ratios except nitrogen remained unchanged. After each adjustment of C/N, it was stable for 2 days, and then the PHAs content of sludge was measured every 30 minutes in a complete cycle.

2.3 Analytical techniques

COD_{Cr}, MLSS, and TP were analyzed by the standard method[17].

The percentage of PHAs in sludge was measured using gas chromatography (Agilent 7980A-5975C GCMS) [18]. The sample was centrifuged firstly in a high-speed refrigerated centrifuge at 8000 r/min for 2 min, and then the supernatant was collected through a filter paper into a sampling cup. After the samples were mixed with deionized water and centrifuged again for 2 min, the samples without supernatant were [illegible]-dryer for drying for 12 h. The freeze-dried sludge was then

mixed with 2ml chloroform, 2ml methanol containing 500 mg/L benzoic acids, and 2ml methanol acidified with H_2SO_4(10% v/v) in sample tubes. The sample tubes were sealed off and heated at 100 ℃ for 4h and cooled to room temperature. Then 2 ml of deionized water was added to the sample tubes and shaken well for 10 minutes. After the separation of the three phases, the organic compounds were then analyzed by gas chromatography.

2.4 Calculations

The PHA content of the sludge (% PHA, mg-PHA/mg-TSS):

$$\%PHA = \%100 \times \frac{PHA_e - PHA_0}{MLSS}$$

The sludge growth rate (S_R, mg-MLSS/h-T):

$$S_R = \frac{MLSS_e - MLSS_0}{V \times T}$$

3. Results And Discussion

3.1 Enrichment of PHAs dominant strains and microbial community succession in activated sludge

3.1.1 Conventional index analysis

Due to the low content of carbon sources in urban sewage in southern China, the activated sludge gradually adapted to a high concentration of carbon sources after recovery and stabilization. Figure 3(a) shows the phosphorus content in the water at the end of the anaerobic-oxygen limiting stages during the enrichment period. PAOs in activated sludge releases intracellular phosphorus during the anaerobic phase and absorbs excess phosphorus in solution during the aerobic phase. The phosphorus content was increasing at the end of the anaerobic phase and was kept below 1 mg/L in effluent at the oxygen-limiting section during the period of sludge recovery. The phosphorus removal rate reached 99.8%, which suggests PAOs were enriched and their phosphorus removal ability was excellent.

With the rector running and the carbon source concentration increasing, SVI decreased from 155 to 72 and MLSS risen from 3800 to 4700. And the color of sludge becoming yellow. As shown in Fig. 3(b), the growth rate of activated sludge shows an increasing trend in the enrichment period, indicating that activated sludge had good adaptability to high carbon sources. It is conducive to the subsequent accumulation of PHAs and the recovery of PAOs.

The growth of activated sludge indicates that the carbon source was absorbed by the PHAs-accumulating bacteria and then assimilated for their growth and proliferation. There are two main ways

ored as internal carbon source PHAs; being assimilated by

cells for their growth and proliferation. For the process of PHAs accumulation, more carbon sources will be used for PHAs synthesis rather than cell proliferation [19].

During the enrichment of PHAs-accumulating bacteria, the increasing COD concentration improves the tolerance of high-load carbon of sludge, and then efficient PHAs-accumulating bacteria can be accumulated. As shown in Fig. 4, acetate was rapidly absorbed by microorganisms in the anaerobic section of this cycle. The COD concentration quickly decreased in the first 0.5h and was almost removed by the end of the anaerobic phase, while the content of PHAs and TP gradually increased. The oxygen-limiting section continues until the end of the cycle, accompanied by the decrease of TP and the consumption of PHAs. Though the conditions of influent substrate concentrations were different, the proportion of PHB is all about 75%. The PHAs synthesized by acetate as a carbon source are mainly PHB, and similar research results have also appeared in the report of Hong Chen[20]. PHV and PHB have the same trend. PHAs content gradually increased in the anaerobic stage, reaching a maximum of 23.9% at the end of the anaerobic phase, and was progressively consumed in the oxygen-limited section, and the final content was 7.28%.

According to the Mino model[21, 22]and Smolders model for acetate absorption[23], during the anaerobic section, the degradation of intracellular polyphosphate and a small amount of glycogen are hydrolyzed by PAOs to produce ATP and reducing power when acetate is the substrate. The energy is used for active transport to absorb external carbon sources such as VFAs. Then the acetate is converted to the precursor of PHB, acetyl CoA, and a small amount of the precursor of PHV, propionyl CoA. A large amount of phosphate that cannot be used for intracellular synthesis is accumulated and then diffuses to the water in the form of active transport, causing the TP to increase in the solution. In the aerobic section, O_2 is used by PAOs to oxidize PHAs as the electron acceptor. The energy produced by the degradation of PHAs and the reducing power provided by reducing coenzyme II ($NADH_2$) are used to actively transport phosphates from the water into the cells and forms polyphosphates. In this way, the effect of phosphorus removal is achieved.

3.1.2 Microbial community analysis

The microbial community in the sludge sample before and after enrichment were analyzed high-throughput 16S rRNA gene sequencing. Phyla *Proteobacteria* was enriched and increased from 32.04–69.66% of total OTUs, was the most abundant phylum. And *Betaproteobacteria*(31.34%), *Gammaproteobacteria*(19.91%), and *Alphaproteobacteria*(11.98%) are the main class. The bacterial cells showed in Fig. 5 are all affiliated with *Betaproteobacteria* and they are all belong to PHA-accumulating bacteria[24]. Phyla *Bacteroidetes* decreased from 32.40–22.03% after enrichment, and other phyla comprised 8.31% of total OTUs. The succession of the community and the change of mixed bacterial function is synchronous. It can be seen that the microbial community structure has a more obvious succession before and after the enrichment section.

