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Effects of phosphorus and nitrogen limitation on PHA production in activated sludge

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Abstract

The effects of phosphorus and nitrogen limitation on polyhydroxyalkanoate (PHA) production and accumulation by activated sludge biomass with acetate as a carbon source were investigated. Pre-selected influent carbon-phosphorus (C:P, *W/W*) of 100, 160, 250, 500 and 750, and carbon-nitrogen (C:N, *W/W*) of 20, 60, 100, 125 and 180 were applied in the phosphorus limitation experiments and the nitrogen limitation experiments, respectively. The maximum PHA accumulation up to 59% of the cell dry weight with a PHA productivity of 1.61 mg PHA/mg COD consumed was observed at the C:N 125 in the nitrogen limitation experiment. This value was much higher than that obtained in previous studies with a normal substrate feeding. The study showed that activated sludge biomass would produce more polyhydroxybutyrate than polyhydroxyvalerate under the stress of nutrient limitation, especially under phosphorus limitation conditions. The experimental result also indicated that both phosphorus and nitrogen limitation may cause sludge bulking.

Key words: polyhydroxyalkanoate; acetate; nitrogen limitation; phosphorus limitation; sludge bulking

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Introduction

Polyhydroxyalkanoates (PHAs) are intracellular energy and carbon storage materials synthesized by numerous microorganisms (Poirer et al., 1995; Lee, 1996). The most common forms of PHA accumulated by microorganisms in activated sludge are polyhydroxybutyrate (PHB) and polydroxyvalerate (PHV) (Satoh et al., 1992). Because PHAs are truly biodegradable (Yu and Si, 2001), they are regarded as potential substitutes for traditional petrochemically produced plastics. However, PHA products are ten times more expensive to produce than polyethylene products (Lee, 1996) due to the high production cost, which is made up of operating expenses. The polyethylene product depends largely on the cost of raw materials, such as pure cultures and substrate media (Kasemsap and Wantawin, 2007) .

With the aim of commercializing PHA, a great deal of effort has been devoted for reducing the production cost by using activated sludge as a mixed culture and a renewable resource (Satoh et al., 1999; Yu, 2001; Chua et al., 2003; Rhu et al., 2003; Kumar et al., 2004; Yan et al., 2006; Khardenavis et al., 2007; Cai et al., 2009). Economic evaluation showed that the production expenses of PHA can be reduced by more than half if renewable waste and activated sludge are used (Serafim et al., 2004). When renewable wastes such as high strength wastewater and activated sludge are involved, energy, such as methane or hydrogen, can be produced from the anaerobic treatment process (Steven et al., 2005; Ren et al., 2006). This process involves the formation of volatile fatty acids, such as acetic and butyric acids, by acidogenic bacteria. Consequently, these fatty acids can be utilized and polymerized into PHA by bacteria usually when there is an essential growth-limiting component such as nitrogen, phosphate, sulfur, oxygen, or the presence of excess carbon as an energy source (Salehizadeh and Van Loosdrecht, 2004). Kumar et al. (2004) reported a maximum 33% of biomass (*W/W*) by using activated sludge with the C:N (*W/W*) range from 24 to 144. Rhu et al. (2003) reported that a maximum PHA content of 51% was obtained with an anaerobic/aerobic cycle with phosphorus limitation (P limitation).

This study aimed to evaluate the effects of P limitation and nitrogen limitation (N limitation) on PHA accumulation in activated sludge by treating a synthetic wastewater in two sequencing batch reactors (SBRs) under the same anaerobic/aerobic operating cycles. The investigation also aimed to determine a feasible nutrient limitation mode for PHA accumulation in future studies.

1 Materials and methods

1.1 Materials

The sludge used as an inoculum was dry sludge obtained from the secondary sedimentation tank of a brewery wastewater treatment plant in Harbin, China. The water

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content of the dry sludge was around 80%. The experimental systems used were two bench scale SBRs operated in parallel with an anaerobic/aerobic cycle.

The SBRs, with a working volume of 4 L each, were operated in 3 cycles a day, with each cycle of 8-hr consisting of influent feeding (0.5 hr), anaerobic treatment (2 hr), aerobic treatment (4 hr), settling period (1 hr) and supernatant decanting (0.5 hr). The temperature of the reactors was between 18 and 20°C and pH in the reactor varied from 7.2 at the start of the cycle to 8.5 at the end. No attempt was made to control pH in the reactor. The sludge retention time (SRT) for both reactors was 10 days to keep the MLSS concentration 3000 mg/L during the operation.