As the results shown in Fig. 5, the proportion of the PHA-accumulating bacteria in the initial sludge was

nt, the abundance of dominant PHA-accumulating bacteria in

sludge was significantly increased, which was reflected by increasing PHAs content. OTU4 (*Hydrogenophaga* genus) is a gram-negative hydrogen oxidizing bacteria with good PHAs synthesis ability. Koller[25] synthesized about 40% PHAs from isolated *Hydrogenovora* DSM 1749 using unhydrolyzed whey lactose as substrate, which verified the PHAs synthesis ability of the strain. In this experiment, the strain was acclimated and enriched (from 0.05–9.57%) under anaerobic and oxygen-limiting conditions. The result shows a certain effect of PHAs synthesis in macroscopic function and indicates that *Hydrogenophaga* is the dominant genus and plays a leading role in the process of PHA synthesis. *Thauera*[26] is a typical aerobic PHAs producing bacterium, which can synthesize PHAs by carbon source in the presence of oxygen. The abundance of OTU43 (*Thauera* genus) in the sludge was significantly improved compared with that in the initial sludge, indicating that there were aerobic PHAs-accumulating bacteria in the sludge after enrichment. They were the potential strains for the synthesis of PHAs in the oxygen-limiting feedstock whereafter.

Dechloromonas[27], *Zoogloea*[28], and *Hydrogenophaga*[29] were all reported as the dominant bacteria in enhanced biological phosphorus removal (EBPR). The changes in the abundance of these bacteria before and after the activity recovery were related to the effect of macroscopic phosphorus accumulation and release. OTU19、OTU53、OTU49、OTU86 all belong to *Zoogloea*. *Zoogloea* can secrete extracellular polymeric substances(EPS) that are composed of extracellular polysaccharides, proteins, nucleic acids, and other biological macromolecules. Then *Zoogloea* with compact structures and the strong ability to adsorb and oxidize organic matters is formed and it is of great significance to the settling ability of sludge.

3.2 Effect of feeding in the oxygen-limiting section on PHA production

3.2.1 Normal nutritional conditions

Figure 6 The changes of TP, COD, and PHAs in one-time and batch feeding when C/N = 20

As shown in Fig. 6, the changes of PHAs appear a different trend from before when the feeding modes were carried out in the oxygen-limiting section. In the experiment of one-time feeding in the oxygen-limiting section, the PHAs storage in the anaerobic section shows an upward trend. Still, the inflection point at the end of the anaerobic section is not the maximum value of this cycle. Acetate has been added again after 1h of oxygen-limiting operation, and the PHAs content increases rapidly again. After adding the carbon source for 0.5H (oxygen-limiting for 1.5H), it reached a new peak and then began to decrease. In the batch feeding operation, the first inflection point appeared after oxygen-limiting for 0.5h, and then PHAs were repeatedly synthesized and consumed with the batch addition of carbon source. The maximum content of PHAs in the two feeding modes is 26.52% and 34.98%, respectively, which were higher than the PHAs content without feeding mode.

The new trends indicate that feeding in the oxygen-limiting section can promote the increase of the content of PHAs, and this operation performs at not only the end of the anaerobic section but also the

end of a cycle. It has been documented[30] that under the anaerobic-aerobic mode, a kind of PAOs existing in activated sludge is in a "dormant state" in the anaerobic section, and does not degrade the substrate and synthesize PHAs. In contrast, in the aerobic stage, this kind of bacteria can quickly absorb carbon sources to synthesize PHAs and, at the same time, accumulate phosphorus to achieve phosphorus removal. However, according to the changes of TP and COD showed in the figure, it can be concluded that the bacteria that perform the PHAs synthesis in the oxygen-limiting section are not this kind of PAOs because the degradation of COD still occurred in the anaerobic section. And the phosphorus content in the microbial communities system was decreased compared with no feeding operation, which indicates that the overall activity of PAOs is reduced under the anaerobic-oxygen limiting feeding process. It is speculated that aerobic PHAs-accumulating bacteria such as Thauera plays the role of PHAs synthesis in the oxygen-limiting stage at this time. This bacteria is sensitive to the carbon source and can continue to synthesize PHAs within 30 minutes after feeding. It has been pointed out that PHB is the primary monomer component of PHAs synthesized by activated sludge using sodium acetate as the carbon source under aerobic conditions[30]. In this experiment, the main monomer of PHAs synthesized under the mode of feeding in the oxygen-limiting section was also PHB.

Under the average nutrient ratio (C/N/P = 100/20/1), the COD degradation rate and phosphorus removal capacity of the two feeding modes were all reduced, but the influence on PAOs of batch feeding in the oxygen-limiting section was more pronounced. Due to the existence of external carbon sources almost in the whole oxygen-limiting section, the heterotrophic bacteria in the sludge can grow and proliferate by degrading external carbon sources in the presence of oxygen. At the same time, the process of PAOs to decompose PHAs and synthesize polyphosphate would be inhibited. It would not only lead to the increase of effluent phosphorus content and the loss of total phosphorus content in the microbial communities system (effluent phosphorus reached 7.46mg/L, which is similar to phosphorus in inflow). And it would result in less phosphorus release in the anaerobic section of the next cycle. But also it leads to the incomplete degradation of carbon source in the effluent. In the one-time feeding mode, though the process of O_2 being used by PAOs to oxidize PHAs as the electron acceptor and the process of the energy being used by PAOs to absorbs the phosphorus is inhibited in the oxygen-limiting stage. And other heterotrophic bacteria absorb carbon sources to proliferate and compete with PAOs. PAOs still show a good effect on phosphorus removal and COD degradation.

Although PAOs have been affected to a certain extent in batch feeding mode, the comparison in Fig. 6 shows that the maximal content of PHAs obtained by batch feeding is significantly higher than that of onetime-feeding under normal nutrient conditions. Since PHAs-accumulating bacteria are domesticated and enriched in the screening environment of "Feast-Famine", the condition that carbon source always exists in the water under batch feeding mode makes the process of bacteria decomposing PHAs and continuing to grow and proliferate inhibit. The lower consumption and degradation of PHAs in the oxygen-limiting section leads to the accumulation of PHAs in the cell, which increases the initial content of PHA in the next cycle and makes PHAs continuously get higher accumulation.

al conditions (nitrogen-limited)

As shown in Fig. 7, the effects of two feeding modes in the oxygen-limiting section on the synthesis of PHAs were compared under three different nitrogen-limiting conditions (C/N = 60, 100, 150).

In the operation of one-time feeding in the oxygen-limiting section, the trend of PHAs content was also increased in the anaerobic section. PHAs were consumed first in the oxygen-limiting section and then generated again after feeding. In the process of increasing the C/N ratio, all the peak values and final values of PHAs contents are rising.

Under nitrogen-limiting conditions, the synthesis of essential proteins related to growth and proliferation in microorganisms, such as the synthesis of DNA and RNA, will be blocked. That would negatively affect the tricarboxylic acid cycle(TCA cycle) of cells. Under optimal microbial growth conditions with average nutrient ratios[31], the CoASH produced in the TCA cycle inhibits the production of 3-ketoacyl-CoA thiolase. And 3-ketoacyl-CoA thiolase is essential for the synthesis of PHAs because it can catalyze the formation of the precursor acetyl-CoA of PHAs. The imbalance of microbial growth caused by nitrogen restriction would increase intracellular $NADH^{+}$, which would bring CoASH to the non-inhibitory level and then remove the restriction of PHAs accumulation. At the same time, the increase of $NADH^{+}$ can also help the synthesis of PHAs by inhibiting the activity of citrate synthase in microorganisms and promote the accumulation of PHAs in microorganisms[32].

The increase of the C/N ratio within a specific range would stimulate the accumulation of PHAs[33]. In addition, it was observed that the variation of the PHAs content in the anaerobic section showed a decrease in PHAs when C/N is 100 and 150. This phenomenon also appears in the study reported by Wen [33], indicating that it is related to the degree of nitrogen restriction. Under the condition of a high C/N ratio, the metabolism of microorganisms was greatly affected, and there were complex mixed bacteria in activated sludge. It is speculated that the synthesis of protein and nucleic acid is blocked in some microorganisms due to the lack of nitrogen[34], which affects the influence of cell growth and then decomposes their cellular materials under endogenous respiration. Therefore, the synthesis and decomposition PHAs fluctuated in different microorganisms during the whole operation cycle. And the addition of carbon sources made the non-PHAs accumulating bacteria also absorb carbon sources for life activities, which affected the PHAs-accumulating bacteria and increased the complexity of the change of PHAs content. In addition, the settling ability of activated sludge was poor at the high C/N ratio, and the SVI of sludge reached 124 and 165 when the C/N was 100 and 150, respectively.

The nitrogen-limited reactor was operated by gradually reducing the amount of nitrogen source. At the high C/N ratio, the sludge-water mixture was difficult to separate, and the color of the sludge in the reactor started to turn white. Part of the sludge was discharged with the supernatant after the end of the reaction, resulting in the reduction of the sludge volume in the system. It has been reported[35] that sludge bulking of activated sludge would appear when the condition of a high C/N ratio. Due to the lack of nitrogen source, the growth of bacteria in the mixed culture system is inhibited, and a large number of bacterial micelles are in the endogenous metabolic period. Filamentous bacteria with heavy concentrations and high DO affinity peculiarities compete for carbon source and oxygen electron

acceptor under nutrient-limited conditions. And then, they proliferate to become dominant bacteria which would lead to sludge bulking. In conclusion, increasing the C/N ratio can effectively promote the accumulation of PHAs in microbial communities system. But the problem of the sludge being discharged in the process of removing the supernatant caused by the deterioration of sludge settling ability cannot be ignored. Therefore, the C/N ratio is not as high as possible.

In batch feeding mode, the change of PHAs becomes more complex with the increase of the C/N ratio. Under nutrient imbalance, the PHAs content did not show a continuous upward trend in the anaerobic section. PHAs were decomposed in the anaerobic section, and the maximum content of PHAs appeared at 0.5h after the end of the anaerobic section. In the subsequent oxygen-limiting section, there was also a higher PHAs content when the C/N ratio was higher. But the variation of the PHAs content was also more changeable, and there were multiple inflection points in the operation process.

The variation of the PHAs content has been fluctuating when C/N = 60 in batch feeding mode, indicating that some PHAs-accumulating bacteria in mixed microbial cultures began to carry out endogenous respiration. PHAs were synthesized and decomposed at the same time, which makes the change rule of PHAs intricate. And the settling ability of activated sludge also started to get worse and SVI was 102. The trend of PHAs content became gentle from C/N = 100 in the oxygen-limiting section. There was no significant increase in the content of PHAs because the degradation and synthesis of PHA kept a balance. Due to the presence of external carbon sources throughout the cycle in batch feeding mode, the PHAs-accumulating bacteria could not eliminate other non-PHAs synthetic bacteria under the condition of "Feast-Famine". It indicates that the PHAs-accumulating bacteria have lost their dominant position in the uptake of carbon sources and the utilization of oxygen in the environment. It has been reported[34] that the overall PHAs content of the system can reach a higher level in activated sludge with more abundant and dominant PHAs-accumulating bacteria because both the yield and biomass of PHAs are stimulated to different degrees under the condition of nutrient restriction. The accumulation of PHAs has reached saturation when the C/N ratio is between 100 to 150 because PHAs content was no significant increase. Similar to one-time feeding, there was sludge bulking in the sludge system. The poor settling ability (SVI = 205) leads to part of the sludge was discharged with the supernatant and the PHAs-accumulating bacteria lose their dominant position in the sludge system.

In the process of increasing the C/N ratio, the maximum content of PHAs in the two feeding methods has been improving continuously. However, the activity of PAOs in the one-time feeding mode is less affected than in batch feeding mode when the C/N ratio is high, and the PHAs-accumulating bacteria can still occupy the dominant position in the screening environment of "Feast-Famine" and obtain more PHAs. But the PHAs-accumulating bacteria lost the dominant position in the batch feeding mode. Therefore, the mode of one-time feeding in the anaerobic-oxygen limiting process has a higher PHAs synthesis potential, and the PHAs accumulation performance is better under the nitrogen-limited condition with C/N = 150.