Acetate was used as the sole carbon source to simulate the effluent of food processing wastewater that has been used for fermentation. The chemical oxygen demand (COD) was 2000 mg/L. The synthetic substrate was made of 3000 mg/L acetate, 100 mg/L $MgSO_4$ and 1 mL/L of mineral solution that contained (mg/L): KI 1.18, H_3BO_3 0.15, $CuSO_4$ 0.03, $CaCl_2$ 5, $ZnSO_4 \cdot 7H_2O$ 0.12, $FeCl_3 \cdot 6H_2O$ 1.5, $MnCl_4 \cdot H_2O$ 0.094, $Na_2MoO_4 \cdot 2H_2O$ 0.06, and 3 g/L EDTA. NH_4Cl and KH_2PO_4 were also added in varying concentrations in accordance with the carbon-nitrogen-phosphorus weight ratios required for the experiments.

1.2 Experimental procedure

1.2.1 Acclimation phase

The dry sludge from the brewery wastewater treatment plant was initially activated and enriched in a synthetic wastewater media using acetate as carbon source. Nitrogen and phosphorus, according to the weight ratio of C:N:P = 100:5:1, was supplied at this stage for cell growth. After enrichment, the activated sludge was inoculated into the 1#SBR reactor for acclimation prior to the P limitation experiment and into 2#SBR for acclimation prior to starting the N limitation experiment. The carbon-nitrogen-phosphorus weight ratio at the acclimation phase before P limitation experiment was controlled at 100:5:0.5 with COD 2000 mg/L. The carbon-nitrogen-phosphorus weight ratio at the acclimation phase before N limitation experiment was maintained at 100:2.5:1 with COD 2000 mg/L. Sludge samples were withdrawn at an interval of 30 min throughout the cycle and were analyzed for PHA content and biomass concentration.

1.2.2 Phosphorus and nitrogen limitation phase

The systems were initially operated at growth phase by providing enough nitrogen and phosphorus for cell nutrition and growth. P limitation was then introduced in the first SBR (1#SBR). The system went through an acclimation phase where half the standard phosphorus concentration was supplied, before entering the P limitation phase. During the P limitation phase, the carbon-phosphorus (C:P) weight ratio of the influent was controlled at 100, 160, 250, 500 and 750. COD, mixed liquor suspended solids (MLSS), glycogen concentration, phosphate concentration and PHA were measured every hour during the cycle study. Measurements were conducted on day 10 (1 SRT) after phosphorus concentration was changed each time. N limitation was investigated in the second SBR (2#SBR). The system went through an acclimation phase where half of the standard nitrogen concentration was supplied before entering the N limitation phase. During the N limitation phase, the carbon-nitrogen (C:N) weight ratio was controlled at 20, 60, 100, 125 and 180. The measurement process for the N limitation experiment was the same as that for the P limitation experiments.

1.3 Analytical methods

The following parameters were measured daily throughout the growth and nutrient limitation cycles: pH, oxidizing reduction potential (ORP), dissolved oxygen (DO), glycogen, orthophosphate, PHA and MLSS. The PHB and PHV fractions of PHA were determined separately. MLSS, orthophosphate and COD analyses were performed according to the Chinese SEPAC Standard Methods (SEPAC, 1997); pH and ORP were measured using a pH meter (HI8424, HANNA, Italy); DO was measured using a DO meter (DO200, YSI, USA); determination of glycogen was performed according to the method described by Jenkins et al. (1993). The determination of PHA was performed using gas chromatography after methanolytic decomposition as described by Satoh et al. (1996). A gas chromatograph (GC6890N /FID, Agilent, USA) with an HP-5 column (30 m length, 320 μm internal diameter, 0.25 film thickness) was used for all gas chromatography.

2 Results and discussion

2.1 PHA production with P limitation

2.1.1 Acclimation phase

The yield of PHA was approximately 5% of sludge dry weight under the balanced nutrient condition. After enrichment, the percentage contents of PHA produced with operation time are plotted in Fig. 1. The PHA accumulation percentage in the sludge increased with the time of operation, and the maximum percentage PHA accumulation in the acclimation phase for P limitation was observed during day 11 to day 13, equivalent to 1.3 sludge ages. The best result of PHA accumulation in the acclimation phase was 34% (*W/W*) in 1#SBR.