4. Conclusion

Two modes of feeding in the oxygen-limiting section can effectively increase the content of PHAs. Both Hydrogenophaga and Thauera that enriched in the microbial communities system have the potential to synthesize more PHAs in the oxygen-limiting section. The process of PHAs degrading is inhibited when the carbon source is constantly present in the environment, which makes the synthesis effect of PHAs better in the batch feeding operation. However, in the process of increasing the C/N ratio, the PAOs activity is less affected in the one-time feeding method, which showed a more significant accumulation potential of PHAs than in the batch feeding method. Nitrogen restriction can increase the content of PHAs, but sludge bulking appeared in both feeding modes when the C/N ratio is high.

Declarations

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Ethical Approval and Consent to Participate

Not Applicable.

Consent to Publish

Not Applicable.

Competing Interests

The authors declare no competing interests.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors Contributions

Design of the study and preliminary preparation of experiment: Qian Fang, Yihan Xie, Kequan Zhang.

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References

1. Gasser, E., et al., *Microbial production of biopolymers from the renewable resource wheat straw.* J Appl Microbiol, 2014. **117**(4): p. 1035-44.
2. Van Wegen, R.J., Y. Ling, and A.P.J. Middelberg, *Industrial Production of Polyhydroxyalkanoates Using Escherichia Coll: An Economic Analysis.* Chemical Engineering Research and Design, 1998. **76**(3): p. 417–426.
3. Bhalerao, A., R. Banerjee, and R. Nogueira, *Continuous cultivation strategy for yeast industrial wastewater-based polyhydroxyalkanoate production.* J Biosci Bioeng, 2020. **129**(5): p. 595–602.
4. Albuquerque, M.G., et al., *Mixed culture polyhydroxyalkanoate (PHA) production from volatile fatty acid (VFA)-rich streams: effect of substrate composition and feeding regime on PHA productivity, composition and properties.* J Biotechnol, 2011. **151**(1): p. 66–76.
5. Serafim, L.S., et al., *Strategies for PHA production by mixed cultures and renewable waste materials.* Appl Microbiol Biotechnol, 2008. **81**(4): p. 615 – 28.
6. Majone, M., et al., *Influence of storage on kinetic selection to control aerobic filamentous bulking.* Water Science and Technology, 1996. **34**(5–6): p. 223–232.
7. Pijuan, M., et al., *Aerobic phosphorus release linked to acetate uptake: Influence of PAO intracellular storage compounds.* Biochemical Engineering Journal, 2005. **26**(2–3): p. 184–190.
8. Pijuan, M., C. Casas, and J.A. Baeza, *Polyhydroxyalkanoate synthesis using different carbon sources by two enhanced biological phosphorus removal microbial communities.* Process Biochemistry, 2009. **44**(1): p. 97–105.
9. Ahmadi, F., A.A. Zinatizadeh, and A. Asadi, *The effect of different operational strategies on polyhydroxyalkanoates (PHAs) production at short-term biomass enrichment.* Journal of Environmental Chemical Engineering, 2020. **8**(3).
10. Valentino, F., et al., *Polyhydroxyalkanoate (PHA) storage within a mixed-culture biomass with simultaneous growth as a function of accumulation substrate nitrogen and phosphorus levels.* Water Res, 2015. **77**: p. 49–63.
11. Liu, C., et al., *The effect of anaerobic-aerobic and feast-famine cultivation pattern on bacterial diversity during poly-beta-hydroxybutyrate production from domestic sewage sludge.* Environ Sci Pollut Res Int, 2016. **23**(13): p. 12966-75.
12. Madhusoodanan, G., R.C. Hariharapura, and D. Somashekara, *Dissolved oxygen as a propulsive parameter for polyhydroxyalkanoate production using Bacillus endophyticus cultures.* Environment, Development and Sustainability, 2021.
13. Kapagiannidis, A.G., I. Zafiriadis, and A. Aivasidis, *Comparison between aerobic and anoxic metabolism of denitrifying-EBPR sludge: effect of biomass poly-hydroxyalkanoates content.* N Biotechnol, 2013. **30**(2): p. 227 – 37.
14. Liu, W.T., et al., *Internal energy-based competition between polyphosphate- and glycogen-accumulating bacteria in biological phosphorus removal reactors–effect of P/C feeding ratio.* Water Research, 2015. **31**(6): p. 1430–1438.

15. Fan, Y., et al., *Optimization of phthalic acid batch biodegradation and the use of modified Richards model for modelling degradation.* International Biodeterioration & Biodegradation, 2004. **53**(1): p. 57–63.
16. Cao, T.-T., L. Wang, and Y.-M. Li, *Operation of the AAO Process Under Low Dissolved Oxygen Conditions and Its Simulation.* Huan jing ke xue = Huanjing kexue, 2018. **39**(1): p. 219–226.
17. Zhang, D., et al., *Effect of thermal hydrolysis pretreatment on volatile fatty acids production in sludge acidification and subsequent polyhydroxyalkanoates production.* Bioresour Technol, 2019. **279**: p. 92–100.
18. Wen, Q., et al., *Bulking sludge for PHA production: Energy saving and comparative storage capacity with well-settled sludge.* Journal of Environmental Sciences, 2012. **24**(10): p. 1744–1752.
19. Salehizadeh, H. and M.C. Van Loosdrecht, *Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance.* Biotechnol Adv, 2004. **22**(3): p. 261 – 79.
20. Chen, H., et al., *Polyhydroxyalkanoate production from fermented volatile fatty acids: effect of pH and feeding regimes.* Bioresour Technol, 2013. **128**: p. 533-8.
21. Mino, T., et al., *EFFECT OF PHOSPHORUS ACCUMULATION ON ACETATE METABOLISM IN THE BIOLOGICAL PHOSPHORUS REMOVAL PROCESS - ScienceDirect.* Biological Phosphate Removal from Wastewaters, 1987: p. 27–38.
22. Sudiana, I.M., et al., *Metabolism of enhanced biological phosphorus removal and non-enhanced biological phosphorus removal sludge with acetate and glucose as carbon source.* Water Science and Technology, 1999. **39**(6): p. 29–35.
23. Smolders, G.J., et al., *Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence.* Biotechnology and bioengineering, 1994. **43**(6): p. 461 – 70.
24. Huang, L., et al., *Insights into Feast-Famine polyhydroxyalkanoate (PHA)-producer selection: Microbial community succession, relationships with system function and underlying driving forces.* Water Res, 2018. **131**: p. 167–176.
25. Koller, M., et al., *Potential of Various Archae- and Eubacterial Strains as Industrial Polyhydroxyalkanoate Producers from Whey.* Macromolecular Bioscience, 2007. **7**(2): p. 218–226.
26. Colpa, D.I., et al., *Thauera aminoaromatica MZ1T Identified as a Polyhydroxyalkanoate-Producing Bacterium within a Mixed Microbial Consortium.* Bioengineering (Basel), 2020. **7**(1).
27. McIlroy, S.J., et al., *Filamentous members of cluster III Defluviicoccus have the in situ phenotype expected of a glycogen-accumulating organism in activated sludge.* FEMS Microbiol Ecol, 2010. **74**(1): p. 248 – 56.
28. Li, H., et al., *Simultaneous nitrogen and phosphorus removal by interactions between phosphate accumulating organisms (PAOs) and denitrifying phosphate accumulating organisms (DPAOs) in a sequencing batch reactor.* Sci Total Environ, 2020. **744**: p. 140852.
29. Chung, B.S., et al., *Hydrogenophaga caeni sp. nov., isolated from activated sludge.* Int J Syst Evol Microbiol, 2007. **57**(Pt 5): p. 1126–1130.

30. Cui, Y.W., et al., *Effects of carbon sources on the enrichment of halophilic polyhydroxyalkanoate-storing mixed microbial culture in an aerobic dynamic feeding process.* Sci Rep, 2016. **6**: p. 30766.
31. Tan, G.-Y., et al., *Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review.* Polymers, 2014. **6**(3): p. 706–754.
32. Jayakrishnan, U., D. Deka, and G. Das, *Influence of inoculum variation and nutrient availability on polyhydroxybutyrate production from activated sludge.* Int J Biol Macromol, 2020. **163**: p. 2032–2047.
33. Wen, Q., et al., *Effects of phosphorus and nitrogen limitation on PHA production in activated sludge.* Journal of Environmental Sciences, 2010. **22**(10): p. 1602–1607.
34. Chen, Z., et al., *Reconsideration on the effect of nitrogen on mixed culture polyhydroxyalkanoate production toward high organic loading enrichment history.* Frontiers of Environmental Science & Engineering, 2019. **13**(4).
35. Guo, J.H., et al., *Filamentous and non-filamentous bulking of activated sludge encountered under nutrients limitation or deficiency conditions.* Chemical Engineering Journal, 2014. **255**: p. 453–461.

Figures

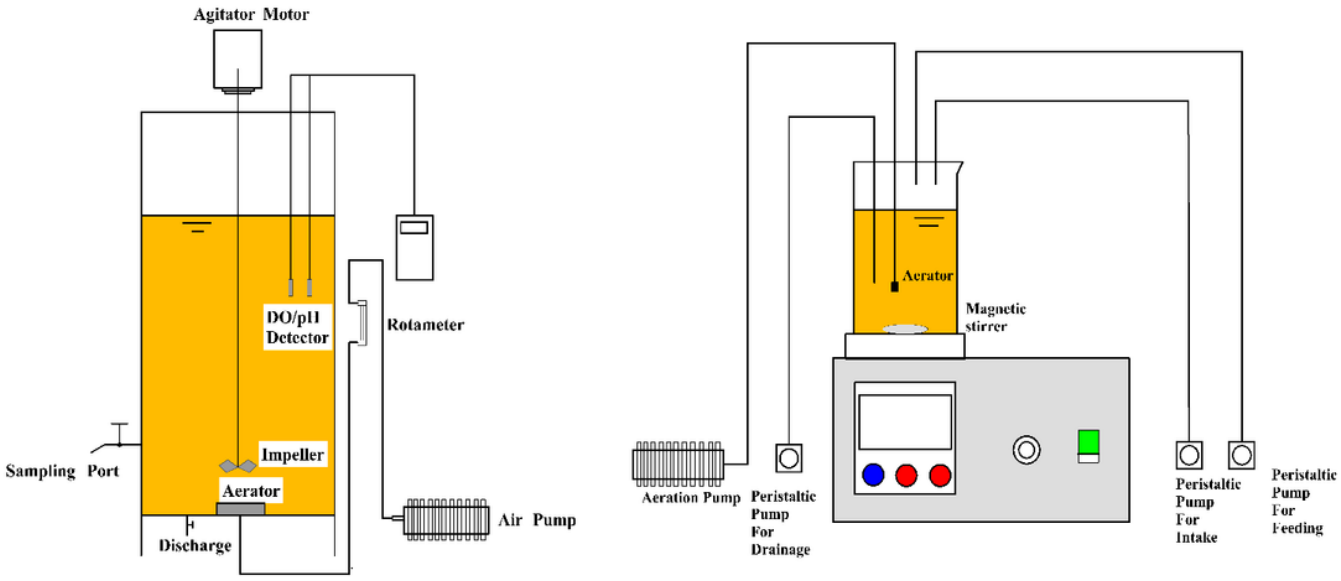

Figure 1

The devices of enrichment reactor (left) and accumulation reactor (right)