2.1.2 Phosphorus limitation phase

After the acclimation phase, the phosphorus content of the influent feed was gradually reduced to acquire C:P (*W/W*) of 100, 160, 250, 500 and 750. PHA accumulation measurements were conducted on day 10 (1 SRT) after phosphorus concentration was changed each time. The profile of PHA contents within one cycle under the different C:P weight ratios are shown in Fig. 2.

Figure 2 shows that the PHA accumulation percentage increased with the increasing C:P weight ratio. For most cases, the maximum accumulation occurred either at the end of the anaerobic phase or at half an hour after the aeration phase commenced. This is probably because when the system switched to the aerobic phase, the actual DO concentration in the reactor was still low, equal to around 0.5 mg/L, leading to a lag phase for PHA synthesis. When the C:P weight ratio was equal to 250, PHA accumulation achieved 25% of cell dry weight at the end of the anaerobic phase. However, the maximum percentage of PHA accumulation (37%), which represents PHA productivity of 0.79 mg PHA/mg COD consumed, was found with an influent C:P weight ratio 750 after 2.5 hr aeration. The results showed that PHA was continuously synthesized and accumulated by the activated sludge in the aerobic phase in our study. Saito et al. (1995) found that sludge accumulated more PHB under aerobic conditions than under anaerobic conditions. In the aerobic metabolism model, PHA accumulators are selected regardless of the ability of microorganisms to accumulate poly-P or glycogen, and the selected PHA accumulators will have a lower tendency to accumulate glycogen (Salehizadeh and Van Loosdrecht, 2004). Punrattanasin (2001) investigated three types of oxygen operating conditions–the absence of oxygen (anaerobic), oxygen limitation and fully aerobic. It was found that the two-stage bioprocess approach was the most successful strategy for PHA production, and fully aerobic conditions were better and less complex for PHA production.

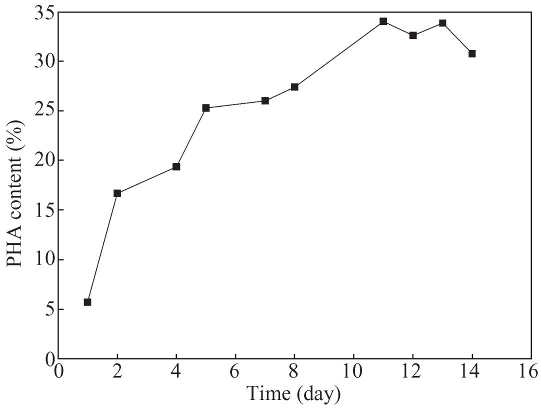

Fig. 1 Profile of PHA production in 1#SBR during the acclimation phase for phosphorus limitation.

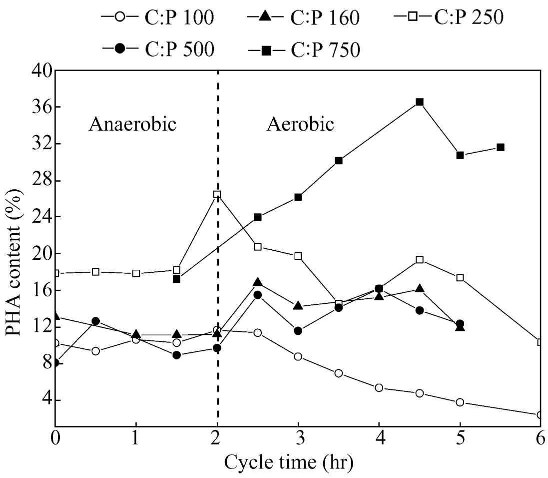

Fig. 2 Profile of PHA production at different C:P weight ratios in one cycle.

PHA includes PHB, PHV, 3H2MB (3-hydroxy-2-methylbutyrate) and 3H2MV(3-hydroxy-2-methylvalerate), and PHB and PHV are the most common constituents. PHB has characteristics that increase crystallinity, stiffness and brittleness of the end product, while PHV increases softness and flexibility (Rhu et al., 2003). It is generally understood that the PHB content of the end product may be increased when the carbon sources with even numbers of carbon groups (acetate, butyrate) are used, while the 3HB-co-3HV is increased when odd numbers of carbon groups (propionate, valerate) are used (Saito et al., 1995; Punrattanasin, 2001). As shown in Table 1, the PHB/PHA ratios found in this experiment varied from 0.70 with an influent C:P (*W/W*) of 100 to 0.99 with an influent C:P (*W/W*) 750. The higher PHB/PHA ratio in our study was due to the use of acetate as a carbon source. It was noticed that the PHB/PHA ratio increased with the increasing P limitation in the substrate. This shows that activated sludge biomass tends to produce more PHB than PHV under the stress of P limitation.