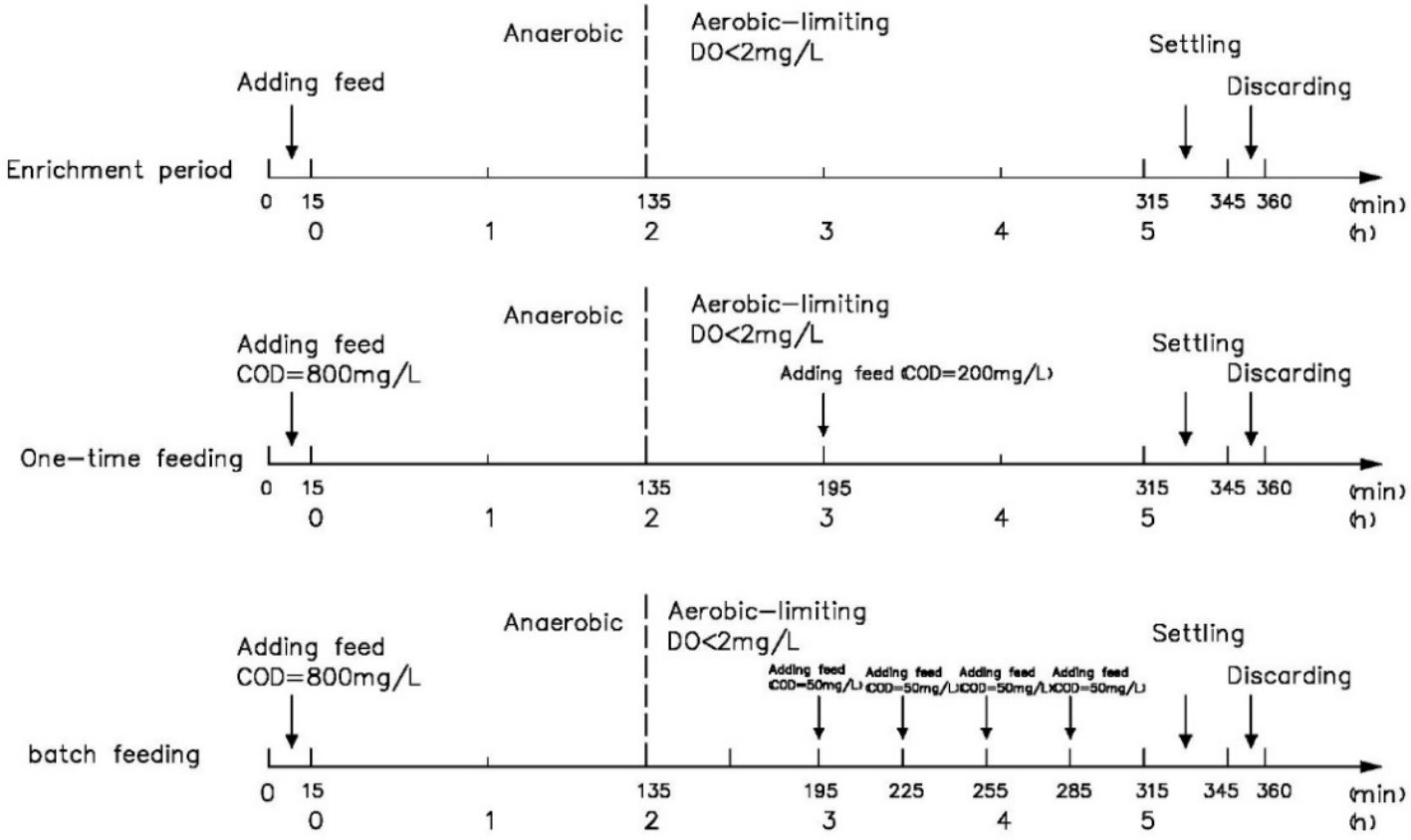

Figure 2

Operation method of enrichment period, one-time feeding and batch feeding

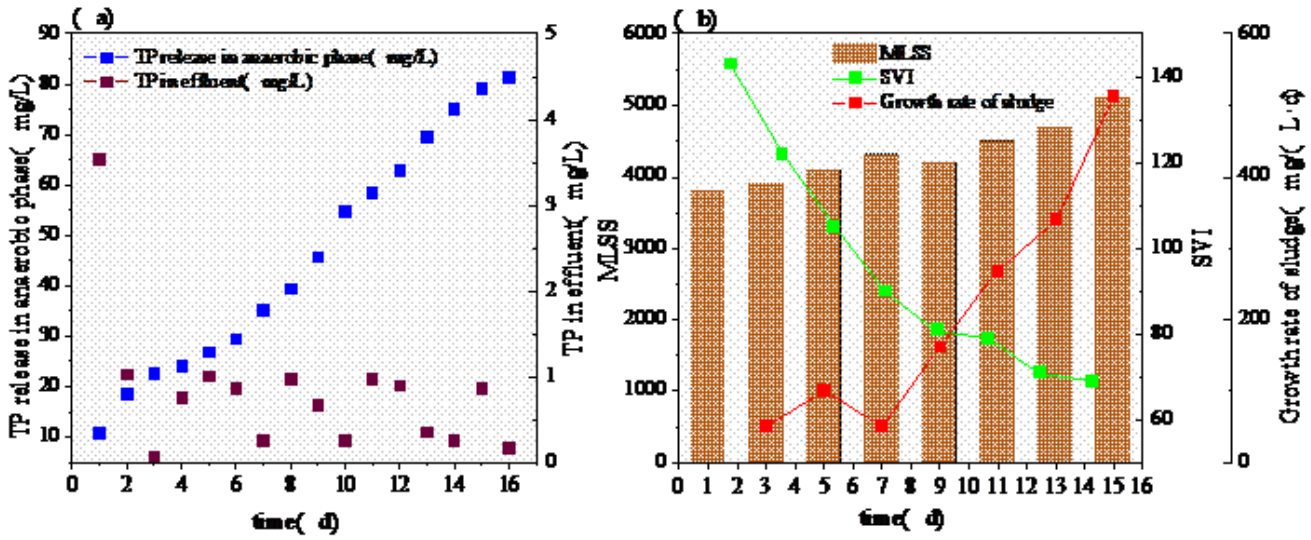

Figure 3

TP indicators in the enrichment period (a) and changes in MLSS, SVI, and sludge growth rates during enrichment(b)

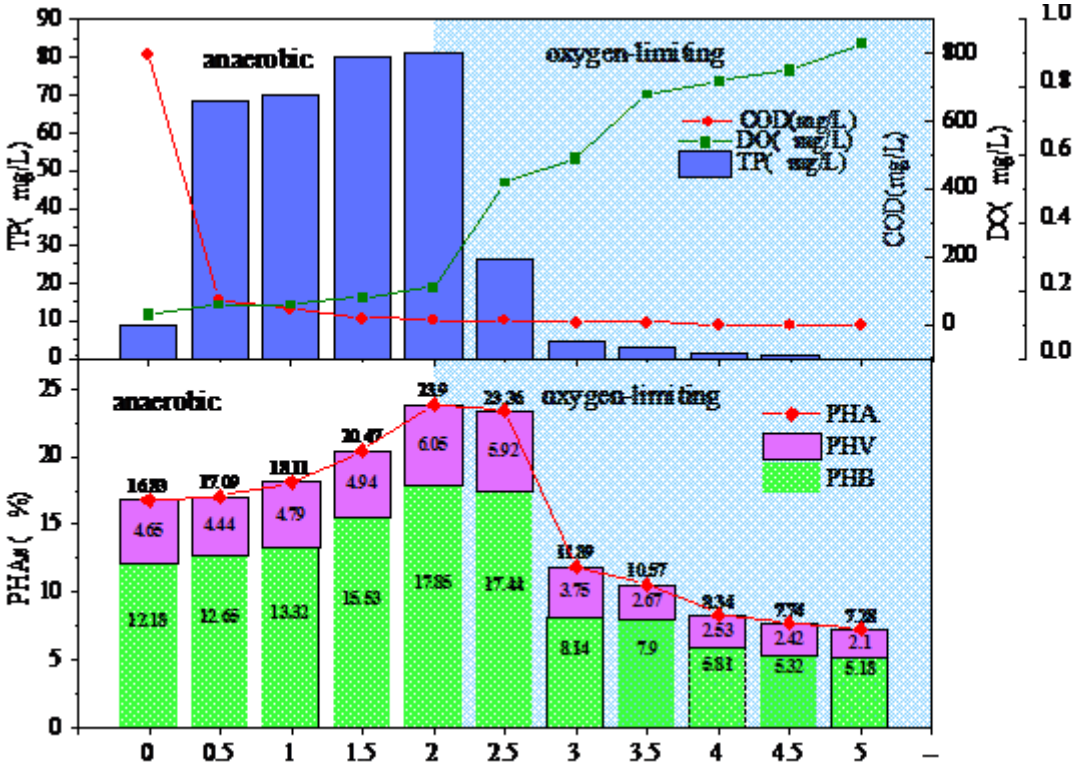

Figure 4

The changes of COD, TP, DO, and PHAS in the enrichment period

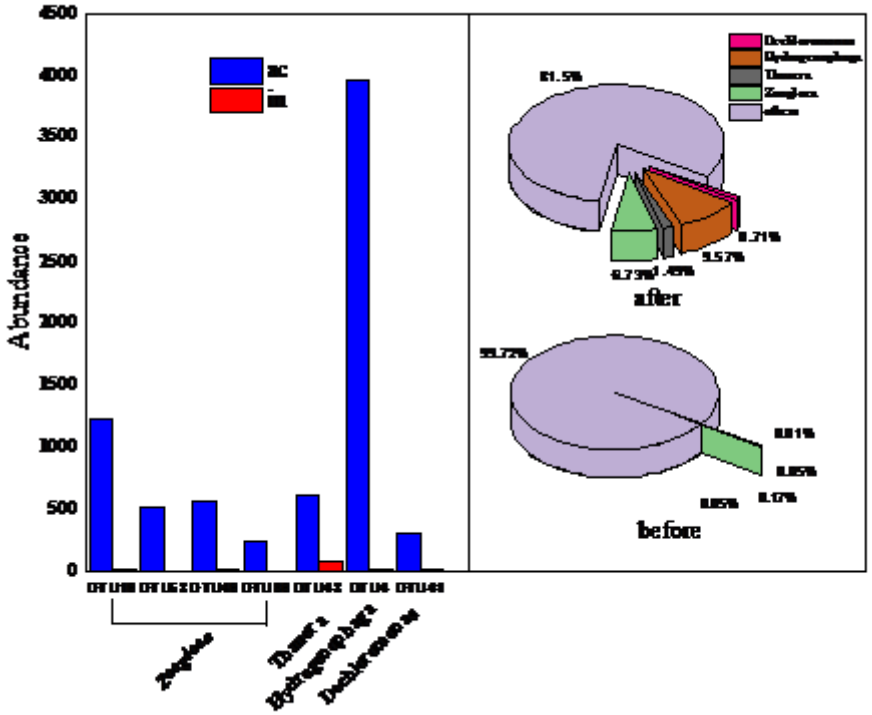

Figure 5

The main OUT sequence number and taxonomic proportion of dominant bacterial genera before and after enrichment of PHAs in sludge