For the typical anaerobic-aerobic activated sludge process, microorganisms consume energy sourced from polyphosphate or glycogen to absorb organic substrates such as short chain fatty acids under anaerobic conditions and they temporarily store the organic substrates, such as PHB, until the conditions become aerobic. Under aerobic

Table 1 PHB and PHV production at different C:P weight ratios (%)

Running time	C:P = 100			C:P = 160			C:P = 250		
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA
Anaerobic beginning	7.14	3.08	0.70	11.07	2.04	0.84	13.67	4.18	0.76
Anaerobic end	8.51	3.14	0.73	9.41	1.79	0.84	22.14	4.34	0.84
Aerobic beginning	8.63	2.72	0.76	15.05	1.75	0.89	17.28	3.47	0.83
Aerobic for 2 hr	3.71	1.62	0.70	13.82	1.39	0.91	12.90	3.29	0.79
Aerobic end	1.84	0.56	0.77	NA	NA	NA	8.79	1.34	0.87

Running time	C:P = 500			C:P = 750		
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA
Anaerobic beginning	7.56	0.51	0.94	NA	NA	NA
Anaerobic end	9.05	0.64	0.93	18.98	0.45	0.98
Aerobic beginning	14.47	1.00	0.93	23.70	0.27	0.99
Aerobic for 2 hr	15.48	0.70	0.96	34.67	0.44	0.99
Aerobic end	NA	NA	NA	30.08	0.39	0.99

NA: not available.

conditions, they grow and regenerate polyphosphate and glycogen while aerobically utilizing the temporal carbon storage, PHB (Cech and Hartman, 1993). In this study, the glycogen content of the biomass and the phosphate in the bulk phase were measured and the results are plotted in Fig. 3. As shown in Fig. 3, the glycogen and phosphate contents showed obvious change when the conditions in the SBR changed from anaerobic to aerobic. The content of phosphate in the bulk phase increased under anaerobic condition, while decreased under aerobic. The results showed that the reducing power required for the conversion of acetate into PHA is provided by the intracellular glycolysis or polyphosphate, and the microorganisms responsible for the synthesis of PHA is partially from polyphosphate accumulating organisms (PAOs) or glycogen accumulating organisms (GAOs).

The maximum percentage PHA accumulation in the activated sludge in our study was observed when the influent C:P was equal to 750, however, one week after that, sludge bulking occurred. The MLSS concentration in the system dropped from 3000 mg/L to less than 1000 mg/L. Similar problems have been found by Chinwetkitvanich et al. (2004) when the activated sludge was placed under conditions of P limitation. It is well known that activated sludge microbes will secrete excess extracellular biopolymers with high moisture when the C:P weight ratio in the supplied substrate is significant high, which triggers non-filamentous activated sludge bulking. PHA production in this experiment was thereby reduced by large amounts of biomass lost from the system. It is recommended that future investigations into the processes of P limitation utilize a method to avoid the loss of biomass, such as the use of membrane separation.

2.2 PHA production with N limitation

2.2.1 Acclimation phase

The percentage contents of PHA produced with operation time are plotted in Fig. 4. The PHA accumulation percentage in the sludge increased dramatically with operation time. More than 30% (*W/W*) of PHA accumulation was observed by day 5, which was equivalent to 0.5 times the sludge age. The PHA content increased slowly with

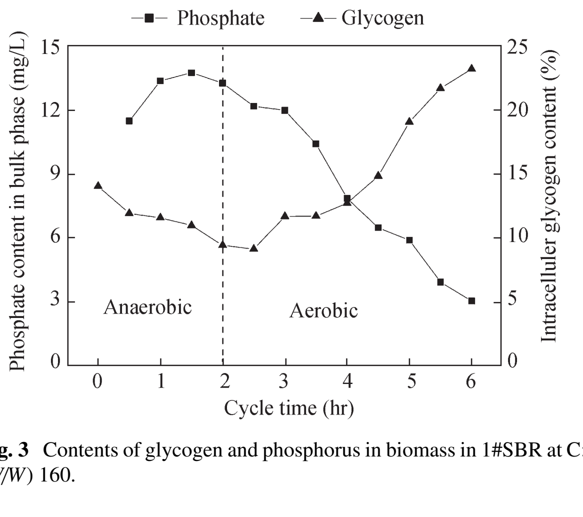

Fig. 3 Contents of glycogen and phosphorus in biomass in 1#SBR at C:P (*W/W*) 160.