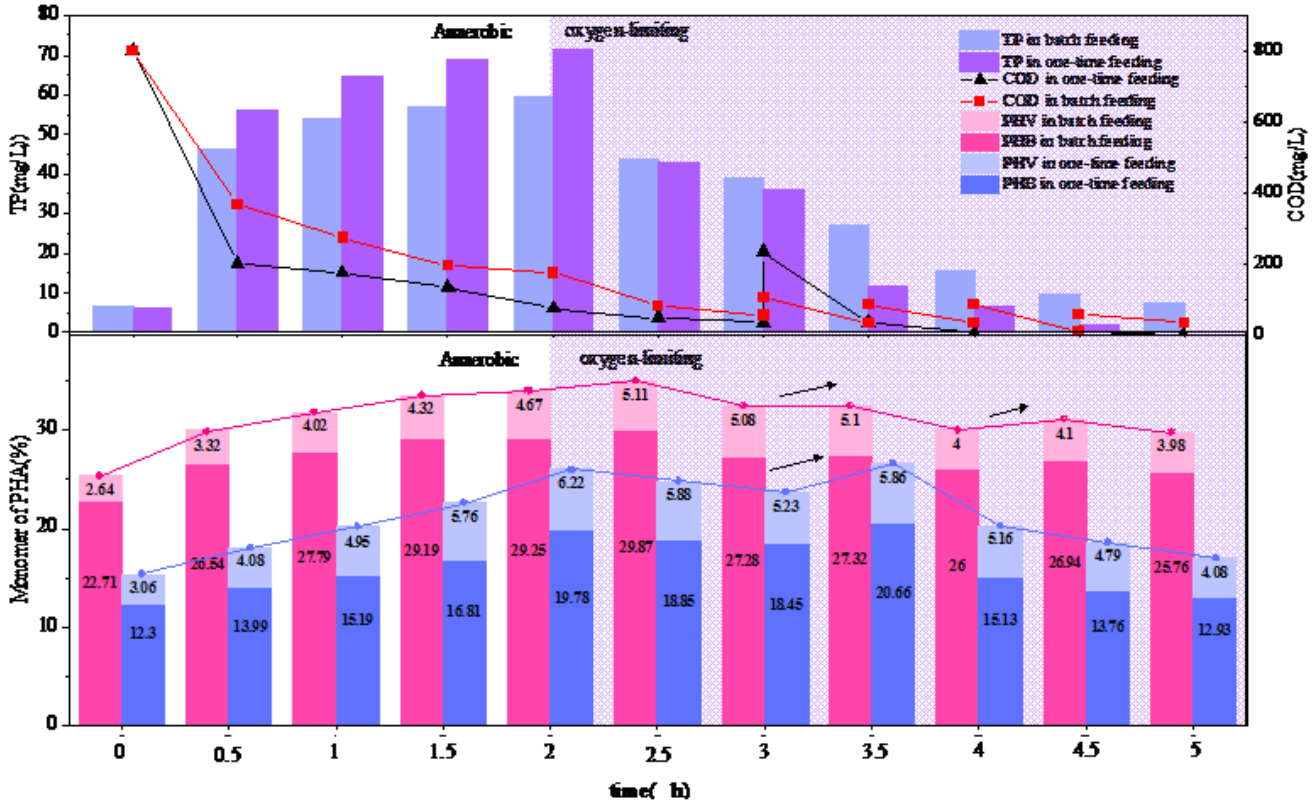

Figure 6

The changes of TP, COD, and PHAs in one-time and batch feeding when C/N=20

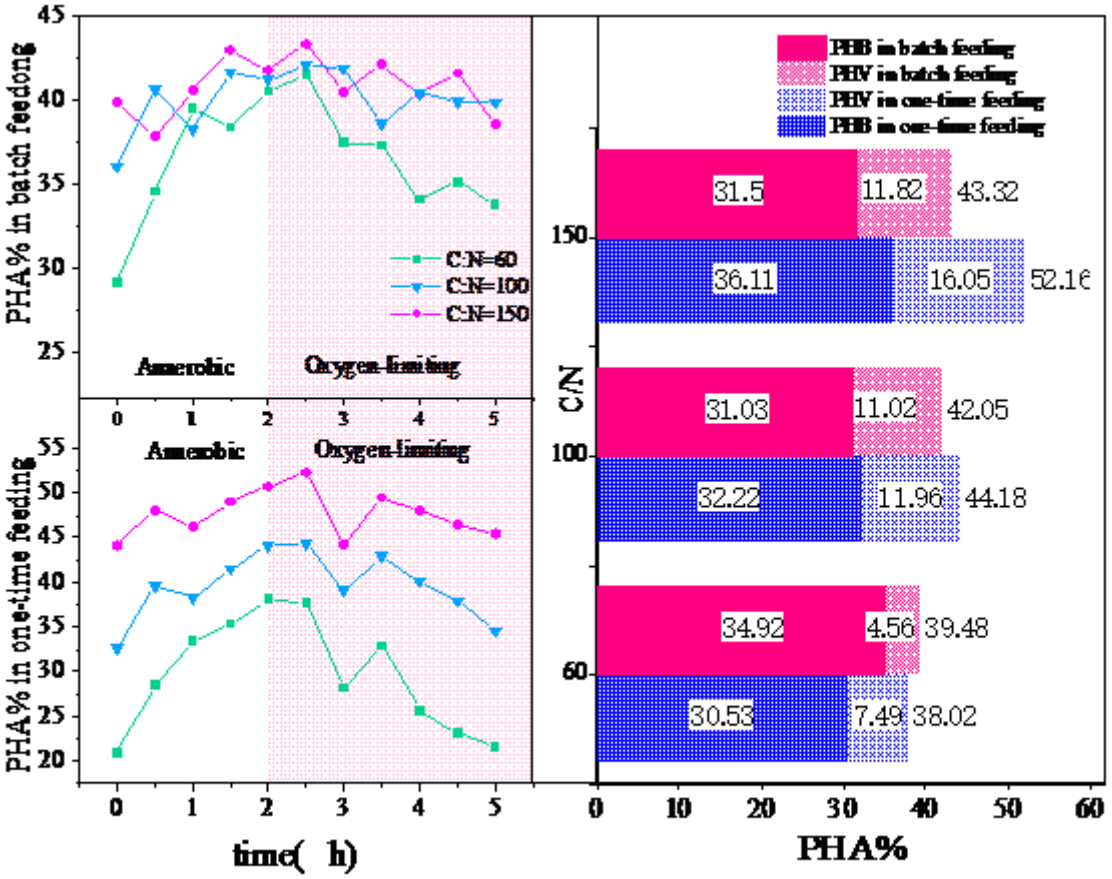

Figure 7

The changes of PHAs content in one-time feeding mode and batch feeding mode and the comparison of monomer when PHAs content was at maximum under different C/N