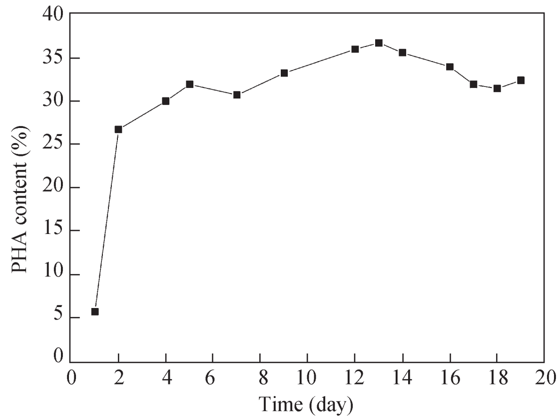

Fig. 4 Profile of PHA production in 2#SBR during the acclimation phase for nitrogen limitation.

the operating time. The best result for percentage PHA accumulation in the acclimation phase for N limitation was observed on day 13. The maximum PHA accumulation found in the acclimation phase was 37% (*W/W*). The result indicated that the activated sludge system acclimated faster to the condition of N limitation than condition of P limitation, as noted by Chinwetkitvanich et al. (2003). The PHA production was also higher.

2.2.2 Nitrogen limitation phase

After the acclimation phase the concentration of nitrogen was gradually reduced in the influent feed for N limitation. The weight ratio of C:N in the influent were adjusted to 20, 60, 100, 125 and 180. Samples were taken after 10 days operation (1 SRT) with each nitrogen concentration to measure the PHA production. The profile of PHA content within one cycle under different C:N weight ratios is shown in Fig. 5.

Figure 5 shows that as the C:N weight ratio in the influent was increased from 20 to 100, the PHA accumulation percentage in the activated sludge biomass increased from 12% to 41% of the dry cell weight. The maximum PHA accumulation percentage was observed with a C:N weight ratio 125, and occurred at the end of anaerobic phase. At the C:N weight ratio of 125, a maximum of 59% PHA

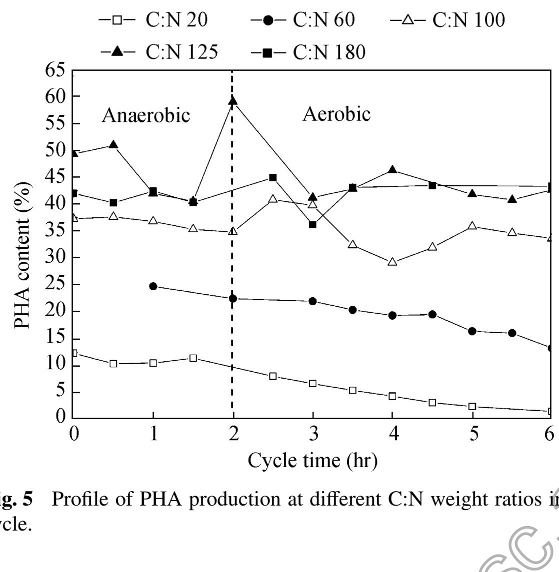

Fig. 5 Profile of PHA production at different C:N weight ratios in one cycle.

Table 2 PHB and PHV production at different C:N weight ratios (%)

Running time	C:N = 20			C:N = 60			C:N = 100		
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA
Anaerobic beginning	7.90	4.44	0.64	NA	NA	NA	32.80	4.49	0.88
Anaerobic end	NA	NA	NA	17.07	5.39	0.76	30.49	4.34	0.87
Aerobic beginning	5.49	2.58	0.68	NA	NA	NA	36.10	4.70	0.88
Aerobic for 2 hr	2.93	1.45	0.67	14.94	4.40	0.77	25.52	3.62	0.87
Aerobic end	1.25	0.30	0.80	10.89	2.37	0.82	30.86	2.79	0.92

Running time	C:N = 125			C:N = 180		
	PHB	PHV	PHB/PHA	PHB	PHV	PHB/PHA
Anaerobic beginning	45.31	3.97	0.92	37.69	4.31	0.89
Anaerobic end	54.11	4.88	0.92	NA	NA	NA
Aerobic beginning	NA	NA	NA	40.36	4.54	0.90
Aerobic for 2 hr	43.34	2.91	0.93	NA	NA	NA
Aerobic end	40.21	2.48	0.94	39.37	3.92	0.91

NA: not available.

accumulation was obtained, with a PHA productivity equal to 1.61 mg PHA/mg COD consumed. This is much higher than previous studies under a normal substrate feeding (Rhu et al., 2003; Kumar et al., 2004; Yan et al., 2006).

As shown in Table 2, the PHB/PHA ratios varied from 0.64 to 0.94 with C:N weight ratio in the influent increased from 20 to 180. The higher PHB/PHA ratio was again due to the use of acetate as carbon source. It is noted that PHV production is higher under conditions of N limitation than under P limitation (Tables 1 and 2). Since the PHV increases softness and flexibility of the PHA, this information may be helpful for the further studies.

Although the percentage accumulation of PHA in the activated sludge system was high under nitrogen limitation, the MLSS concentration in the system reduced sharply by the end of the experiment. A large population of filamentous microorganisms was observed in the system. This may be attributed by the high C:N weight ratio applied in the influent, which is lower than the minimum C:N weight ratio required for the biomass to continue cell growth and anabolic metabolism.

2.3 Comparison of nitrogen limitation and phosphorus limitation

Similar to previous studies (Chinwetkitvanich et al., 2003), although the experiments with P limitation resulted in an increase in accumulation of PHA, it was still significantly lower than PHA accumulation obtained in the N limitation experiments. There are two probable causes for the higher rate observed with N limitation. On one hand, when the system undergoes N limitation, it may inhibit the synthesis of microbial protein and allow PHA to develop as a major product due to the lack of nitrogen. On the other hand, the feed contained comparably high levels of nitrogen throughout the P limitation experiment, which enabled the biomass to continue cell growth and anabolic metabolism. Researchers (Du et al., 2001; Kessler and Witholt, 2001) have previously explained that under nitrogen-rich conditions, intracellular concentrations of acetyl-CoA (a precursor for PHB synthesis) are presumably low as it mostly enters the tricarboxylic acid cycle (TCA cycle). The citrate synthase reaction results in a liberation of large amounts of free coenzyme A (CoASH), which could inhibit the 3-ketothiolase condensation reaction of acetyl-CoA to aectoacetyl-CoA and thus inhibit PHB synthesis.

From the commencement of supernatant decantation until the end of the influent feeding of the next cycle with new P and N conditions, the SBR systems were under anaerobic conditions. The accumulation of PHA was observed during these periods of time (data not shown). When the systems switched to the aerobic phase, the accumulation of PHA could not stop at the begining of the aeration, and continued for at least 0.5 hr. There are two probable causes for this. First, the actual low DO concentration in the system, around 0.5 mg/L, may lead to a lag phase for PHA synthesis. Second, at the end of the anaerobic phase, the biomass still contains phosphorus which will be decomposed.

In addition, the results of this experiment show that both P limitation and N limitation can cause sludge bulking. It is not surprising to observe the decline of the MLSS during the nutrient limitation because both N and P are essential nutrients required for cellular growth by organisms. P limitation caused a non-filamentous activated sludge bulking due to the expansion of excess viscous water attached, while N limitation resulted in a filamentous bulking. PHA production was thereby reduced by the large amounts of biomass loss from the system.

3 Conclusions

Although the experiments with P limitation resulted in a large accumulation of PHA, it was still significantly lower than those obtained in the N limitation experiments. The maximum percentage of PHA accumulation (59%) was observed with a C:N weight ratio equals to 125. The ratio of PHB/PHA produced varied from 0.64 to 0.99, showed that the activated sludge biomass tends to produce more PHB than PHV under the stress of nutrient limitation, especially under conditions of P limitation. Both P limitation and N limitation could cause sludge bulking. P limitation caused a non-filamentous bulking, while N limitation resulted in a filamentous bulking.

Acknowledgments

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